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Neuroregeneration after spinal cord injury and in amyotrophic lateral sclerosis - possibilities for stem cell therapy

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMSC	adipose-derived mesenchymal stromal cells
ASIA	American Spinal Injury Association grading system for the evaluation of neurological deficits after acute spinal cord injury
BBB	blood-brain barrier
BBB-test	Basso-Beattie-Bresnahan test
BDNF	brain-derived neurotrophic factor
BMSC	bone marrow mesenchymal stromal cells
C 1-8	cervical level of the vertebral column and spinal cord
ChaseABC	chondroitinase ABC
CHAT	cholinacetyltransferase
CNS	central nervous system
CSF	cerebrospinal fluid
CSPG	chondroitin sulphate proteoglycans
DNA	Deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
ESC	embryonic stem cells
FGF	fibroblast growth factor
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrilarry acidic protein
GFP	green fluorescent protein
GVHD	graft versus host disease
HAS	hyaluronan synthase

HD	Huntington’s disease
hESC	human embryonic stem cells
i.m.	intramuscular
i.p.	intraperitoneal
IGF-1	insulin-like growth factor 1
IL	interleukin
iPS	induced pluripotent stem cells
KO	knockout animals
L 1-5	lumbar level of the vertebral column and spinal cord
MN	motoneurons
MP	methylprednisolone
MPP	matrix metalloproteinase
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MSC	mesenchymal stromal cells
NASCIS	National Acute Spinal Cord Injury Study
NF	neurofilament
NGF	neural growth factor
NI	neural induction
NMJ	neuromuscular junction
NPC	neural stem/progenitor cells
NSC	neural stem cells
NT-3	neurotrophin-3
OEG	olfactory ensheathing cells
OMgp	oligodendrocyte myelin glycoprotein
pAMSC	predifferentiated adipose-derived mesenchymal stromal cells
PBS	phosphate-buffered saline

PD	Parkinson’s disease
PET	positron emission tomography
PNN	perineuronal net
PNS	peripheral nervous system
PV	parvalbumin positive cells
S.E.M.	standard error of the mean
SC	spinal cord
SCs	stem cells
SCI	spinal cord injury
TBI	traumatic brain injury
Th 1-13	thoracic level of the vertebral column and spinal cord
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WFA	wisteria floribunda agglutinin

CONTENTS

1. INTRODUCTION

1.1. Spinal cord anatomy

The spinal cord is the part of the CNS that transmits sensory information to the brain and regulates motor and autonomic functions. It has a cylindrical appearance and is approximately 1 cm in diameter, 42- 45 centimeters-long. Topographically, it leaves the skull through the foramen magnum and extends down the vertebral canal to the upper margin of the second lumber vertebra. The spinal cord is divided in the craniocaudal direction into the pars cervicalis, pars thoracica, pars lumbaris and pars sacralis with two enlargements at the cervical and lumbar regions, called the *intumescentia cervicalis and lumbaris*. The terminal part of the SC starts to narrow down into a wedge-shape and is denoted as the *conus medullaris* with its caudal extension called the *filum terminale*, which is surrounded by a bundle of nerves from the lumbar and sacral levels called the *cauda equina*. Generally, the SC contains 31 pairs of spinal nerves with the corresponding 31 segments (8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal). The spinal cord is located in the spinal canal surrounded with three layers of coverings: the *dura mater*, the *arachnoid membrane* and innermost, the *pia mater*. Between the inner surface of the bone and the dura mater there is a space filled mainly with fatty tissue and venous plexuses called the epidural space, which has an important clinical application in the use of epidural anesthesia. Another important anatomical structure is the subarachnoid space, i.e. the space between the *arachnoid membrane* and the *pia mater*, filled with the cerebrospinal fluid (CSF). This bulge is routinely accessed for sampling the CSF via a lumbar puncture and could be used for the intrathecal application of different agents.

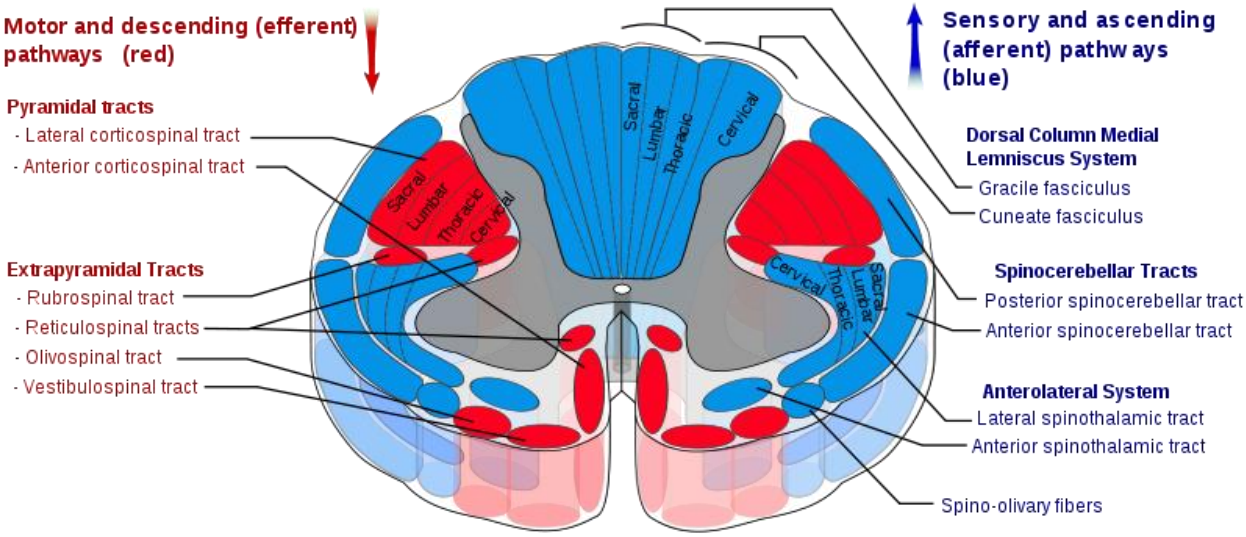

Figure 1. Spinal cord tracts (Häggström, 2010)

In cross-section the SC shows a central butterfly-shaped gray matter surrounded by peripherally located white matter and a central canal in the centre. The gray matter mainly consists of glial cells and neuronal bodies giving rise to three main types of connections – ascending, descending and local (interneurones) (Figure 1) – while the white matter is built from myelinated and unmyelinated ascending and descending axons and glia. **Ascending tracts**: *posterior funiculi* (responsible for kinesthesia and discriminative touch); *posterior spinocerebellar tracts* (responsible for proprioception); *spinothalamic tracts* (responsible for pain and thermal sensation). Below the sixth thoracic segment, ascending tracts form the *fasciculus gracilis*, whereas above that level they form the *fasciculus cuneatus*. **Descending tracts:** *lateral and anterior corticospinal tracts* (mediate voluntary and precise movements in the skeletal muscles); *extrapyramidal tracts - rubrospinal* (mediation of voluntary movement), *lateral vestibulospinal* (regulates extensor muscle tonus aimed at maintaining an upright position). Medial *vestibulospinal tract* (controls head position), reticulospinal tracts (modulates voluntary muscle movements, tone, reflex activity and breathing capacity). The autonomic nervous system originates in the hypothalamus and sends its sympathetic and parasympathetic descending

tracts mainly through the lateral funiculi to the preganglionic sympathetic and parasympathetic neurons in the brain stem and T1-L2 and S2-S4, respectively.

The spinal cord is supplied with blood from the *vertebral arteries* that give rise to one *anterior* and two *posterior* branches from each. The anterior branches fuse to form the *anterior spinal artery* that supplies the anterior two-thirds of every spinal cord segment. The *posterior spinal arteries* supply the posterior one-third of the SC. Additionally, the *vasocoronal arteries* arising from anterior and posterior spinal arteries provide blood circulation to the peripheral part of the lateral funiculi and play a role in the anastomosis between the anterior and posterior spinal arteries. The radicular arteries arise from the intercostals vessels and form a bifurcation with the anterior and posterior spinal arteries, thus forming the circumferential arterial supply to the SC. Veins that drain blood from the SC to the anterior and posterior epidural plexus have a similar pattern as that of the spinal arteries (Brodal, 2010, Holtz A., 2010).

1.2. General features of spinal cord injury and amyotrophic lateral sclerosis

Diseases of the central nervous system still remain among the most challenging pathologies known to mankind. This is because neurological disorders are typically devastating to the affected patients and their families, the individuals are often robbed of the qualities that are strongly associated with being human, and because the vast majority of neurological and neurodegenerative disorders lack effective therapies (La Spada and Ranum). Today, there are several known risk factors leading to an increase in the number of these maladies, among them the urbanisation and aging of the population in developed countries, living in or exposure to a toxic environment, genetic polymorphisms, bad habits and an inapropriate diet, traumatism and a variety of stresses and risk factors in our daily life. Due to these and many other reasons, it has been shown that an increasing number of people are affected every year by neurological diseases

such as stroke, brain and spinal cord traumatic injury (SCI), neurodegenerative diseases and CNS tumors. Some of these disorders are strictly inherited, such as Huntington's disease, while others are predominantly sporadic although with a minority of hereditary cases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease, which occur frequently in human populations (Carlesi et al.). In some cases neurodegenerative diseases arise in a particular geographic region and are associated with cultural and enviromental factors, e.g. Guam-type ALS/parkinsonism dementia complex (ALS/PDC), which is caused by the presence of β-methylaminoalanine in an indigenous plant commonly used as food by the Chamorros in Guam (Ince and Codd, 2005).

Neurodegeneration is a term used for diseases characterized by complex, progressive, multifaceted processes leading to the loss of structure and/or the function of brain and spinal cord neurons, including death. All of these occur due to the impairment of adaptive processes (cell division, neurotrophic factor signaling, anti-apoptotic mechanisms, antioxidant enzymes, ion homeostasis and many others) that take place in the nervous tissue under normal conditions. Many neurodegenerative diseases occur as a result of degenerative processes in selected areas of the CNS with specific symptoms and affects different levels of the neuronal circuitry, accompanied by the atrophy of central and peripheral nervous system structures. As research has progressed, many similarities have appeared which relate these diseases to one another on a sub-cellular level. For example, excitotoxicity, neuroinflamation and oxidative stress are common processes that play a crucial role in both SCI and neurodegenerative diseases. This work aims to focus closely on recent progress made in the understanding of neuroplasticity and treatment of SCI and ALS and, thus making it possible to use lessons from both pathologies in order to develop successful approaches to treatment or at least to stabilizing the processes responsible for disease progression.

1.3. SPINAL CORD INJURY (SCI)

1.3.1. Epidemiology of SCI

Currently in the European Union there are at least 330 000 people living with spinal cord injury and about 11 000 new cases every year (Onose et al., 2009). According to the National Spinal Cord Injury Statistical Center (NSCISC), the number of people in the United States who were alive in 2010 and have SCI has been estimated to be approximately 265,000 persons, with a range of 232,000 to 316,000 persons, with approximately 12 000 new cases annually. It is estimated that the annual incidence of spinal cord injury in the population, excluding those who died at the scene of an accident, is approximately 40 cases per million (NSCISC, 2010). The above statistics suggest that this is a serious problem that affects countries worldwide with almost the same frequency of new cases annually. Even though the level of medical care has increased in the last decades and life expectancy for people exposed to traumas and various types of neurodegenerative diseases has significantly grown, there has still been very limited progress in improving the neurological symptoms and defects of the affected patients as there are still large gaps in our understanding of the processes that take place after injury or the onset of degeneration. Due to these gaps in understanding, it remains unclear what kind of treatment approach should be employed and when treatment should be implemented.

1.3.2. Pathophysiology of Spinal Cord Injury

Human SCIs are very heterogeneous. Generally, traumatic injury to the spinal cord is defined by two broad components: a primary component (also called primary SCI), attributable to the mechanical impact and shear forces themselves, and a secondary component (secondary SCI) that consists of a series of systemic and local neurochemical changes that occur in the nervous tissue after

the initial traumatic shock (Klussmann and Martin-Villalba, 2005). According to the time after injury and the changes that occur during that period, SCI can also be divided into three phases: acute, intermediate and chronic. It is hard to delineate one phase from another, as there are many common traits, but nonetheless each stage has its own features and processes that dominate.

Acute phase: About 27% of human SCIs are characterized by the direct impact of penetrating objects (rapid flexion/rotation, distortion, laceration or foreign body stabbing). The remaining 73% of clinical cases are caused by temporary compression of the cord due to bone fragments, intervertebral disks etc. Any mechanical deformation of the spinal cord leads to the rupture of neuronal cell membranes with the release of the intracellular contents, hemorrhage, vasospasm, localized edema, breakdown of the blood-brain barrier etc. As a result, a cascade of vascular, biochemical and cellular processes leads to necrosis and apoptosis, causing the massive death of neuronal, glial and endothelial cells and a shift of metabolism towards anaerobic glycolysis (Katoh et al., 1996, Emery et al., 1998). Meanwhile, there is also an activation of macroglial cells and ongoing demyelination involving oligodendroglial cells (Beattie et al., 2000, Farooqui et al., 2004). Morphologically and clinically, the extent of post-traumatic damage usually correlates with the degree of force and pressure applied on the spinal cord, the time elapsed after the initial damage, and the kinetic energy that was absorbed by the nervous tissue. It is also interesting to note that most of the time, damage to the spinal cord is limited to a single or not even a whole segment, and only rarely does complete damage (transection) to the spinal cord occur. The transection of axons in the adult mammalian CNS also induces the degeneration of the axons separated from the cell bodies (Wallerian degeneration); meanwhile the proximal endings that remain connected to their perikarya form terminal retraction bulbs. After the initial

phase of SCI, axonal dieback occurs up to several millimeters more proximal from the site of the lesion (Seif et al., 2007).

Intermediate phase: The intermediate phase begins minutes after the initial injury and persists for weeks after SCI. This phase is characterized by secondary damage to the nervous tissue caused by vascular defects with related hypoxia, the release of glutamate from intracellular stores that causes excitotoxicity (Katayama et al., 1990) via an increased influx of Ca^{2+} into the neurons along with a depletion of ATP regeneration (uncoupled mitochondrial electron transport), the production of free radicals with subsequent lipid peroxidation, local inflammation (Taoka et al., 1997, Carlson et al., 1998) and changes in ionic homeostasis (Figure 2). All of these processes trigger a chain of events that are accompanied by an inflammatory reaction leading to secondary necrotic cell death (mainly of oligodenrocytes) at the core of the injury site and apoptotic cell death in the surrounding areas (Crowe et al., 1997, Popovich et al., 1997), reaching its highest levels at about 1 week after injury (Farooqui et al., 2004). The demyelination (Waxman, 1989, Bunge et al., 1993) and degeneration of the fiber tracts also leads to neuronal death in areas remote from the primary lesion site, such as the motor cortex in the brain (Lee et al., 2004). At this stage a number of oligodendrocytes and astrocytes die in the core of the injury (Crowe et al., 1997), meanwhile there is an activation of astrocytes at the edge of the primary injury site. These astrocytes display increased metabolism (the number of mitochondria and endoplasmic reticula inside cells is increased), and they start to form long neurites, aiming to prevent the spread of an aggressive environment further in both directions (Nathaniel and Nathaniel, 1977, Eddleston and Mucke, 1993). These cells are also called gemistocytes, and the peak of tissue infiltration by these cells is reached two to three weeks after injury. Importantly, this infiltration of activated astrocytes subsequently acts to block regeneration after SCI due to the formation of a barrier to axonal sprouting across the lesion (Fawcett, 2006). Another important factor that blocks

the regeneration of the injured axons at this stage is the activation of oligodendrocytes, which results in the formation of myelin membranes and the synthesis of oligodenrocyte-myelin glycoprotein (OMG) and myelin-associated glycoprotein (MAG), both of which have neurite growth inhibitory activity (Schwab and Caroni, 1988, Domeniconi et al., 2002, Oertle et al., 2003) (see below).

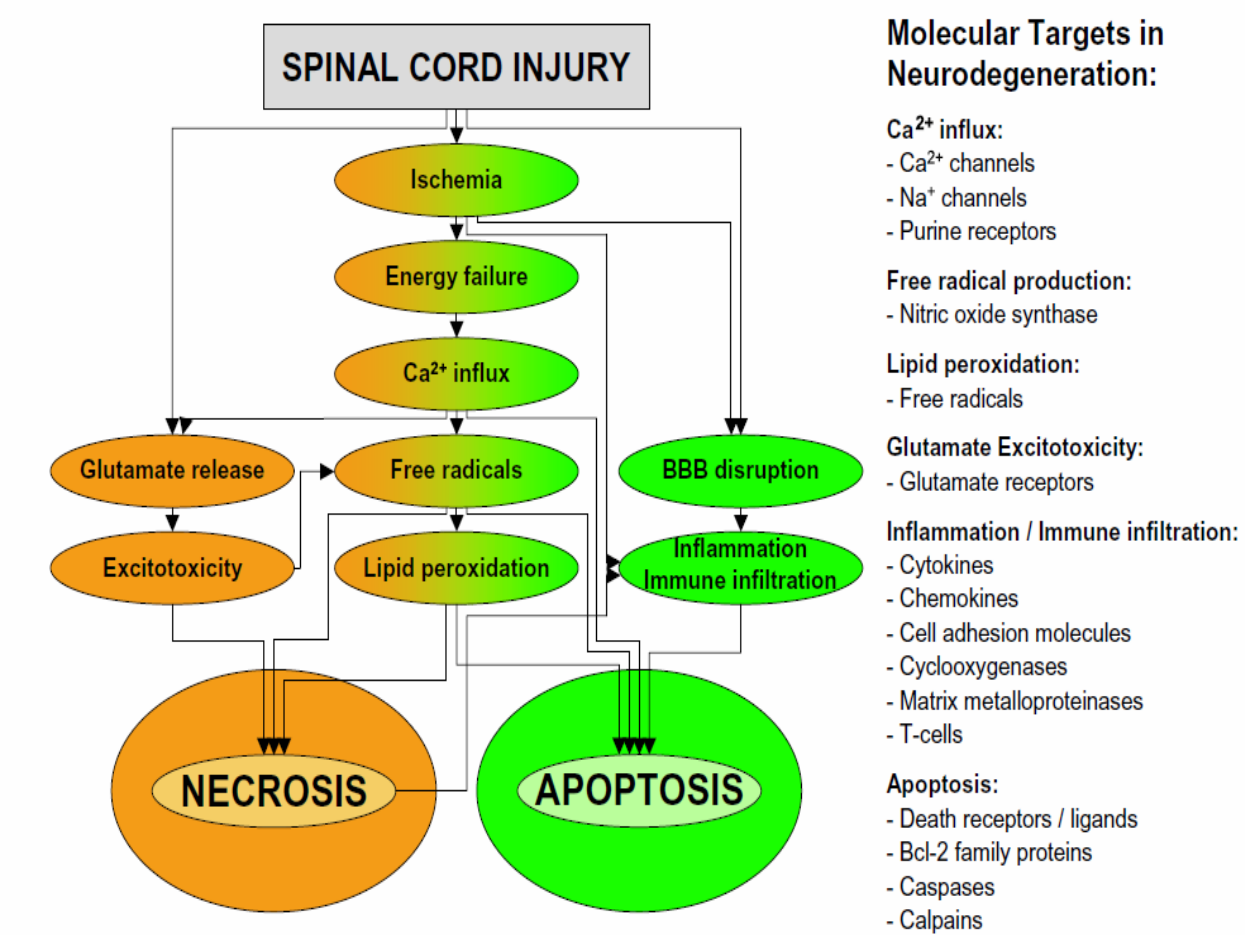

Figure 2. Neurotoxic events that follow injury to the spinal cord and molecular targets for intervention (Klussmann, S. and A. Martin-Villalba, 2005)

Chronic phase: The chronic phase starts days after injury and can last for years. The main feature of this phase is ongoing demyelination (Bunge et al., 1993, Schwab and Bartholdi, 1996, Totoiu and Keirstead, 2005), local inflammation and apoptosis (Fleming et al., 2006), a decrease in the number of activated macrophages, and the formation of a glial scar and pseudocysts (also

called syringomyelia) (Windle and Chambers, 1950, Fawcett and Asher, 1999, Nielsen et al., 1999, Perrouin-Verbe et al., 1999). This phase of SCI presents a major challenge to doctors and scientists and attracts the greatest research interest, as most SCI patients remain in this phase, to a greater or lesser extent, for the rest of their lives. Targeting the main components of the glial scar in the chronic phase could provide effective therapy for patients in the future (Figure 3).

1.3.3. Clinical Symptoms of SCI

The clinical symptoms of SCI vary with the site of damage, because of the way the spinal nerves are distributed in an orderly arrangement down the length of the SC. Neural systems that can be permanently disrupted below the level of injury involve not only the obvious control of limb muscles, but also various types of sensation and the normal function of the internal organs. Anatomically, the spinal cord comprises ascending (gracile and cuneate fascicules, dorsal and ventral spinocerebellar, lateral and ventral spinothalamic tracts) and descending (lateral corticospinal, hypothalamosoinal, rubrospinal, vestibulospinal and ventral coricospinal tracts) pathways that transfer sensory and motor signals, respectively.

Depending on which anatomical structures are affected, patients after SCI will have corresponding functional deficits. The common symptoms include complete/incomplete loss of motor (para-/tetra paresis, muscle hyperreflexia, hypertonicity, Babinski sign and clonus), sensory (hypo-/anesthesia, paresthesia, dyseshtesia, hyperpathie and allodynia) and autonomous functions (urinary retention due to detrusor/sphincter dyssynergia/areflexia, deficits in the cardiovascular system, breathing, sweating, bowel control, impairment of body temperature regulation, persistent loss of the bulbocavernosus reflex, ileus, sexual dysfunction etc.). These deficits lead to a succession of secondary

problems, such as pressure sores, chronic neurologic pain or paresthesias, urinary tract infections, spontaneous hyperactivity of motoneurons resulted in stiffness and uncontrolled, spastic muscle spasms. In order to systematize and grade the severity of neurological loss and symptoms, clinicians have long used different scales. The most widely used system for the evaluation of functional recovery was devised at Stokes Manville before World War II and popularized by Frankel in the 1970s (UAB-SCIMS, 2011). The scale is based on motor and sensory deficits and consists of five grades (table 1).

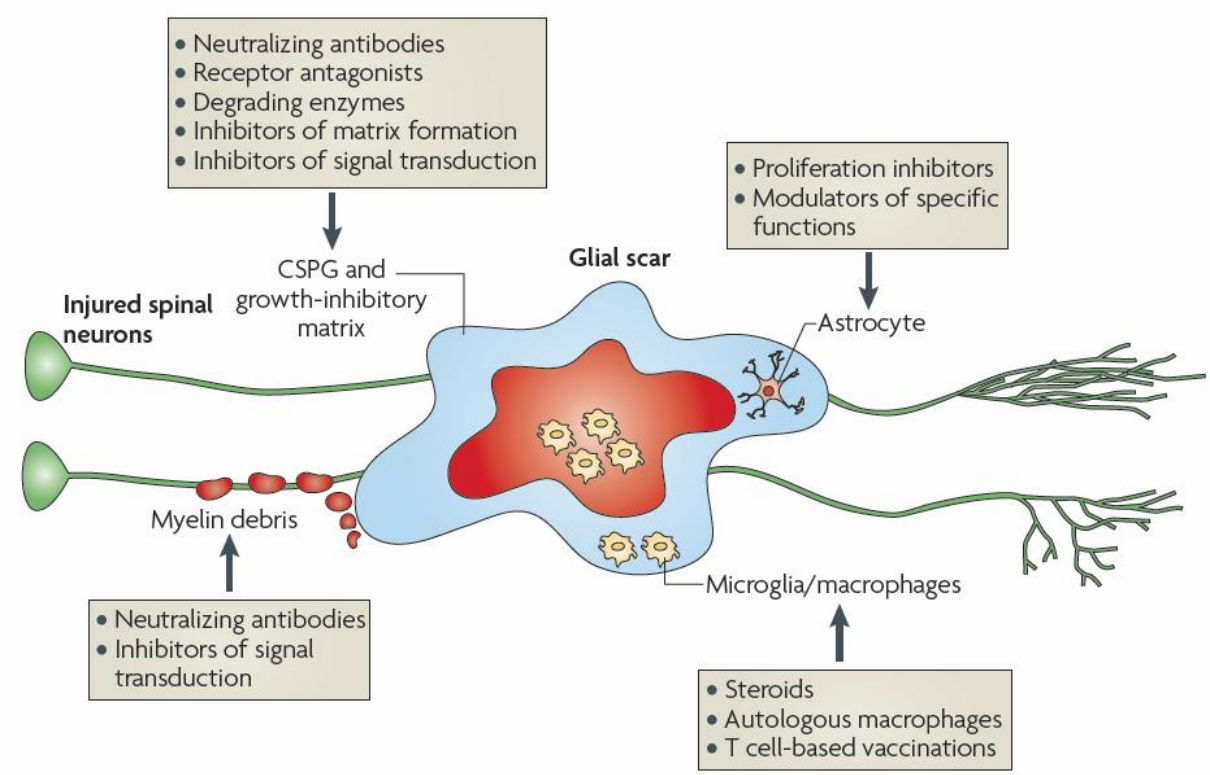

Figure 3. Scar components and potential therapeutic interventions (Rolls et al., 2009)

Nowadays, a newer impairment scale has been offered by the American Spinal Injury Association (ASIA) to classify SCIs. It is based on the Frankel scale, but differs in several important respects (table 1 and 2a, b). First, instead of no function below the injury level, ASIA A is defined as a person with no motor or sensory function preserved in the sacral segments S4-S5. ASIA B is essentially identical to Frankel B but adds the requirement of preserved sacral S4-S5

function. It should be noted that ASIA A and B classification depends entirely on a single observation, i.e. the preservation of motor and sensory function of S4-5. The ASIA scale also adds quantitative criteria for C and D, whereas the original Frankel scale has a subjective element in evaluation, but ignores arm and hand function in patients with cervical spinal cord injury. To get around this problem, ASIA stipulated that a patient would be an ASIA C if more than half of the muscles evaluated had a grade of less than 3/5. If not, the person was assigned to ASIA D. ASIA E is of interest because it implies that somebody can have a spinal cord injury without having any neurological deficits, at least none detectable on a neurological examination of this type. These changes in the ASIA scale have significantly improved the reliability and consistency of the classification.

A	**Complete paralysis**
B	**Sensory function only below the injury level**
C	**Incomplete motor function below injury level**
D	**Fair to good motor function below injury level**
E	**Normal function**

Table 1. Frankel Grade Function

1.3.4. Current Therapy

Therapeutic approaches for SCI fall into three separate time frames. The first could be described as acute neuroprotection after an SCI. This is directed at interrupting the cascade of secondary injury processes, thus limiting tissue damage. The chief architect of the treatable secondary damage concept was A.R. Allen, with his innovative studies of spinal injuries in dogs (Allen, 1911).

A	**Complete: no motor or sensory function is preserved in the sacral segments S4-S5.**
B	**Incomplete: sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.**
C	**Incomplete: motor function is preserved below injury level, and more than half of the key muscles below the neurological level have a muscle grade less than 3**
D	**Incomplete: fair to good motor function below injury level, half of the key muscles below the neurological level have a muscle grade more than 3**
E	**Normal: motor and sensory functions are normal**

Table 2a. American Spinal Injury Association (ASIA) impairment scale

Over the intervening ninety years, speculation and experiments on the nature of the secondary pathology have been dominated by a succession of preoccupations: compromised blood flow, edema, catecholamine, oxidative damage, excitotoxicity, inflammation, nitric oxide and apoptosis (Blight, 2002). All of these have led to a series of controlled clinical trials. Several pharmacological groups have organized phase I-III human clinical trials to test their drugs of interest: steroids (methylprednisolon, Tirilazard), gangliosides (GM-1, Sygen®), opiate receptor antagonists (naloxone), noncompetitive NMDA antagonists (dizocilpine, gacyclidine), the potassium channel blocker 4-aminopyridine (fampridine, Acorda Therapeutics), autologous cellular therapy (stimulated homologous macrophages, Proneuron) etc. (Jones et al., Faden et al., 1981, Bracken et al., 1990, Geisler et al., 1991, Hao et al., 1992, Rapalino et al., 1998, Knoller et al., 2005). Of these, methylprednisolon (MP) has been the only drug that resulted in significant improvement of motor and sensory functions not just in animal studies, but also in patients after SCI in the NASCIS-3 human trial. The effect is observed when a bolus injection of 30mg/kg body weight of

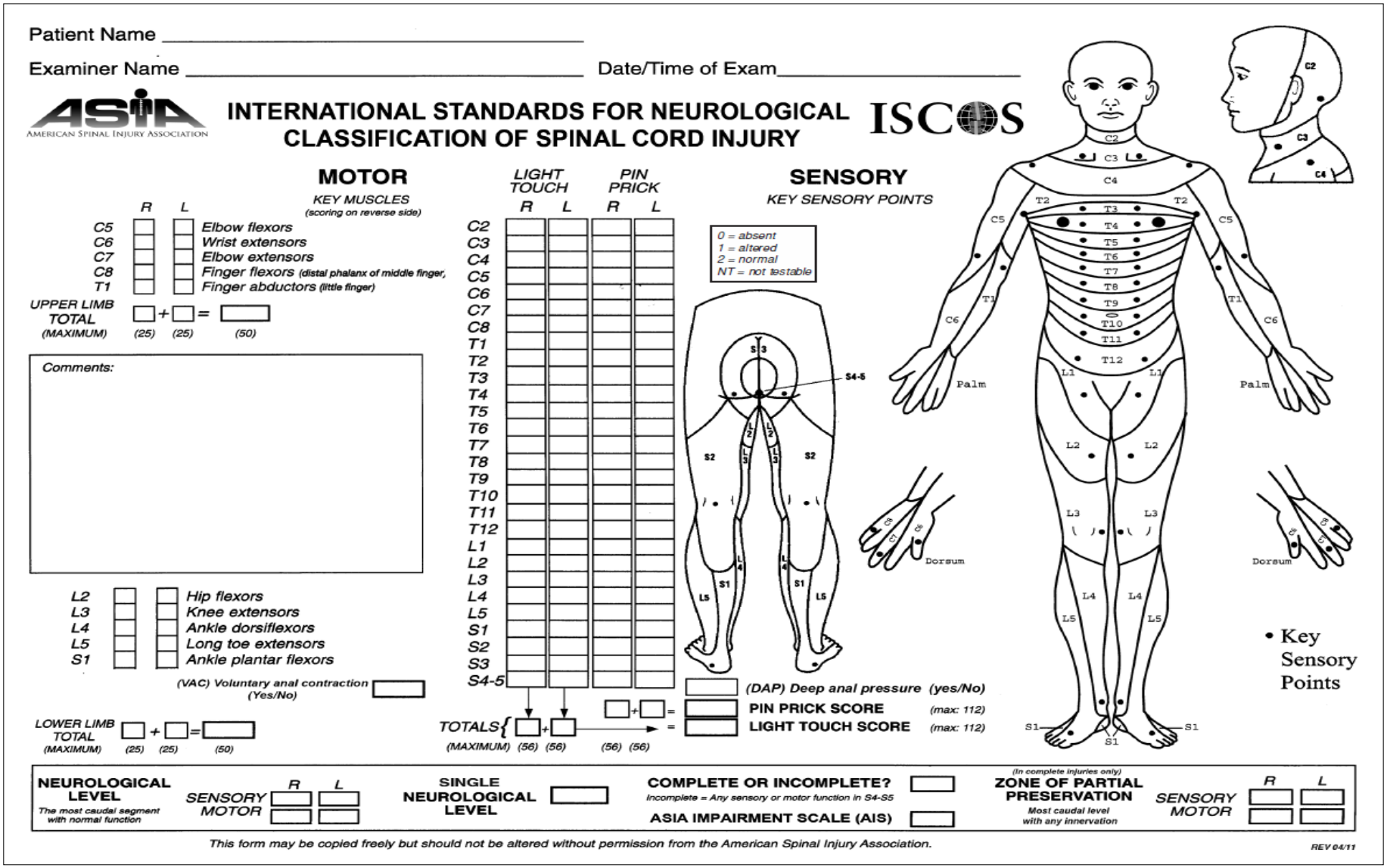

Table 2b. ASIA protocol

methylprednisolone initiated within 3-8 hours after the injury with (the therapeutic window), followed by an infusion of 0.9% NaCl over 45 minutes, followed by 5.4mg/kg/h of MP maintained for 48 hours (Bracken et al., 1990, Bracken et al., 1997, 2003, Vaquero et al., 2006). Interestingly, until now there is ongoing discussion about the mechanism of action of MP and also about the effectiveness of steroids after SCI, especially considering the possible side effects of steroids (sepsis, pneumonia and other infections) and the fact that many animal studies have failed to show a neuroprotective effect in the early phase of SCI (Bracken et al., 1990, Bartholdi and Schwab, 1995, Hurlbert, 2000, Vaquero et al., 2006). Nevertheless, MP remains the only drug that has been proven to have an effect if administered to patients with SCI. Generally, after a trauma that could possibly involve SCI, the common approach seeks to arrest or even reverse sensor and motor dysfunctions via the prevention of all active and passive movement of the spine, support of the cervical and lumbar lordoses, and limitation of head movement with a collar. All patients should be transferred to a specialized hospital able to handle trauma victims. Currently, the only standard possibility to treat patients with SCI is surgical intervention, high doses of MP and symptomatic therapy (control or management of urinary and cutaneous infections, pain, spasticity, bladder and bowel management, sexual and reproductive function) followed by rehabilitation. The second therapeutic phase deals with the consequences of SCI. Rehabilitative efforts aim to stabilize the current status and to train reflexes and residual circuits to achieve optimal living conditions for the patient who has a given deficit. In the future, a third treatment approach will be directed towards the enhancement of axonal regeneration (2003).

1.3.5. Experimental Models of SCI

In order to study the mechanisms underlying the processes that take place after injury as well as the effect of different therapeutic strategies, a proper and

reproducible experimental model should be used. In view of both ethical and economic issues, most research is performed using rodents, in particular rats and mice, which have a quite similar anatomy and course of spinal cord regeneration as that of humans. Moreover, their high resistance to infections makes them perfect candidates for experimental work. Currently, several traumatic models are used to study SCI. Hulsebosch suggested classifying damage to the SC into four types, according to morphological changes (Hulsebosch, 2002, Holtz A., 2010):

1. Cord maceration (extradural clip compression)
2. Laceration injury (hemisection, transection)
3. Contusion/compression injury (extradural/balloon compression lesion, weight drop technique etc.)
4. Solid cord injury (aortic occlusion)

Complete and dorsal hemisection, when the ipsilateral spinal tracts are surgically severed, are used either alone or in combined studies related to anatomical analysis of SC tracts, axonal regeneration, sprouting and transport by means of antero-/retrograde tracers, as well as to test the biocompatibility of synthetic materials etc. (Hejcl et al., Teng et al., 2002, Massey et al., 2006). Clinically, a hemisection of the SC is manifested as Brown-Séquard syndrome, in which ipsilateral hemiplegia and contralateral pain and temperature sensation deficits are found. In rodents this intervention causes relatively moderate functional deficits with no need for extra care in the postoperative period.

The more severe model of a stab SCI is surgical transections of the spinal cord with a complete and permanent lose of function below the level of the injury. This results in complete paraplegia or tetraplegia depending on the level of injury. Animals after a transaction of the SC require special care for 2-3 weeks post-injury in order to restore normal bladder function and to prevent urinary infections, autophagia etc. This model is useful in testing biomaterials (that could be grafted immediately in case of hemi-transections), evaluating the

regenerative effects of different therapeutic agents (e.g., stem cells, CSPG or NOGO), and behavioral testing of motor and sensory recovery.

There are also experimental models that mimic the symptoms of ischemic SCI. Ischemic SCI represents a serious complication associated with the transient cross-clamping of the descending thoracic or thoracoabdominal aorta. The symptoms are related to a selective loss of small inhibitory interneurons but with the continuing presence of ventral α-MN and supraspinal input. Depending on the duration of the ischemic interval, ischemic SCI might result in paraparesis or fully developed spastic plegia with or without a rigidity component (Taira and Marsala, 1996, Cizkova et al., 2007). This model presents a stable and powerful tool that is used to study the mechanisms underlying neural death, as well as to evaluate the efficacy of anatomical and functional regeneration of the spinal cord.

Contusive and compressive models of SCI have several advantages when compared to laceration/maceration techniques. They have very similar characteristics and time course to a majority of clinical cases, especially those in which the nervous tissue has been damaged by fragments of vertebras or intervertebral discs. The oldest contusive model of SCI is the "weight drop model", introduced by Allen. His kinetic model reflects the initial damage to the SC that takes place in humans (Allen, 1911). The same approach to SCI, but utilizing a more sophisticated tool, has been developed by researchers at New York and Ohio State Universities (Bresnahan et al., 1987, Gruner, 1992). Another model of SCI is a mechanical compression of the nervous tissue. A balloon-induced compression lesion deserves special attention because of its clinical relevance (Tarlov et al., 1953, Vanicky et al., 2001, Urdzikova et al., 2006). Another advantage of such a lesion is that there is no need to perform a laminectomy or to perforate the *dura mater*, resulting in glial scar formation only at the site of the balloon-induced compression itself. Two weeks after

injury, the centre of the lesion is dominated by necrosis, bleeding and tissue edema. Subsequently, pseudocysts form and the nervous tissue atrophies.

1.3.6. Regenerative Strategies

The adult mammalian spinal cord contains powerful inhibitory substances that prevent axonal growth and are vital under normal conditions. However, these same factors create a major obstacle for functional recovery after SCI. Regeneration of the SC is limited due to weak neuronal plasticity, an umbrella term referring to a variety of compensatory processes (spontaneous regeneration of affected axons, dendrites remodeling, changes in neuronal and synaptic strength etc.) that are taking place inside the spinal cord after the trauma in order to restore lost structures and function. (Carulli et al., Zorner and Schwab). In 1911 Santiago Ramón y Cajal showed for the first time that adult CNS neurons are able to regrow if they are provided with the permissive environment of a conditioned sciatic nerve (Ramón y Cajal, 1928). He showed that the inability of adult neurons to regenerate is due not just to the intrinsic differences between PNS and CNS neurons, but also to the damaged environment (Horner and Gage, 2000). Another breakthrough study in the field of neural regeneration was published in 1981 by David and Aguayo, who suggested that axonal regeneration might be achieved only by targeting both intrinsic cellular and CNS environmental factors (David and Aguayo, 1981). Since that time a vast increase has been achieved in our understanding of the cellular and molecular compounds that restrict/inhibit regenerative pathways, and this knowledge has become the basic platform for scientists in their efforts to regenerate the CNS.

Regeneration in the adult CNS requires a multi-step process. On one hand, regenerative strategies after SCI are focused either on the protection of the injured neurons and glial cells (by directly influencing the cells or the environment) or even on the partial replacement of lost cells (by the transplantation of embryonic stem cells, bone marrow mesenchymal stromal

cells, neural precursor cells, induced pluripotent cells, olfactory ensheathing cells etc.). On the other hand, following SCI or TBI, regenerating axons must overcome a number of structural or functional challenges that restrict and inhibit neurite outgrowth and the ability to re-establish functional neural connections, among them: the formation of a glial scar at the injury site, the synthesis of inhibitory proteoglycans (chondroitin sulphate proteoglycans), netrin-1, semaphorin 4D and ephrinB3, inflammation, alterations in the excitatory-inhibitory balance within local circuits, the instability of synaptic connections, the remyelination of spared nerve fibers, the replacement of lost cells and the activation of myelin-associated glycoproteins (MAG), oligodendrocytemyelin glycoprotein (OMgp), Nogo-A etc. (Dusart and Schwab, 1994, Rhodes and Fawcett, 2004, Schwab, 2004, Silver and Miller, 2004, Hofstetter et al., 2005, Fawcett, 2006, Bavelier et al., 2010). Several of these proteins are up-regulated by cells that form the glial scar, and the remainder are expressed by oligodendrocytes, which form the insulating myelin membrane (Carulli et al., 2005, Nash et al., 2009). Once contact is made, the axon needs to be re-myelinated and functional synapses need to form on the targeted neurons (Figure 4). Thus, targeting these components of the host tissue could have a significant regenerative effect.

1.3.6.1. The perineuronal net (PNN) is a layer of condensed pericellular matrix that aggregates and wraps around the soma and proximal dendrites of some neurons in the CNS (Kwok et al.). The condensed extracellular matrix of the PNN (Figure 5) is formed mostly around parvalbumin-positive neurons and is built around a hyaluronan backbone, to which several types of CSPG through cartilage link protein (Crtl1) are bound, and with tenascin-R binding to the CSPG core proteins: neurocan, aggrecan, phosphacan, brevican and neurocan (Carulli et al., Kwok et al., Dityatev et al., 2007, Kwok et al., 2008). Through specific interactions during the late period of development, known as the critical

period, when neuronal connections are formed in the mammalian CNS, these extracellular matrix molecules form large and stable aggregates (Carulli et al.).

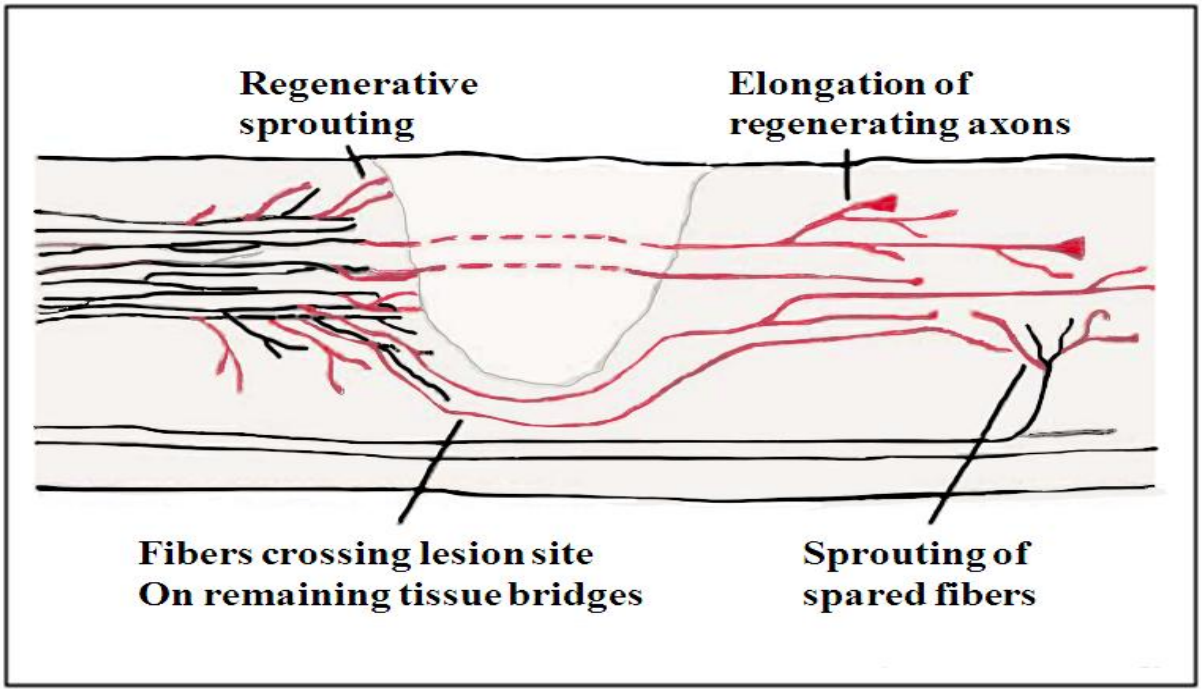

Figure 4. Axonal sprouting through the lesion after SCI (Adopted from Schwab M.E., 2004)

When the matrix is already organized it appears as a coat on the neuronal surface and controls the formation of synapses and connections that are important for plasticity. In particular, **chondroitin sulphate proteoglycans (CSPG)** have been shown to play an important role in axonal guidance during development and regeneration (Kwok et al., Bruckner et al., 2000, Kwok et al., 2008). On the other hand, in vitro and in vivo experiments have shown that after an injury to the spinal cord, CSPG and particularly chondroitin sulphate chains are up-regulated in activated astrocytes, leading to the restriction of anatomical as well as synaptic plasticity and axonal outgrowth into the site of injury (Lin et al., Smith-Thomas et al., 1994, Smith-Thomas et al., 1995, Chung et al., 2000).

One easy and convenient way of manipulating the ECM in both the glial scar and PNN in the CNS is by the application of **chondroitinase ABC (chaseABC)**. ChaseABC is a bacterial enzyme isolated from Proteus vulgaris, which digests the chondroitin sulfate chain into its basic disaccharide units, hence removing the glycosaminoglycan chains from the core proteins (Kwok et

al., Yamagata et al., 1968). It has been shown that if CSPG are digested after a trauma to soluble disaccharides with chaseABC, then the axons are able to regenerate and sprout through the glial scar more readily, suggesting that enzymatic digestion of CSPG can reactivate plasticity in the adult CNS (Carulli et al., Davies et al., 1997, Lemons et al., 1999, Pizzorusso et al., 2002, Galtrey et al., 2007).

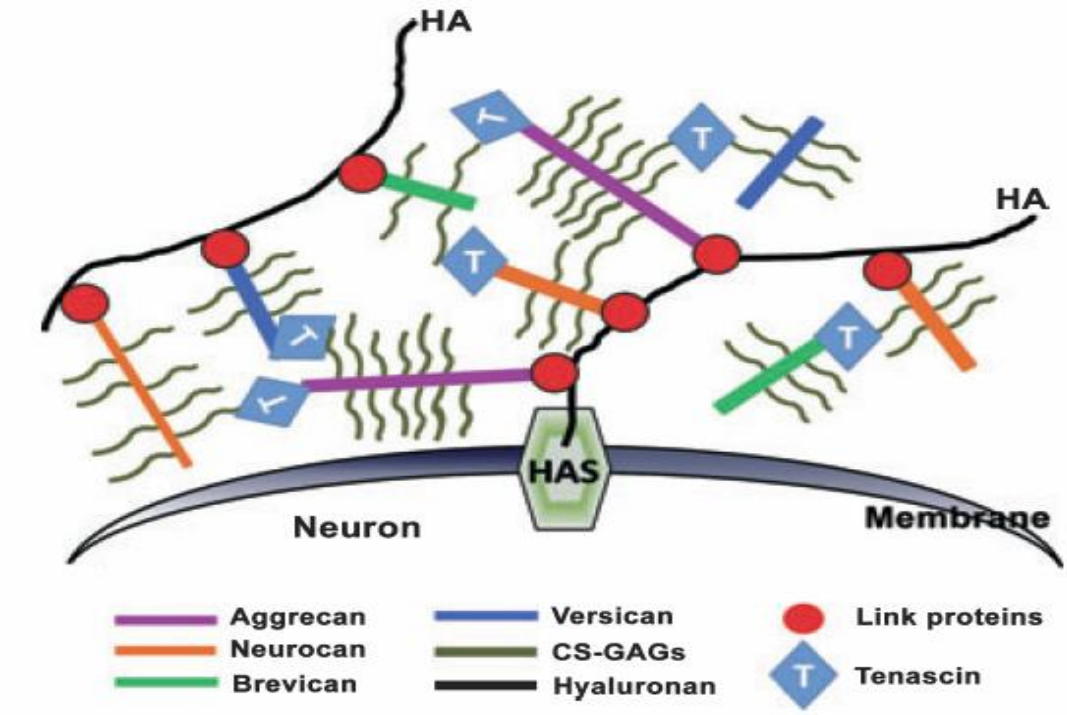

Figure 5. The schematic structure of an extracellular neuronal PNN (adopted from Kwok JC, J Neurochem. 2010)

Interestingly, chaseABC mono/combined therapy is effective not only in the early post-injury period, but also in the chronic stage after SCI. It has been shown that chaseABC therapy initiated 1 month after injury of the rubrospinal tract not only promoted the sustained rescue of neuronal atrophy, but also partially reduced the already established atrophy of the rubrospinal neuronal cell bodies (Carter et al.). Moreover, the sustained infusion of chaseABC into the chronically injured spinal cord followed by the application of NPCs promoted the integration and extensive migration of the cells within the host SC (Karimi-Abdolrezaee et al.). There is also evidence that the application of chaseABC

after SCI might also enhance remyelination (Siebert et al., 2011). However, there are several disadvantages to the use of chaseABC, one of which is that it loses its enzymatic activity rapidly at 37 °C, necessitating the use of repeated injections or local infusions for a period of days to weeks (Bradbury and Carter). Recently, it has been reported that this problem can be overcome by the creation of thermostabilized chaseABC, which showed significant differences in CSPG digestion, axonal growth and functional recovery following the sustained local release of thermostabilized chaseABC delivered by a hydrogel-microtube scaffold system versus a single injection of chaseABC (Lee et al.). Furthermore, no adverse effects have been noted after the intrathecal or intraspinal application of chaseABC in either rodents or in larger mammals such as cats, rabbits and pigs, thus making it a potential candidate for clinical trials in human (Bradbury and Carter, Olmarker et al., 1991, Olmarker et al., 1996, Tester and Howland, 2008).

1.3.6.2. Matrix metalloproteinases (MPP) are a family of endopeptidases, enzymes that are capable of breaking down the axon growth-restricting components in the CNS including CSPG, tenascin-C, brevican, versican and NOGO-66 (Belien et al., 1999, Walmsley et al., 2004, Yong, 2005, Pizzi and Crowe, 2007). In the developing CNS, MPPs create a fluid extracellular environment that could also play an important role in the regeneration of injured mammalian tissue. After a neurotrauma, MMPs are either secreted from cells (influx of different leukocyte subsets, microglia, neural and endothelial cells) or anchored to the plasma membrane. Various MPPs are expressed differently during CNS pathologies. Concerning spinal cord injury, Duchossoy and colleagues have shown that a hemisectioned rat spinal cord upregulates MMP-2 and MPP-9 production at the site of injury (Duchossoy et al., 2001). Functional recovery in the chronically injured spinal cord was evaluated in MPP-2 and MPP-9 knockout mice by Hsu, who found that MPP-2 deficient mice displayed a more severe glial scar, fewer serotonergic fibers caudal to the injury and

significantly reduced motor recovery when compared to wild-type animals; meanwhile, MPP-9 deficient mice exhibited significant locomotor recovery and the attenuation of neutrophil infiltration compared to wild-type animals (Duchossoy et al., 2001, Hsu et al., 2006, Hsu et al., 2008). Currently, the scant but suggestive data for MPPs (especially MPP-2, 9 and 12) may point to a promising therapeutic target during spinal cord regeneration. In vivo studies have shown that early and short term application (for only 3 days after the spinal cord injury) of the MPP inhibitor GM6001 improved neurological function in rodents (Noble et al., 2002).

1.3.6.3. Myelin-associated glycoproteins and Nogo are among the inhibitory molecules that have attracted great interest in recent years in the context of the restricted neuroplasticity and axonal regeneration observed after injury of the fiber tracts in the adult CNS. Only Nogo-A and its receptor (NgR1) have been shown to be strongly expressed in the nervous system and to play a crucial role in neurite growth and branching during CNS development, in growth-restricting function during maturation and in wiring stabilization in the adult CNS (Schwab, Oertle et al., 2003). It is expressed by differentiated oligodendrocytes and by developing neurons. The function of the other two isoforms with regard to neuroplasticity is unknown (Zorner and Schwab). Under pathological conditions such as a spinal cord lesion or a section of the corticospinal tracts in adult rats and primates, inhibition of Nogo-A with a Nogo-A-specific antibody leads to enhanced axonal regrowth and compensatory sprouting, accompanied by increased motor recovery (Bregman et al., 1995, Liebscher et al., 2005, Freund et al., 2006, Freund et al., 2007). Encouraged by the beneficial effects observed in *in vitro* and *in vivo* studies, a Phase I clinical trial was initiated in patients with acute spinal cord injuries utilizing a human Nogo-A antibody (ATI-355; Novartis) (ClinicalTrials.gov: NCT00406016). In this trial more than 50 subjects with SCI have been treated, showing excellent tolerance with no side effects ascribed to the anti-Nogo-A antibody. A phase II trail to test the efficacy

of anti-Nogo-A antibody in the treatment of severe paraplegic and tetraplegic patients is underway (Schwab, Zorner and Schwab). Interestingly, patients with amyotrophic lateral sclerosis have been shown to have increased Nogo-A expression in their muscles in the early stages of the disease, and this expression has been proposed to serve as a possible diagnostic marker for the disease (Jokic et al., 2005).

1.3.6.4. Stem cells are pluripotent cells with unlimited self-renewal capacities. They are able to differentiate into diverse specialized cell types, including neuronal and glial cell lineages (Nistor et al., 2005, Lee et al., 2007). Stem cells are classified by their source and the tissue they are typically generated from. Based on when they appear during the lifetime of the organism, they include: 1) human embryonic stem cells (hESC), which have been shown to possess pluripotent abilities as they give rise to all tissues in an organism; 2) somatic (adult-derived) stem cells, multipotent and found in different tissues in the fully developed organism and in umbilical cord blood as well; and 3) induced pluripotent stem (iPS) cells, recently discovered and capable of regaining there pluripotent properties after the artificial introduction of transcriptional factors into the somatic cell (Takahashi and Yamanaka, 2006). Considering the ability of stem cells to provide an enormous source of cells and to differentiate into glial and neuronal cells, they are excellent candidates to replace lost cells after SCI and to promote regeneration in neurodegenerative diseases; in addition, they are capable of long-term survival following transplantation. Currently, different kinds of stem cells have been used to promote regeneration after experimental SCI, including bone marrow mesenchymal stromal cells (BMSC), olfactory ensheathing cells (OEG), predifferentiated adipose-derived mesenchymal stromal cells, neural stem/progenitor cells, embryonic stem cells etc (Amemori et al., Arboleda et al., McDonald et al., 1999, Ogawa et al., 2002, Urdzikova et al., 2006, Parr et al., 2008). However, ethical considerations, a high risk of tumorigenesis and restricted access limit the clinical utility of fetal and

embryonic stem cells. However, Geron Corporation (Menlo Park, CA 94025 USA) has recently initiated a phase I clinical trial evaluating the use of hESC-derived oligodenrocyte progenitor cells (GRNOPC1) in patients with SCI (http://clinicaltrials.gov, #NCT01217008). Considering the above concerns, as well as the quite regular usage of bone marrow to treat hematological diseases, BMSC seem to be an ideal candidate for the cellular therapy of SCI and neurodegenerative diseases of the CNS.

1.3.6.5. Mesenchymal stromal cells (MSC) can be isolated by a relatively simple procedure. Currently, the properties of bone marrow-derived MSC (AMSC) are among the best characterized and these cells among the most widely used in clinical practice. However, MSC can also be isolated from alternative organs, the most promising of which are adipose-derived MSC isolated from fat tissue. After harvesting a patient's MSC are easily expanded and cultured. Extensive growth in culture makes it possible to obtain the required number of cells for transplantation and also to graft autologous cells, thus eliminating the risk of graft-versus-host disease (GVHD) and avoiding the use of cytostatics. Despite the tissue of origin, all MSC can differentiate *in vitro* into chondrocytes, osteocytes, muscle cells, adipocytes, or even neurons and glia (Prockop, 1997, Mezey et al., 2000, Krause, 2002). It has also now been demonstrated that the plasticity (ability of the cell to change its default fate) and tissue regenerative potential of BMSC may far exceed their use in hematopoietic diseases. The administration of MSC can induce the secretion of several growth factors by host cells (paracrine function), such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), neural growth factor (NGF), glia cell-line derived neurotrophic factor (GDNF) and IGF-1, that play a crucial role in neuroregeneration (Gu et al., Uccelli et al., Li et al., 2002, Zhang et al., 2004, Vercelli et al., 2008). Indeed, some scientists believe that AMSC are more suitable cells for allogenic transplants and tissue engineering as they retain a stem cell phenotype and mesenchymal pluripotency through higher passages

(over 25 passages) and they are easier to work with (Zhu et al., 2008). Transplanted BMSC have also been shown to migrate to the lesion site following induction by local signals. In addition, they are immunopotent, do not stimulate alloreactivity and escape lysis by cytotoxic T-cells and natural killer (NK)-cells (Le Blanc, 2003, Aggarwal and Pittenger, 2005, Urdzikova et al., 2006). In vivo experiments (performed in the Institute of Experimental Medicine in Prague and also by other groups) using different SCI models and different routes of BMSC administration, including in combination with biomaterials, revealed significant functional recovery (increased motor activity and sensation) of the paralyzed limbs, reduced cavity formation in the spinal cord and better axonal regrowth through the glial scar (Gu et al., Hejcl et al., Zeng et al., Ohta et al., 2004, Urdzikova et al., 2006, Sykova and Jendelova, 2007, Parr et al., 2008). The unique immunologic and survival properties of MSC, and increasing knowledge from *in vivo* studies, will facilitate the transfer of preclinical findings from bench to bedside.

1.3.6.6. Biomaterials that is, materials that are used and adapted for a medical application and thus intended to interact with a biological system - have become increasingly important in the development of drug delivery systems and tissue engineering approaches and can play key roles in overcoming the inherently insufficient protection, repair and regeneration of the nervous tissue (Orive et al., 2009). Many researchers are focusing their efforts on creating physical pathways for regenerating axons, especially after SCI. These include the creation of a mechanical scaffold from natural or newly synthesized materials that will provide a growth platform for host cells and guide the axons through the glial scar and posttraumatic pseudocysts to form new connections. Polymers and natural materials of many types and different combinations can be used for this purpose as well as to create drug vehicles capable of providing sustained delivery of potentially neuroprotective agents into an affected area such as

proteins, enzymes, antibodies, genes and oligonucleotides etc. Despite the lack of an "ideal material" to be used as a scaffold in order to promote the desired reparative processes after mammalian SCI, all candidates should possess several characteristics, as follows (Liu and Cao, 2007, Tabesh et al., 2009):

1) Biocompatibility (that is, neither cytotoxic nor systemically toxic).

2) Immunologically inactive.

3) Controlled biodegradability or bio-resorbability.

4) Possessing inter-connecting stable pores of appropriate size to promote integration, diffusion and vascularisation.

5) Mechanically similar to the extracellular environment, thus making the scaffold suitable for implantation into the intended site and permitting its fabrication in a variety of shapes and sizes.

6) Should not induce any adverse response including additional mechanical damage to the nervous tissue.

7) Should lessen glial scar formation while facilitating cell adhesion and axonal sprouting.

Up to now, several types of **natural polymers** have been successfully used in different experimental models of SCI. Collagen and fibronectin, as major protein components of the extracellular matrix, have been considered to be ideal scaffold materials by many scientists for nervous system repair. In vitro studies utilizing dorsal root ganglia (DRG) explants have shown glial cell migration and axonal growth on collagen/poly-epsilon-caprolactone nanofibers (Schnell et al., 2007). Injectable forms of fibrin and fibronectin have been shown to promote axonal growth and integration after acute injury to the spinal cord (King et al.). Suzuki has shown that grafting a frozen alginate after spinal cord transection in rats led to/resulted in the sprouting of myelinated and nonmyelinated fibers into the hydrogel with the formation of synapses between

the neurons located on both sides of the cavity (Suzuki et al., 1999, Kataoka et al., 2001, Suzuki et al., 2002).

Achievements of modern chemistry have made it possible to use **synthetic biomaterials** in regenerative medicine. Synthetic biomaterials, if compared with natural polymers, have been shown to possess several advantages when they are used as a scaffold component to treat SCI:

- Synthetic materials offer better control of their physical and mechanical properties, porosity and biodegradability of
- The possibility of combining different types and shapes of synthetic materials make them more suitable for use in nervous tissue regeneration
- Synthetic materials can be produced in large amounts
- It is easier to combine synthetic materials with other therapeutic strategies such as neurotrophins, stem cells, drugs, enzymes etc (Hejcl et al., Holan et al.).

The implantation of a porous poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA) hydrogel and tubes made of it into the transected spinal cord showed a significant improvement of locomotor function, good biocompatibility, neurofilament growth into the gel and decreased cytochrome oxidase C activity four weeks after the injury (Kubinova et al., Bakshi et al., 2004, Reynolds et al., 2008). Other synthetic hydrogels with biodegradable properties made of poly (lactic co-glycolic acid) (PLGA), poly(varepsilon-caprolactone fumarate) (PCLF), oligo(polyethylene glycol) fumarate (OPF) or positively charged OPF (OPF^+) were compared as scaffolds seeded with Schwann cells 1 month after a transection injury. Significantly greater axonal ingrowths was found into hydrogels made of PCLF and OPF^+ compared to that seen with PLGA hydrogels. OPF^+ polymers showed more centrally distributed axonal regeneration within the hydrogel channels, while other polymers (PLGA, PCLF and OPF) tended to show more evenly dispersed axons within the channels (Chen et al.). Nondegradable hydrogels, such as

PHPMA or PHEMA, have been reported to be well-tolerated and to provide therapeutic benefit in several preclinical studies on chronic SCI, and these materials are presently also undergoing evaluation in pre-clinical trials (Hejcl et al., Woerly et al., 2001a, Woerly et al., 2001b). An overview of recent advances in the application of synthetic materials, alone or in combination with stem cells/growth factors, to treat SCI is presented in Table 3 (Kubinova and Sykova, 2012).

1.3.6.7. Erythropoietin (EPO) and its analogues in the treatment of SCI. The cytokine erythropoietin (EPO) is a glycoprotein mediating cytoprotection in a variety of tissues, including the spinal cord, through the activation of multiple signaling pathways. Studies with recombinant human erythropoietin in animal models of traumatic spinal cord and ischemic injuries showed that, if administered immediately after injury, it inhibits lipid peroxidation and neuronal apoptosis, reduces inflammation, protects neurons against glutamate toxicity and restores vascular integrity (Morishita et al., 1997, Kontogeorgakos et al., 2009, Matis and Birbilis, 2009, Onose et al., 2009). It has been reported that the administration of EPO immediately after SCI facilitates the early recovery of function, as well as a remarkable reduction of lesion size along with improved locomotor recovery (Gorio et al., 2002, Vitellaro-Zuccarello et al., 2008). However, the risk of polycythemia, hyperviscosity syndrome and hypertension caused by the chronic administration of high doses of EPO in order to stimulate neuroprotection limits its usage. Carbamylated erythropoietin (CEPO) is a newly developed, distinct isoform that does not bind to the classical erythropoietin receptor, has no hematopoietic properties and has been shown to possess neuroprotective effects in animal models of SCI (Leist et al., 2004, Coleman et al., 2006, Mennini et al., 2006, Lapchak, 2008). A phase III clinical trial assessing the safety and efficacy of erythropoietin versus methylprednisolone in acute SCI has been initiated and is currently ongoing (http://clinicaltrials.gov, identifier # NCT00561067).

Table 3. Stem cell-seeded scaffolds for SCI treatment (adopted from (Kubinova and Sykova, 2012)

Cell type	Scaffold	Degradability	Additional compounds	Lesion type/behavioural study	Type of transplantation/ analysis	Described effect	Citation
SCs	PAN/PVC guidance channels	No	-	Transection/x	Immediate/4 weeks	Improved axonal myelination and regeneration of propriospinal and sensory axons.	(Xu et al., 1995b, Xu et al., 1997)
SCs	PAN/PVC guidance channels	No	BDNF, NT-3	Transection/x	Immediate/6 weeks	Improved regeneration of long descending axons.	(Xu et al., 1995a)
SCs	PAN/PVC guidance channels	No	Methylprednisolone	Transection/x	Immediate/6 weeks	Improved axonal myelination and axonal extension of long distant neurons.	(Chen et al., 1996)
SCs	PAN/PVC guidance channels	No	-	Hemisection/ x	Immediate/2, 4 weeks	Axonal regeneration and their re-entry into distal host tissue.	(Xu et al., 1999, Hsu and Xu, 2005)
SCs	PAN/PVC guidance channel	No	BDNF, NT-3 (intraspinal infusion)	Hemisection/ x	Immediate/4 weeks	Axons crossed the lesion and penetrated into the distal host spinal cord	(Bamber et al., 2001)
SCs	PAN/PVC guidance channel	No	GDNF	Hemisection/ x	Immediate/4 weeks	Synergistic effect of combined therapy on axonal regeneration and myelination.	(Iannotti et al., 2003)
SCs	PAN/PVC guidance channel	No	ChaseABC	Hemisection/ x	Immediate/4 weeks	Improved axonal growth across the graft-host interface.	(Chau et al., 2004)
SCs	poly-(beta-hydroxybutyrate) tubular conduit	Yes	-	Hemisection/ x	Immediate/4 weeks	Axonal regeneration of long spinal tracks.	(Novikova et al., 2008)
SCs	PLGA multichannel scaffold	Yes	-	Transection/x	Immediate/1 or 2 months	Facilitation of axonal regeneration.	(Moore et al., 2006, Chen et al., 2009a)
SCs	PLGA multichannel scaffold	Yes	-	Transection/x	Immediate /1,2 and 3 months	Scaffolds with 450um diameter channels promoted greater axonal regeneration than 660 um diameter channels.	(Krych et al., 2009)
SCs	PLGA, PCLF, OPF & OPF+	Yes	-	Transection/-	Immediate /4 weeks	Comparison of the regenerative capacity of polymer scaffolds with different stiffness.	(Chen et al., 2011)

	multichannel scaffold						
SCs	PLA tube	Yes	-	Transection/x	Immediate /1, 2 and 4 months	Collapse of the tube reversed the regenerative effect.	(Oudega et al., 2001)
SCs and SCs producing BDNF and NT-3	Freeze-dried PLA macroporous tubular scaffold	Yes	-	Transection/-	Immediate /1, 2 and 6 weeks	Limited axonal ingrowth into the scaffold. Low cell survival. No significant differences in BBB score between cell groups.	(Hurtado et al., 2006)
SCs or NSCs	PLGA multi-channel scaffold	Yes	-	Transection/-	Immediate/4 weeks	Greater axonal regeneration in the SC-treated group. No significant differences in BBB score.	(Olson et al., 2009)
SCs and NSC	PLGA	Yes	-	Hemisection/+	Immediate/1, 2, 3 and 6 months	Co-transplantation promoted functional recovery.	(Chen et al., 2010)
SCs or NPC	SA peptide nanofiber	Yes	-	Hemisection/x	Immediate/6 weeks	Survival of transplanted cells. Greater axonal regeneration in the SC-treated group.	(Guo et al., 2007)
Peripheral nerve graft	Chitosan guidance channels	Yes	-	Chronic clip SCI/-	Immediate/14 weeks	Axonal regeneration and myelination. No functional improvement.	(Nomura et al., 2008a)
MSC	PHPMA-RGD macroporous hydrogel	No	-	Chronic compression lesion/+	Delayed/6 months	Prevention of tissue atrophy, axonal regeneration and significant functional improvement with combined treatment.	(Hejcl et al., 2010)
MSC	PHEMA hydrogel	No	-	Hemisection/x	Immediate/6 weeks	Axonal ingrowth into the scaffold. In vivo MRI visualization of superparamagnetic iron-oxide nanoparticles-labelled MSC.	(Sykova and Jendelova, 2005)
MSC	Serotonin modified PHEMA	No	-	Hemisection/x	Immediate/4 weeks	Transplanted cells survived and migrated out of the scaffold into the spinal cord tissue.	(Kozubenko et al., 2010)
MSC	PLGA/small intestine mucosa	Yes	BDNF	Transection/+	Immediate/4 and 8 weeks	Axonal regeneration and significant functional improvement with combined treatment.	(Kang et al., 2011)
MSC	Gelatine sponge in PLGA tube	Yes	-	Transection/x	Immediate/1 and 8 weeks	Reduced inflammation, promoted angiogenesis and reduced cavity formation	(Zeng et al.)

MSC	Fibrin gel	Yes	-	Hemisection/ +	Immediate/4 weeks	Significant functionnal improvement.	(Itosaka et al., 2009)
MSC	PLGA	Yes	-	Transection/x	Immediate/10 weeks	Development of complete transection model.	(Min et al., 2011)
NSC /NSC overexpressing NT-3	PCL	Yes	ChaseABC	Hemisection/ +	Immediate/9 weeks	Enhanced axonal regeneration and significant functional improvement with combined treatment.	(Hwang et al.)
NSC	PLGA with complex guidance architecture	Yes	-	Hemisection/ +	Immediate/2 and 4, 12 months	Enhanced axonal regeneration and significant long term functional improvement.	(Teng et al., 2002)
NSC	PLGA with complex guidance architecture	Yes	Peroxynitrite scavengers	Hemisection/ x	Immediate/24 hours	Anti-apoptotic effect of free radical scavengers on grafted NSCs.	(Yu et al., 2009)
NSC	Chitosan guidance channels	Yes	-	Transection/-	Immediate/14 weeks	Survival of transplanted cells and axonal regeneration. No functional improvement.	(Nomura et al., 2008b)
mESC-NP	Fibrin gel	Yes	NT-3, PDGF	Hemisection/ x	Delayed/2 weeks	Enhanced survival and neural differentiation of neural progenitor cells with combined treatment.	(Johnson et al., 2010)
hESC-NP	Collagen	Yes	-	Hemisection/ +	Delayed/5 weeks	Neural and glial *in vivo* differentiation, functional improvement.	(Hatami et al., 2009)
Astrocytes	Collagen gel	Yes	-	Hemisection/ +	Immediate/4 weeks	Increased axonal ingrowth. Mild functional improvement.	(Joosten et al., 2004)

Abbreviations: "x"- behavioral studies were not performed;"+"- functional improvements; "-" no functional improvements; SC- Schwann cells.

1.4. AMYOTROPHIC LATERAL SCLEROSIS (ALS)

1.4.1. Epidemiology of ALS

Amyotrophic lateral sclerosis (also called Charcot, motor neuron (MND) or Lou Gehrig's disease) is a devastating, progressive neurodegenerative disease that affects motoneurones (MN) in the brain and the spinal cord. ALS was described for the first time in 1848 by Aran, who reported 11 cases of the malady, including a 43-year-old man who presented with focal wasting and paresis of the upper extremities, weakness and cramps and died within 2 years (Aran, 1848). Aran suggested that the disease had been inherited from the man's parents, but in the year 1873 Jean-Marie Charcot reported that ALS was never inherited and that was the main reason for delineating ALS from muscular atrophy (Charcot, 1881). The view that ALS is rarely connected with family history persisted for almost one hundred years before the discovery of a number of genes associated with both familial (FALS) and sporadic ALS (SALS). Currently, it is regarded that around 10-23 percent of all cases of ALS have a family history, whereas the rest are sporadic (Andersen and Al-Chalabi, Bento-Abreu et al., van Es et al.). Nowadays, mutations in at least 15 genes have been found to cause FALS, with the most common being mutations in the superoxide dismutase 1(SOD1) gene, followed by *FUS, TARDBP, Senataxin, Angiogenin, Ubiquilin 2, Alsin, SIGMAR1* etc. (Table 4) (Chen et al., Elden et al., Orlacchio et al., Rosen et al., 1993, Hadano et al., 2001, Hand et al., 2002, Sapp et al., 2003, Chen et al., 2004, Nishimura et al., 2004, Gitcho et al., 2008, Kabashi et al., 2008, Sreedharan et al., 2008, Chow et al., 2009, van Es et al., 2009, Vance et al., 2009). FALS is usually inherited in an autosomal dominant way, but recessive and even X-linked forms also exist (Valdmanis et al., 2009). Despite their different genetic background, SALS and FALS are clinically

indistinguishable. Most commonly, the disease strikes people between the ages of 40 and 70, and its incidence declines thereafter. This feature in is contrast to other neurodegenerative maladies, such as Parkinson's and Alzheimer's diseases, and suggests that ALS is not a disease of aging, but rather a disease for which age is one of a number of risk factors. Its incidence of about 1-2 per 100 000 individuals is fairly uniform, except for a few high incidence foci, such as the Kii peninsula of Honshu island and Guam (Kuzuhara and Kokubo, 2005, Steele, 2005).

Locus (chromosome)	Gene	Protein length (amino acids)	Number of mutations	Analysis type (initial)	Modes of inheritance	Phenotypic MND variants	Other features*	References
ALS1 (21q22.1)	*SOD1*	153×2	166	Linkage	Dominant–cp Dominant–icp Recessive *De novo* mutation	ALS PMA PBP (rare) BFA (rare)	Cognitive impairment (rare) Cerebellar ataxia Autonomic dysfunction (rare) FTD (rare)	Rosen *et al.* (1993)[31]
ALS2 (2q33.2)	*ALS2*	1,657	19	Linkage	Recessive	Juvenile PLS, juvenile ALS or infantile HSP	Unknown	Hadano *et al.* (2001)[42]
ALS3 (18q21)	Not identified	Unknown	None	Linkage	Dominant	ALS	NA	Hand *et al.* (2002)[122]
ALS4 (9q34)	*SETX*	2,677	9	Linkage	Dominant	ALS	AOA2, cerebellar ataxia, motor neuropathy‡	Chen *et al.* (2004)[43]
ALS5 (15q21.1)	*SPG11*	2,443	12	Linkage	Recessive	Juvenile ALS	NA	Orlacchio *et al.* (2010)[123]
ALS6 (16q11.2)	*FUS*	526	42	Linkage Candidate	Dominant–cp Dominant–icp *De novo* mutations Recessive	ALS ALS–FTD	Parkinsonism FTD	Vance *et al.* (2009)[60] Kwiatkowski *et al.* (2009)[61]
ALS7 (20p13)	Not identified	Unknown	None	Linkage	Dominant	ALS	NA	Sapp *et al.* (2003)[124]
ALS8 (20q13.3)	*VAPB*	99	3	Linkage	Dominant	ALS, PBP or PMA	Unknown	Nishimura *et al.* (2004)[66]
ALS9 (14q11.2)	*ANG*	147	17	Candidate	Dominant–cp Dominant–icp?	ALS PBP or ALS–FTD	Parkinsonism	Chen *et al.* (2010)[69]
ALS10 (1p36.2)	*TARDBP*	414	44	Candidate Linkage	Dominant–cp Dominant–icp Recessive (rare)	ALS ALS–FTD	PSP, PD FTD Chorea	Gitcho *et al.* (2008)[47] Sreedharan *et al.* (2008)[48] Kabashi *et al.* (2008)[49]
ALS11 (6q21)	*FIG4*	907	10	Candidate gene	Dominant	ALS PLS	CMT4J‡ Cognitive impairment	Chow *et al.* (2009)[110]
ALS12 (10p15–p14)	*OPTN*	577	5	Homozygosity mapping	Dominant–cp Recessive	ALS	POAG‡	van Es *et al.* (2009)[71]
ALS13 (12q24)	*ATXN2*	1,313	6 (intermediate length)	Candidate gene	Dominant	ALS	NA	Elden *et al.* (2010)[75]

Table 4. ALS-associated genes, adopted from Andersen (Andersen and Al-Chalabi)

ALS is not a rare disease, and its lifetime risk of development approaches 1/400-1/700 with a somewhat more frequent occurrence in men than in women (male to female ratio is ≈ 1.5) (Bento-Abreu et al., Johnston et al., 2006).

1.4.2. Pathophysiology of ALS

As mentioned earlier, most ALS cases start sporadically. Only recently clues have been discovered that help to identify the molecular and cellular mechanisms of ALS. The discovery of mutations in a number of genes related to FALS and the creation of transgenic (Tg) animal models targeting those genes have improved our understanding of the molecular pathways underlying ALS. In 1993, Rosen showed for the first time that mutations in the *SOD1* gene located on chromosome 21q are the most common cause of FALS; the corresponding protein catalyzes the conversion of superoxide free radicals to hydrogen peroxide, which can be further detoxified to water and oxygen by catalase (Bento-Abreu et al., Rosen et al., 1993). A deficit of the SOD1 enzyme leads to changes in redox-sensitive signaling and to the oxidative damage of lipids, proteins and DNA, also called **oxidative stress** (Rao and Weiss, 2004). Various cell types, especially astrocytes and microglia, are implicated in MN dysfunction and death (**Figure 6**) via the development of glutamate-caused **excitotoxicity**, protein misfolding and aggregation (**foldopathy**), altered axonal transport (**dying-back-neuropathy or axonopathy**), **RNA-processing defect** (TARDBP and FUS genes), **mitochondrial impairment** (inner mitochondrial membrane defect, vacuolated cristae), **endoplasmic reticulum (ER) stress** etc. (Duffy et al., Ferraiuolo et al., Dion et al., 2009).

A generally accepted mechanism contributing to the development of ALS is oxidative damage of excitatory amino acid transporter 2 (EAAT2) located on astrocytes, which enables glutamate transport out of the synaptic cleft, thus maintaining a non-toxic concentration of glutamate (Barber and Shaw). Once

EAAT2 does not function properly, a dramatic increase of glutamate inside the synaptic cleft occurs, leading to overstimulation of the calcium-permeable a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type of glutamate receptor, which causes calcium influx and the **excitotoxicity** of MNs (Van den Bosch, 2006, Van Den Bosch et al., 2006).

Considering that spinal MN do not express calcium-binding proteins such as parvalbumin and calbindin, an increase of intracellular calcium causes an overload and depolarization of mitochondria, followed by the generation of oxygen species, leading to the inhibition of glutamate uptake in the neighboring astrocytes, thus establishing a vicious circle (Bento-Abreu et al., Carriedo et al., 2000, Rao et al., 2003). Even more, it has been shown that cultures of astrocytes expressing mSOD1 kill spinal primary and embryonic mouse stem cell-derived motor neurons, triggered by soluble toxic factors through a BAX-dependent mechanism, but do not cause the death of spinal GABAergic or dorsal root ganglion neurons (Nagai et al., 2007). Motoneuronal cell death in ALS also involves the activation of caspases and apoptosis (BCL-2 and BAX genes), and damage to mitochondrial function is likely to contribute to this process (Sathasivam et al., 2005). Degeneration of the skeletal muscles' and motoneurons' mitochondria also leads to the accumulation of large, membrane-bound vacuoles (Wong et al., 1995, Wiedemann et al., 1998, Petrozzi et al., 2007). However, the various mechanisms of mitochondrial involvement are not mutually exclusive and may interact and cooperate. Impaired mitochondrial function pertains not only to defects in energy production, but also to mitochondrial dynamics, communication with other organelles, the activation of the mitochondrial apoptotic pathway, and turnover (see below).

Transgenic animals for human SOD1 develop selective MN degeneration due to a toxic gain of function (Gurney et al., 1994, Boillee et al., 2006b). The generally

accepted hypothesis on the pathobiology of mutant SOD1 (mSOD1) relates to its propensity to aggregate, resulting from the failure of complex chaperone systems to fold proteins normally during their synthesis (Shaw and Valentine, 2007). Under normal conditions these misfolded proteins shift from the ER to the cytosol, where they are degraded by the proteasome system. Mutant SOD1 protein has been shown to aggregate in the mitochondrial membranes and ER and inhibit derlin-1, the protein that transports proteins destined to be degraded from the ER to the cytosol, thus inducing **ER stress** and the vulnerability of MNs. Mutant SOD1 forms insoluble aggregates that could directly damage the mitochondrion through swelling, with the expansion and increased permeability of the outer membrane and intermembrane space, leading to the release of cytochrome C (CytC) and caspase activation; inhibition of the translocator outer membrane (TOM) complex, preventing mitochondrial protein import; and aberrant interactions with mitochondrial proteins such as BCL2. Aggregates of mutant SOD1 and BCL2 are found specifically in the spinal cord, which might relate to the motor neuronal specificity of mSOD1 phenotypes (Figure7). Mutation of the vesicle-associated membrane protein B gene (VAPB), also known as synaptobrevin-associated protein B, is implicated in ER to Golgi transport, as well as in axonal transport that causes the development of classic ALS symptoms. (Bento-Abreu et al., Pasinelli and Brown, 2006, Nishitoh et al., 2008, Kanekura et al., 2009). Israelson has recently established a direct link between misfolded mSOD1 and mitochondrial dysfunction: he showed that mSOD1 binds directly to the voltage-dependant anion channel (VDAC-1) at the outer mitochondrial membrane, leading to diminished survival and accelerating paralysis in $SOD1^{G37R}$ mice (Israelson et al., 2010).

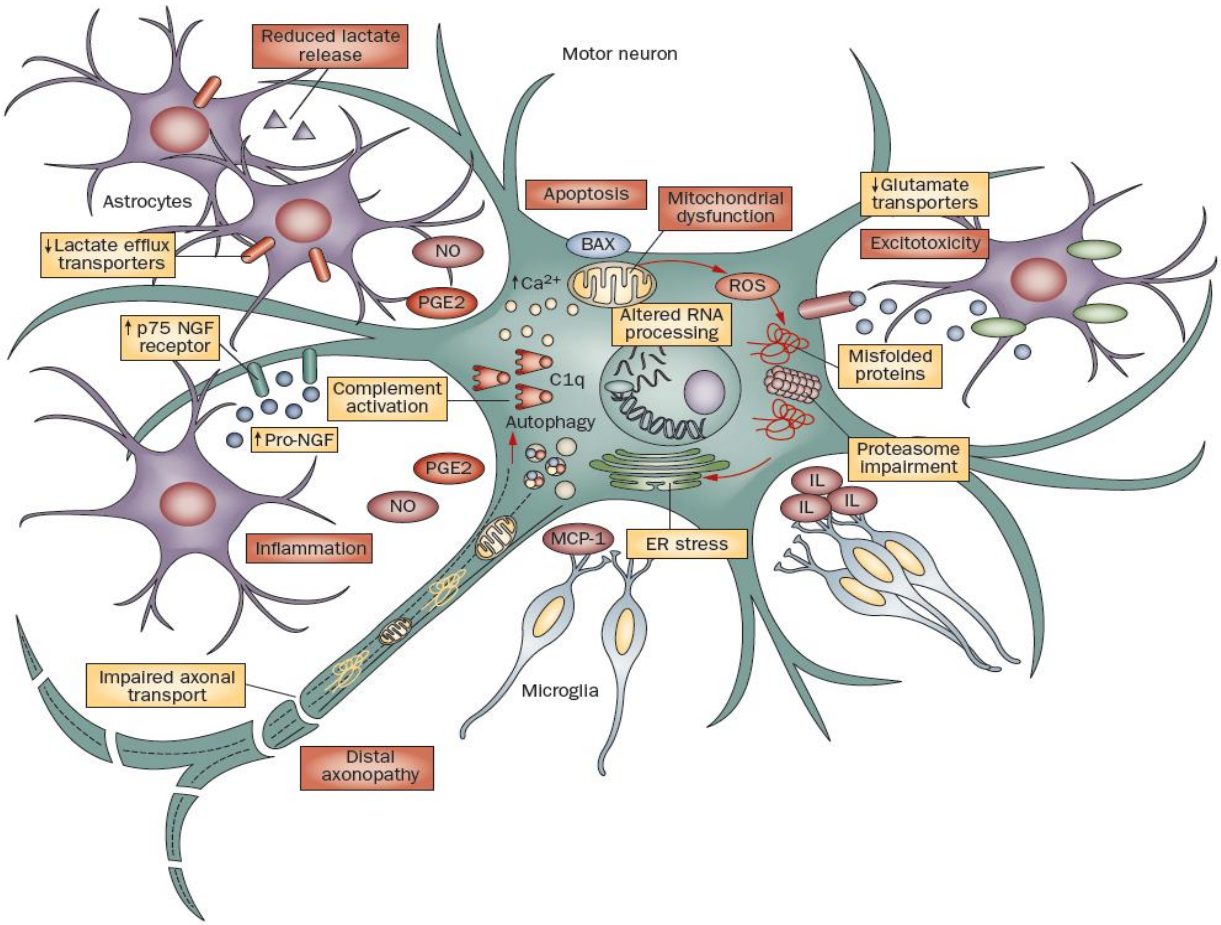

Figure 6. ***Molecular mechanisms of motor neuron injury in ALS, adopted from (Ferraiuolo et al.)***

MN are among the most asymmetric cells in nature, extending axons in humans that can be more than a meter in length. This structure demands active **axonal transport** between the soma and an axon, forming a neuromuscular junction (NMJ) in order to support normal cellular function. Motoneuronal organelles such as lysosomes, peroxisomes, and mitochondria are not positioned statically within cells but are transported on cytoskeletal elements, that is, microtubules and actin cables in association with intermediate filaments. Short-range movement on actin cables requires myosin motors, whereas long-range movement on microtubules requires two other types of motors: dynein/dynactin for retrograde transport and kinesins for anterograde transport (LaMonte et al., 2002). When an inappropriate

accumulation of light heterodymers of neurofilaments (NF-L) occurs, it perturbs axonal transport that subsequently leads to the vulnerability of MNs, especially those possessing large caliber myelinated axons (5µm) (Kawamura et al., 1981, Frey et al., 2000, Fischer et al., 2004, Boillee et al., 2006a).

The exact mechanism is not yet fully understood, but there is evidence of mSOD-induced damage to mitochondria and reduced ATP supply to molecular motors (De Vos et al., 2008). Interestingly, the connection to the muscle at the NMJ is lost long before MN degeneration or death and even before the initiation of symptoms due to a deficiency of motor neurotrophic factors, thus supporting the hypothesis that ALS is a **dying-back-axonopathy** (Fischer et al., 2004). The above observations reveal a remarkable potential connection between the sporadic and familial forms of the disease.

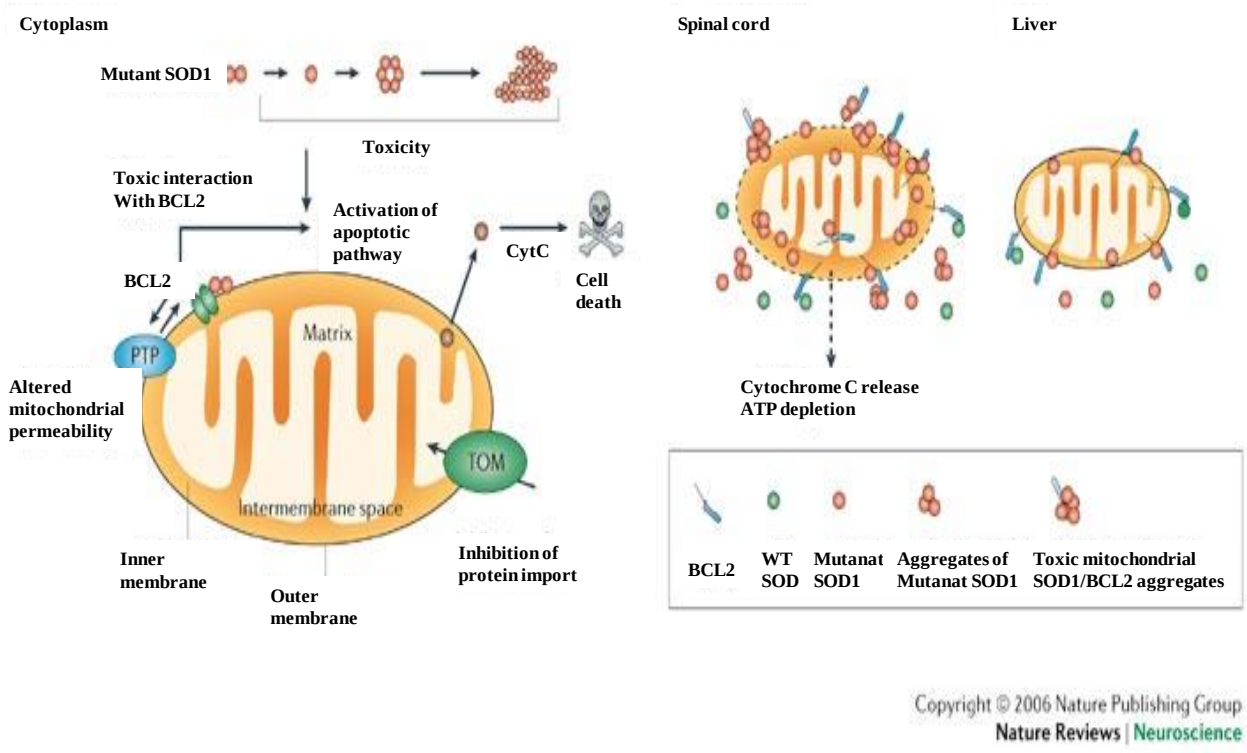

Figure 7. The mitochondrion as a target of mutant SOD1 (Pasinelli and Brown, 2006)

Another emerging theme has to do with the RNA processing encountered in FALS due to mutations in the *TARDBP, FUS/TLS* and optineurin (*OPTN*) genes (Maruyama et al., 2010). *TARDBP* is a gene encoding a TAR DNA-binding protein 43 (**TDP-43**), which only recently has been associated with ALS. Under normal conditions TDP-43 is more abundantly present in the nucleus than in the cytoplasm and is implicated in multiple aspects of RNA processing, including transcriptional regulation, alternative splicing and microRNA (miRNA) processing. An impairment of mitochondrial trafficking and maldistribution, with an excess in the cell bodies of MN and a paucity in distal motor axon terminals, have been shown in humans and in transgenic mice expressing human TARDBP (Schon and Przedborski, Shan et al., De Vos et al., 2007, Sasaki and Iwata, 2007). In about 4 percent of FALS pedigrees, 1.5 percent of SALS patients as well as in cases of frontotemporal lobar degeneration (FTLD), it has been shown that TDP-43 is the major protein constituent of skein-like ubiquitinated inclusions in the cytoplasm of neuronal and glial cells, while it is attenuated or absent in the nucleus (Neumann et al., 2006, Sreedharan et al., 2008). It seems that the cytoplasmic redistribution of TDP-43 plays an important role and is an early pathogenic event in both familial and sporadic ALS (Ferraiuolo et al., Giordana et al.).

A further missense mutation in the gene encoding **FUS/TLS** (fused in sarcoma/translocated in liposarcoma) has been reported recently, providing more evidence for the central role of proteins involved in RNA processing (Vance et al., 2009). The FUS/TLS gene is located in a region on chromosome 16 previously associated with ALS6 (Ruddy et al., 2003). Similarly as TDP-43, FUS/TLS is involved in transcriptional regulation, the maintenance of genomic stability, synaptic plasticity, pre-mRNA splicing as well as in the export of fully processed mRNA to the cytoplasm and thus shuttles between the nucleus and the cytoplasm

(Bento-Abreu et al., Zinszner et al., 1997, Fujii et al., 2005). Mutations in FUS have been identified in 4-5 percent of ALS6 families and in some SALS cases (Kwiatkowski et al., 2009, Corrado et al., 2010, Deng et al., 2010, Ticozzi et al., 2011). Despite their similar functions inside the cell, the absence of TDP-43 inclusions in mutant FUS-ALS cases implies that the FUS disease pathway is independent of TDP-43 aggregation (Vance et al., 2009).

1.4.3. Diagnosis of ALS

The clinical features of ALS consist of progressive neurological deteriorations that reflect the impairment and subsequent loss of muscle functions, resulting from the death of motor neurons in the corticospinal tract, brain stem or the ventral horns of the spinal cord. The disease type and course is often classified by the site of onset and often reveals a combination of upper and lower MN features. The majority of cases (65%) present with limb symptoms (weakness, variable wasting of affected muscles, stiffness, fasciculations), 30% display bulbar dysfunction (in the form of dysarthria, dysphonia, dysphagia, fasciculating tongue) and the remaining 5% of patients have respiratory-onset disease (difficulties in breathing, coughing) (Hardiman et al.).

To date, we do not have a definitive test or examination for the diagnosis of ALS. Clinical diagnosis is based mostly on the symptoms presented, and additionally on negative laboratory tests and imaging studies that exclude other *MNDs* [primary lateral sclerosis, progressive spinal muscular atrophy and progressive bulbar palsy], *hereditary* (spinobulbar muscular atrophy, HD, hereditary spastic paraparesis etc.), *metabolic* (hyperthyroidism, heavy metal intoxication etc.), *immuno-inflammatory* (multiple sclerosis, myasthenia gravis, polymyositis, paraneoplastic disorders etc.) and *neurodegenerative pathologies*

(PD, progressive supranuclear palsy etc.) and *structural disorders* (tumors, syringomyelia etc.).

The loss of lower MNs can be identified by means of electromyography, where fasciculations, fibrillation potentials and positive sharp waves are recorded (Hardiman et al., Eisen, 2001). During the early stages it is hard to make a final diagnosis due to the high number of symptomatic uncertainties, thus the majority of patients are diagnosed with ALS only after 9-15 months from the initial signs. In order to speed up and standardize the diagnosis of ALS, El Escorial diagnostic criteria were proposed by the World Federation of Neurology (Brooks, 1994). Subsequently, these criteria were revised in light of laboratory testing, electrophysiological and neuropathological examinations for research and clinical trials with the House-Awaji-Shima diagnostic algorithm, see Figure 8 (Schrooten et al., Miller et al., 1999, de Carvalho et al., 2008).

Table 2 | Criteria for diagnosis of ALS

Criteria	Definite ALS	Probable ALS	Possible ALS	Suspected ALS
El Escorial criteria, 1994[51]	Upper and lower motor neuron signs in three regions	Upper and lower motor neuron signs in at least two regions, with upper motor neuron signs rostral to lower motor neuron signs	Upper and lower motor neuron signs in one region, upper motor neuron signs alone in two or more regions, or lower motor neuron signs rostral to upper motor neuron signs	Lower motor neuron signs only, in two or more regions
Revised Airlie House criteria, 1998[52] (incorporating the Awaji–Shima criteria, 2008[53])*	Clinical or electrophysiological evidence, demonstrated by the presence of upper and lower motor neuron signs in the bulbar region and at least two spinal regions, or the presence of upper and lower motor neuron signs in three spinal regions	Clinical or electrophysiological evidence, demonstrated by upper and lower motor neuron signs in at least two spinal regions, with some upper motor neuron signs necessarily rostral to the lower motor neuron signs	Clinical or electrophysiological signs of upper and lower motor neuron dysfunction in only one region, or upper motor neuron signs alone in two or more regions, or lower motor neuron signs rostral to upper motor neuron signs	NA

*These revised recommendations emphasize the equivalence of clinical and electrophysiological tests in establishing neurogenic changes in bodily regions.
Abbreviations: ALS, amyotrophic lateral sclerosis; NA, not applicable.

Figure 8. Criteria for the diagnosis of ALS (Hardiman et al.)

However, the most widely used diagnostic scale that is currently used by doctors is The Revised ALS Functional Rating Scale (ALSFS-R, Figure 9). This scale evaluates a patient's bulbar and limb symptoms, as well as mobility and respiratory function, on a scale from 0 to 48 points, where 0 and 48 correspond to total disability and normal function, respectively. A decline in the ALSFRS-R score is a predictor of reduced survival (Cedarbaum et al., 1999, Traynor et al., 2004).

Figure 9. The ALS Functional Rating Scale-Revised (ALSFRS-R) (Cedarbaum et al., 1999)

1. Speech
4 Normal speech processes
3 Detectable speech disturbance
2 Intelligible with repeating
1 Speech combined with nonvocal communication
0 Loss of useful speech

2. Salivation
4 Normal
3 Slight but definite excess of saliva in mouth; may have nighttime drooling
2 Moderately excessive saliva; may have minimal drooling
1 Marked excess of saliva with some drooling
0 Marked drooling; requires constant tissue or handkerchief

3. Swallowing
4 Normal eating habits
3 Early eating problems—occasional choking
2 Dietary consistency changes
1 Needs supplemental tube feeding
0 NPO (exclusively parenteral or enteral feeding)

4. Handwriting
4 Normal
3 Slow or sloppy; all words legible
2 Not all words are legible
1 Able to grip pen but unable to write
0 Unable to grip pen

5a. Cutting food and handling utensils (patients without gastrostomy)?
4 Normal
3 Somewhat slow and clumsy, but no help needed
2 Can cut most foods, although clumsy and slow; some help needed
1 Food must be cut by someone, but can still feed slowly
0 Needs to be fed

5b. Cutting food and handling utensils (alternate scale for patients with gastrostomy)?
4 Normal
3 Clumsy but able to perform all manipulations independently
2 Some help needed with closures and fasteners
1 Provides minimal assistance to caregiver
0 Unable to perform any aspect of task

6. Dressing and hygiene
4 Normal function
3 Independent and complete self-care with effort or decreased efficiency
2 Intermittent assistance or substitute methods
1 Needs attendant for self-care
0 Total dependence

7. Turning in bed and adjusting bed clothes
4 Normal
3 Somewhat slow and clumsy, but no help needed
2 Can turn alone or adjust sheets, but with great difficulty
1 Can initiate, but not turn or adjust sheets alone

0 Helpless

8. Walking
4 Normal
3 Early ambulation difficulties
2 Walks with assistance
1 Nonambulatory functional movement
0 No purposeful leg movement

9. Climbing stairs
4 Normal
3 Slow
2 Mild unsteadiness or fatigue
1 Needs assistance
0 Cannot do

10. Dyspnea (new)
4 None
3 Occurs when walking
2 Occurs with one or more of the following: eating, bathing, dressing (ADL)
1 Occurs at rest, difficulty breathing when either sitting or lying
0 Significant difficulty, considering using mechanical respiratory support

11. Orthopnea (new)
4 None
3 Some difficulty sleeping at night due to shortness of breath, does not routinely use more than two pillows
2 Needs extra pillows in order to sleep (>2)
1 Can only sleep sitting up
0 Unable to sleep

12. Respiratory insufficiency (new)
4 None
3 Intermittent use of BiPAP
2 Continuous use of BiPAP during the night
1 Continuous use of BiPAP during the night & day
0 Invasive mechanical ventilation by intubation or tracheostomy

Abbreviations: ADL, activities of daily living; BiPAP, bi-level positive airway pressure; NPO, nil per os.

The clinical heterogeneity of ALS, its familial pattern and similarities with other diseases involving motor units demand the discovery of diagnostic and prognostic biomarkers that could have value for patient survival and would help clinicians to aid in decision-making and care-planning. Up to now several electrophysiological and neuroimaging indicators of manifested ALS have been developed, but only **candidate biomarkers** have been suggested to have a potential role in the diagnosis and prognosis of ALS, with further testing needed. Possible candidate biomarkers could be found in the tissues (skeletal muscles) or biological fluids (CSF, serum, plasma etc.) of the patients. Some studies have shown raised levels of multiple and individual inflammatory cytokines (MCP-1, IL-

6) and chemokines (IL-8) in the CSF of ALS patients, with a high specificity (91%) and sensitivity (88%) (Ono et al., 2001, Kuhle et al., 2009, Mitchell et al., 2009, Bowser et al., 2011). Other works have reported significantly elevated levels of proteins such as glial-derived proteins [monocyte differentiation antigen (CD14) and astrocytic S100β] and phosphorylated neurofilament heavy chain (pNfH) in the CSF of patient, and suggested that these proteins could have potential diagnostic value. An increased ratio of CD14 to S100β was recommended as a prognostic indicator with 75% sensitivity and 91% specificity (Brettschneider et al., 2006, Sussmuth et al., 2010).

Increases in the concentrations of insulin-like growth factors in serum, along with a concomitant decrease of their binding protein concentrations, were found in patients who had longer survival times (Hosback et al., 2007). The augmented expression of NOGO-A and -B, along with decreased NOGO-C, peptides that traditionally are associated with restricted regeneration after SCI, was found in the lumbar level of spinal cords of (G86R) SOD1 mice as well as in post-mortem and biopsy samples from patients (Dupuis et al., 2002). Subsequently, increased levels of NOGO-A and NOGO-B in muscle biopsies from ALS patients were correlated with the severity of clinical disability and with the degree of muscle fiber atrophy (Jokic et al., 2005).

Advances in neuroimaging techniques in the last decades, such as diffusion tensor imaging (DTI), proton magnetic resonance spectroscopy, resting functional MRI and magnetization transfer imaging (MTI), have been shown to have great potential to diagnose ALS in a noninvasive manner (Senda et al., Unrath et al., 2007, Lule et al., 2009, Turner et al., 2009). The important features revealed by MRI that are consistent with a diagnosis of ALS are hyperintensity along the CST, hypointensity in the motor cortex and atrophy of the precentral gyrus. DTI has revealed a decreased number of CST fibers and a restriction of water diffusion

across highly coherent fiber pathways compared to normal subjects. (Wang and Melhem, 2005). The combination of DT tractography of the CST and whole-brain voxel-based analysis has demonstrated the involvement of the white matter in motoneuron disease and might be used in evaluating the involvement of motor and extra-motor white matter in the early symptomatic stages, differential diagnosis and monitoring the spread of ALS over time (van der Graaff et al., 2011). Even though brain and spinal cord MRI remains the gold standard for the differential diagnosis of ALS from other diseases with a similar course, the search for biomarkers is now underway. So far, the most promising techniques in a multimodal strategy are multiprotein profiling in the CSF, DTI and motor unit number estimation (Turner et al., 2009).

1.4.4. Current Treatment and Management of ALS

As already mentioned, the presymptomatic or at least the early discovery diagnosis of ALS could offer wider possibilities for prevention and for the treatment of this devastating disease. Screening patients with FALS for mutations in the SOD1, TARDBP, FUS and a few other genes might offer some benefit. Unfortunately, until now we do not know how to prevent, cure or even block the development of ALS, and over 60% of all patients die within 3 years of presentation, and of the remaining, up to 10% survive for more than 8 years; death usually results from pulmonary infections (Kiernan et al.). As an outcome of a number of clinical trials, **Riluzole (**100mg/day), which reduces the presynaptic release of glutamate, remains the only effective drug that slows disease progression and extends the average survival of patients by 3-6 months (Bensimon et al., 1994, Lacomblez et al., 1996). Otherwise, all patients receive palliative or symptomatic therapy. If the patient manifests with the early symptoms of respiratory dysfunction or respiratory failure, he or she should be assessed by pulmonary insufficiency tests, overnight pulse oximetry and measurement of early morning blood gases. Noninvasive positive

pressure ventilation (NIPPV) should be considered at the early stage as it improves the patient's quality of life (Hardiman, Mustfa et al., 2006). Symptomatic and supportive treatment may include the prescription of anticholinergic drugs (such as trihexyphenidyl, amitriptyline or atropine) or the use of a portable suction machine if drooling is troublesome. Spasticity may be helped by baclofen or diazepam. Dysphagia could be managed by modifying food and fluid consistencies, postural advice and, in extreme cases of bulbar involvement, gastrostomy or cricopharyngomyotomy as well; however, all of the above therapies just improve the patient's quality of life, but not his or her survival. The main goal of treatment in the terminal stages should be to keep patients as comfortable as possible (McGeer and McGeer, 2005).

1.4.5. Animal models of ALS

The discovery of genes related to familial cases of ALS led to the generation of transgenic (Tg) animal models by the insertion of multiple copies of known human ALS-causing genes into the genome of the animals. The most widely used model of FALS has been created by Gurney by the insertion of multiple copies of the human mutant SOD1 (G93A) gene into the rodents' genome (Gurney et al., 1994). At a certain age, transgenic animals develop selective MN degeneration that presents with a progressive hind limb weakness, leading to paralysis and death, thus replicating in an almost perfect way the disease process in patients. In addition to the original mutant SOD1 (G93A) gene, other mutations (G37R, G85 R, D90A, G86R) were created that result in a similar disease progression (Van Den Bosch, 2011). These models have helped scientists to understand the mechanisms underlying motoneuronal death and the mechanisms by which neuroprotective agents act.

The discovery of the FUS/TLS protein raised the possibility of creating a new rodent model to study the mechanism of action of this gene in ALS6 (table 4).

Interestingly, most FUS/TLS knockout mice died immediately after birth and thus rarely survived to the time of weaning. In an outbred strain, FUS/TLS knockout mice survived but showed male sterility and reduced fertility in females (Hicks et al., 2000, Kuroda et al., 2000). As a result, no FUS/TLS transgenic rodent models are currently available.

Another gene involved into the development of ALS10 (table 4), TARDBP, has been used to create transgenic mice. The mice were created by overexpressing mutant (A315T) TDP-43 under the control of the mouse prion promoter (Wegorzewska et al., 2009). These mice developed gait abnormalities with body weight loss starting from 3 months of age. However, this model did not have MNs containing TDP-43-positive ubiquinated aggregates inside the cells, and the authors were not able to create wild-type TDP-43-overexpressing mice. As a consequence, the question remained whether these findings were the result of the TDP-43 mutation or of the overexpression of TDP-43 (Van Den Bosch, 2011). The creation of a transgenic model by overexpressing a human wild-type TDP-43 driven by the Thy-1 promotor provided an answer to this question (Stallings et al., 2010, Wils et al., 2010). These mice showed that the overexpression of wild-type TDP-43 results in the toxicity of MNs, with the formation of nuclear and cytoplasmic aggregates of ubiquitinated and phosphorylated TDP-43 inside the cells. Other transgenic rodent models of atypical, rare or candidate FALS genes have also been generated to study this devastating disease (table 5).

1.4.6. Neuroprotective strategies

Despite huge progress in understanding the mechanisms and pathobiology of ALS, current clinical management is still extremely limited. Considering that the disease affects MN at different levels of the CNS, a neuroprotective strategy should aim to restore affected tissue homeostasis throughout the entire nervous system. Numerous attempts have focused on antiglutamatergic, antioxidant, anti-apoptotic,

anti-inflammatory and neurotrophic molecules, as well as on gene-therapy and stem cell application. These and other molecules are able to reach the MN after systemic (intravenous, intraarterial) or local (intrathecal, intraspinal) application as it has been shown that the BBB is compromised in ALS (Garbuzova-Davis et al., 2007).

Disease	Gene product	Animal	Genetic modification	Reference
ALS2	Alsin	Mouse	KO (exon 3) KO (stop codon in exon 3) KO (exon 3 and 4) KO (exon 4)	(Cai et al., 2005) (Hadano et al., 2006) (Devon et al., 2006) (Yamanaka et al., 2006)
ALS8	VAPB	Mouse	PrP; VAPB P56S	(Tudor et al., 2010)
ALS	Dynactin	Mouse	Knock-in G59S $p150^{Glued}$ Thy-1; G59 $p150^{Glued}$	(Lai et al., 2007) (Laird et al., 2008)
CMT2E/ 1F	Neurofilament-L	Mouse	NF-L L394P	(Lee et al., 1994)

Table 5. Overview of transgenic mouse models with atypical, rare and candidate FALS genes

1.4.6.1. Antiglutamatergic therapy has currently shown the best results in clinical trials. As mentioned earlier, **Riluzole** is the only anti-ALS medicine approved for the treatment of patients. Recently, the kynurenine pathway (KP) has emerged as a potential contributing factor (Chen et al., 2009b). The KP is a major route for the metabolism of tryptophan, generating neuroactive intermediates in the process. These catabolites include the excitotoxic N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QUIN) and the neuroprotective NMDA receptor antagonist kynurenic acid (KYNA). These catabolites appear to play a key role in the

communication between the nervous and immune systems and also in modulating cell proliferation and tissue function. At the present time, some of the KP inhibitors, for example Teriflunomide (Sanofi-Aventis) and Laquinimod (Teva Neuroscience), have entered clinical trials (Chen et al., 2009b). Another anti-excitatory drug, **Memantine**, a non-competitive excitotoxic N-methyl-D-aspartate (NMDA)-receptor antagonist, has been shown to delay the development of hind limb paralysis and prolong the survival of SOD1(G93A) mice (Wang and Zhang, 2005).

1.4.6.2. Antioxidant therapy aimed at ameliorating oxidative stress could provide a possible healing effect in ALS patients. However, clinical trials examining the application of vitamin E, acetylcysteine, methylcobalamine, glutathione or coenzyme Q10 (CoQ10) indicate that these drugs are ineffective in ALS patients (Levy et al., 2006, Kaufmann et al., 2009). A novel peptide antioxidant (SS-31) that targets the inner mitochondrial membrane, thus improving mitochondrial dynamics, has been shown to result in a significant extension of lifespan, improved motor performance, decreased MN loss and reduced immunostaining for oxidative stress markers in G93A mice (Petri et al., 2006).

1.4.6.3. Immunotherapeutic strategies to combat ALS also could be an attractive therapeutic approach. Active vaccination with misfolded mSOD1 in the G37R SOD1 mouse model of FALS has been tried, resulting in the reduced loss of spinal cord neurons and a modest but statistically significant increase in life expectancy (Urushitani et al., 2007, Brody and Holtzman, 2008). However, much work remains to be done before clinical trials could be started.

1.4.6.4. The discovery of **neurotrophic factors (NTF)** and their anti-apoptotic effect as well as their ability to increase the survival of MNs during development led to the idea that they might ameliorate neurodegenerative disorders (Appel,

1981, Gould and Oppenheim, 2011). The combined evidence from both mouse and human studies suggests that the impaired production of vascular endothelial growth factor (VEGF) by MN, rather than a lack of functional receptors, is associated with ALS. Specifically, the exogenous delivery of VEGF has been shown to cause a direct neuroprotective effect via the expression of VEGF-receptors in MNs (Van Den Bosch et al., 2004). At the present time, there is encouraging evidence that the intrathecal, intracerebroventricular or intramuscular delivery of VEGF, as well as of insulin-like growth factor-1 (IGF-1), leads to an improvement in disease progression and overall survival in rat models of ALS (Kaspar et al., 2003, Azzouz et al., 2004, Nagano et al., 2005, Storkebaum et al., 2005, Wang et al., 2007). However, clinical trials utilizing the subcutaneous delivery of IGF-1 failed to show a beneficial effect, mainly because of the reduced bio-availability of IGF-1 when injected systemically (Sorenson et al., 2008, Howe et al., 2009). Subsequently, this problem appears to have been solved by the combined usage of IGF with IGF-binding protein 3 (IGFBP), also called IPLEX, which significantly increases the serum half-life of IGF-1; furthermore, approval was granted by the FDA for an early-phase clinical trial in human patients. There is still a great deal of debate surrounding the effectiveness of IPLEX in treating ALS (Williams et al., 2008, Bedlack et al., 2009, Gould and Oppenheim, 2011). However, there is an ongoing clinical trial in Sweden examining the delivery of VEGF protein into the CSF of ALS patients and the results will be forthcoming (http://clinicaltrials.gov, identifier # NCT01384162 and NCT008005501).

An interesting observation is that MNs exhibit trophic heterogeneity, that is they respond to a distinct type/s of NTFs during their development (Kanning et al., 2010). For instance, glial cell line-derived neurotrophic factor (GDNF) knockout mice showed a dramatic and restricted loss of small myelinated axons (γ-MNs) in their lumbar ventral roots, whereas large myelinated axons (α-MNs) were

completely unaffected (Gould et al., 2008). Hence, a monotherapeutic strategy might bring some improvements in motor activity or even extend survival, but this demands distinct NTFs that will target specific types of MN. This concern might be resolved by using cocktails of NTFs delivered in such a way so that they would be able to pass through the BBB in an efficient and controlled way or, alternatively, by using stem cells, which are well known to have paracrine properties, thus enabling the protection of different motoneuronal types.

1.4.6.5. Cell-based therapies, could provide either restoration or preserving of upper and lower MNs, and new neurons must be integration into existing neural circuits (Lindvall and Kokaia, 2006). Alternatively, grafted cells might serve as a vehicle for the delivery of NTFs that is also a very promising strategy to treat ALS. However, ALS is a malady that affects MNs throughout the CNS, and the replacement, even theoretically, of all impaired MNs is hard to accomplish. Moreover, successful MN replacement would necessitate the formation of long tracts of axonal outgrowth and the formation of NMJ by the grafted cells. So far, only a few studies after acute injury of the peripheral nerves of wild type animals (but not FALS animal models) have demonstrated newly formed functional connections between the grafted embryonic stem (ES) cells and the host muscles after transplantation (Deshpande et al., 2006, Yohn et al., 2008, Gowing and Svendsen, 2011). Thus, the generation and grafting of support cells aimed at protecting the remaining host MNs might be more realistic and effective. The discovery of **stem cells** and the characterization of their properties (vis chapter 1.3.6.4 and 1.3.6.5) raised the question of their use as a therapeutic agent during neurodegeneration bringing new hope for ALS patients.

Past *in vitro* and *in vivo* studies have generated MNs from animals and human ESC that maintain typical motoneuronal phenotype and showed functional incorporation after intraspinal transplantation into rodents with MN deficiencies

(Wichterle et al., 2002, Papadeas and Maragakis, 2009). Various cell types, such as human neural stem/progenitor cells (hNSC) and glial restricted precursors (GRP), have been shown to ameliorate ALS, reduce MN degeneration, extend survival and even structurally integrate into the segmental motor circuitry of mutant SOD1 rats, via the formation of functional synapses with the host neurons (Xu et al., 2006, Lepore et al., 2008, Xu et al., 2009). Based on the above results, the Food and Drug Administration approved a phase I clinical trial testing the feasibility and safety of the direct intraspinal transplantation of NSI-566RSC, produced by neural stem cells, into ALS patients; this trial is currently ongoing (http://clinicaltrials.gov, identifier # NCT01348451) (Gowing and Svendsen, 2011, Lunn et al., 2011).

2. AIMS AND HYPOTHESES OF THE WORK

The major aim of this work was to study the potential regenerative/neuroprotective effect of mesenchymal stromal cells *in vivo*, in animals after spinal cord injury and in the SOD1 (G93A) rat model of amyotrophic lateral sclerosis. Additionally, experiments were performed with the aim of identifying the molecular trigger responsible for initiating the maturation of perineuronal nets, a process that results in the downregulation of plasticity in the adult CNS.

The major goals are summarized below:

1. To optimize protocols and evaluate the safety of stem cells delivery into a recipient organism.
2. To examine the effect of *in vitro* predifferentiation on the *in vivo* survival and fate of the transplanted predifferentiated and naive adipose derived mesenchymal stromal cells in the balloon-induced compression model of spinal cord injury.
3. To elucidate the role of perineuronal nets components in the control of plasticity in the adult CNS.
4. To investigate plasticity in the somatosensory system after a cervical dorsal hemisection in animals lacking the link protein Ctrl1 (Hapln1).
5. To evaluate the effect of combined intraspinal and intravenous application of rat bone marrow mesenchymal stromal cells on the course and prognosis of ALS.
6. To explore the survival, biocompatibility and fate of grafted rat bone marrow mesenchymal stromal cells in a rat model of ALS.
7. To study the effect of the intrathecal delivery of human bone marrow stromal cells on the motor function and overall survival of rats after the appearance of the first symptoms of ALS.

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL SPINAL CORD INJURY

3.1.1. Laboratory animals

3.1.1.1. Adult male Wistar rats weighting 280-310g were used in the experiments involving spinal cord injury (balloon compression lesion).

3.1.1.2. We used age-matched adult male (2-4-month-old) link protein 1 knockout mice or CD1 mice (Charles River Laboratories) to study role of cartilage link protein Crtl1 (Hapln1) in neural plasticity. All animals were housed under standard laboratory conditions: a 12:12 h dark:light cycle, room temperature of 23ºC, 2 rats in one cage, with food and water supply *ad libitum.*

3.1.2. Anesthesia

Prior to any manipulation that could cause any pain to the animal, we used different types of general anesthesia. All animals received initial anesthesia. Rats were placed into a plastic box with an approximate diameter of 16.5 cm and a height of 13 cm and closed by a cover in order to maintain the gas concentration. Then a mask connected to an Isoflurane Vapor 19.3 apparatus (Drägerverk AG Lübeck, Germany) was introduced into the box, and isoflurane (Forane, Abbot Laboratories, Ltd., Queenborough, Great Britain) at a concentration of 5% vapor inhalation in air was administered at a flow of 300ml/min for 5-7 minutes. Those rats that were used in the balloon compression lesion model were further anesthetized with 3% vapor inhalation of isoflurane, applied by means of a custom-made nose mask. Those animals that were used for hemisection/transaction with subsequent hydrogel implantation were, after the initial anesthesia, injected intraperitoneally with pentobarbital (Sigma-Aldrich, Inc.) at a concentration of 10mg/ml and a dose of 6ml/kg. The animals were then left for 15 minutes in order to achieve a sufficient

level of anesthesia. Prior to the surgery and 7 days after SCI, the animals received an intramuscular bolus injection of antibiotics (ampicillin (Biotika) 0.3ml or gentamicin (Sandoz) 0.05ml). In order to control salivation and other vagal functions, we injected 0.2 ml of atropine (Hoechst-Biotika) subcutaneously. At the thoracic level the skin was depilated. In order to overcome a small analgesic effect of pentobarbital, a local anesthetic, mesocain, was additionally used (0.3ml subcutaneously and intramuscularly). All surgeries were performed using a surgical microscope (Zeiss) at 15-25x magnification under aseptic conditions.

3.1.3. Balloon-induced compression lesion

As a model of acute spinal cord injury, we used a protocol of a balloon compression lesion that was described by the Department of Neurobiology, University of Kosice and developed in our laboratory (Vanicky et al., 2001, Urdzikova et al., 2006). Adult male Wistar rats weighing 280– 300 g were anesthetized by isofluorane vapor inhalation (3%). After a 3 cm cut of the skin at the Th10-11 level, the vertebral column was exposed after the bilateral retraction of the muscles. Access to the epidural space was achieved by a partial laminectomy of the 10^{th}-11^{th} thoracic vertebrae. When the hole was large enough, a 2F Fogarthy catheter (Baxter Healthcare Corporation, Irvine, CA) filled with distilled water and connected to a 50ul Hamilton syringe was introduced into the epidural space and moved cranially 1 cm up; thus, a balloon was placed at the Th 8 level of the spinal cord (Figure 10). Subsequently, the balloon was rapidly inflated with 15 µl of water by means of a micromanipulator controlling the Hamilton syringe and was left for 5 minutes inside the vertebra column canal. Subsequently, the water was withdrawn from the balloon and the catheter removed from the epidural space. During the surgical procedure hemostasis was controlled and the animals' body temperature was checked and maintained at 37°C with a heating pad. The muscles and skin were sutured in layers with single knots. The animals were injected subcutaneously

with 2ml of saline solution to maintain water balance in the post-operative period. The animals were assisted with manual urination twice a day until the reflex returned.

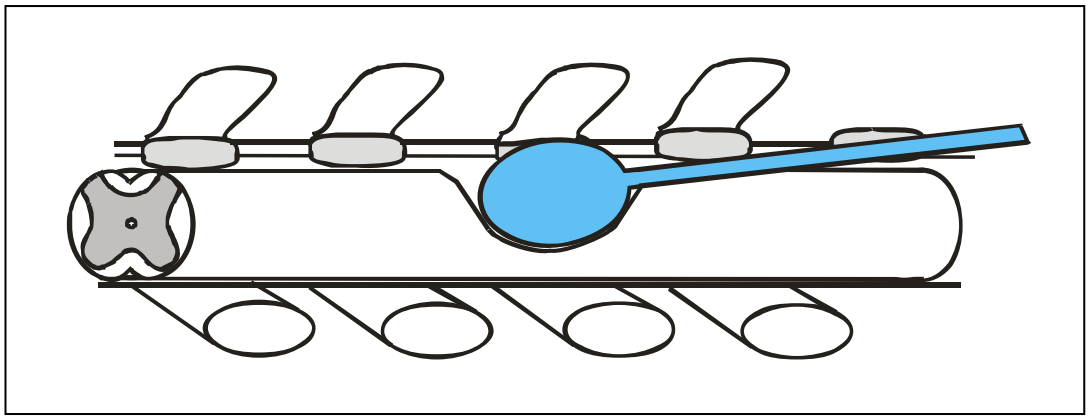

Figure 10. Balloon-induced compression lesion (Vanicky et al., 2001)

3.1.4. Cell isolation and preparation

3.1.4.1. Adipose-derived mesenchymal stromal cells (AMSC)

Adipose tissue from the inguinal pads was dissected, mechanically minced, and treated with 0.2% (w/v) collagenase type I (Worthington Biochemicals, Lakewood, NJ) for 1 h at 37ºC. The isolated cellular fraction was resuspended in proliferation medium consisting of DMEM/F12 + Glutamax (Gibco) supplemented with 10% FBS and 0.2% antibiotics (Primocin) and plated in culture flasks. Cells were harvested once they reached 90% confluence and replated up to the second passage. Cells from the second passage were then either induced to form spheres or were used as undifferentiated cells for transplantation into a spinal cord lesion. To assess the multipotency of AMSC, the cells were differentiated into adipogenic, osteogenic, and chondrogenic phenotypes.

3.1.4.2. Predifferentiation and neural induction of AMSC

After reaching 80% confluence, cultured AMSC (passage 2) were plated in 10 cm^2 Petri dishes and induced to form spheres by replacing the proliferation medium

with sphere induction medium consisting of DMEM/F12+ Glutamax (3:1 ratio, v/v) with B27 supplement (Gibco, Grand Island, NY), 20 ng/ml EGF (R&D Systems Inc., Minneapolis, MN), 40 ng/ml bFGF (R&D Systems Inc.), and 1% Primocin. Four days later, the formed spheres were collected in a 15 ml Falcon tube. They were dissociated by accutase, plated on laminin (Sigma-Aldrich)-coated dishes, placed in differentiation medium consisting of Neurobasal media (Gibco) with B27 supplement (Gibco, Grand Island, NY), 10 ng/ml NGF (R&D Systems Inc., Minneapolis, MN), 20 ng/ml bFGF (R&D Systems Inc.), and 1% Primocin and kept in culture for 6 days. Growth factors were added every second day.

3.1.5. Intraspinal cell implantation

Cell transplantation was carried out 7 days after SCI according to a previously published procedure (Amemori et al.). Cell viability was assessed by the Trypan Blue method. Cells for intraspinal implantation were suspended at a concentration of $5x10^4$ cells per 1 µl of PBS. All rats were anesthetized by isofluorane (3%) vapor inhalation in air. Each animal received intraspinal grafts into the rostral, central and caudal part of the lesion site. The cells were injected at a depth of 1 mm from the dorsal surface, 1mm laterally from the midline. The injections were made at a rate of 1µl/min using a Nano-Injector (Stoelting Co.) by means of a glass pipette. The pipette was kept inside the tissue for one minute, thus preventing the transplanted cells from leaking out of the host tissue.

3.1.6. Post-operative care of the animals

In order to achieve the best possible rehabilitation after the spinal cord injury and cell implantation, as well as to minimize any social stress, all animals were housed in individual cages, two rats per cage. Food and water were provided *ad libitum*. To avoid one of the most common complications after a spinal cord injury, caused by detrusor-sphincter dyssynergie (urinary retention), all animals were assisted with manual urination twice a day until the reflex returned, while ampicillin and

gentamicin were administered by intramuscular injection twice a day for 3 days. All rats were immunosuppressed by intraperitoneal injection of cyclosporine (Sandimmun, Novartis Pharama AG, Basel, Switzerland), 10 mg/kg 24 hours before transplantation and daily until the end of the experiment.

3.1.7. Transmission electron microscopy

For electron microscopy, immunolabeled tissue sections were rinsed in 0.1 M PB and then post-fixed for 1 h in 2% osmium tetroxide in 0.1 M PB. They were subsequently dehydrated through a graded series of ethanols followed by propylene oxide, propylene oxide:epon (50:50), and 100% epon (Agar Scientific Ltd., Stansted, UK), before embedding in fresh Epon between sheets of Aclar plastic (Agar Scientific Ltd., Stansted, UK) (Leranth C, 1989). Epon polymerization was carried out by incubation at 60ºC over 48 h. Following polymerization, regions of interest were cut from the flat embedded tissue and mounted on the tips of Epon blocks. Ultrathin sections of these regions were cut with a diamond knife (Diatome; TAAB, Gillingham, UK) at a thickness of approximately 70 nm and examined by a transmission electron microscope (Morgagni, Philips, Eindhoven, The Netherlands). For the detection of GFP^+ cells in TEM images, sections were incubated in a 1:300 dilution of polyclonal rabbit anti-GFP (Sigma-Aldrich, UK) for 72 h at 4ºC in 0.1% BSA/ 0.1 M TBS/0.25% TRITON X-100. The specificity of the antibody has been previously demonstrated using immunohistochemistry and western blotting (Halliday et al., 1996),(Rodriguez et al., 2008). Subsequently, the primary antibody was detected using an immunoperoxidase procedure (Chan et al., 1990). For immunoperoxidase labeling, sections were washed in 0.1 M TBS and placed in a 1:400 dilution of biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK) for 4 h, followed by two washes in 0.1 M TBS and incubation in a 1:200 dilution of biotin–avidin complex (Vector Laboratories Ltd., Peterborough, UK) for 30 min, followed by washes in

0.1 M TBS. All antisera dilutions were prepared in 0.1 M TBS/0.1% BSA, and the incubations were carried out at room temperature. The peroxidase reaction product was visualized (on TEM images, visible as a black color) by incubation in a solution containing 0.022% 3,30-diaminobenzidine (Sigma-Aldrich, Gillingham, UK) and 0.003% peroxide in 0.1 M TBS for 3 min, followed by washes in 0.1 M TBS and finally in 0.1 M PB. To check for non-specific background labeling or cross-reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of the primary and/or secondary antibodies from the incubation solutions resulted in a total absence of target labeling.

3.1.8. RT-PCR Analysis

At each stage of differentiation, mRNA was isolated from lysed cells using TRI reagent (Sigma-Aldrich) according to the manufacturer's directions. The expression of target and reference genes was determined by one-step real time RT-PCR using a 7500 Real Time-PCR System (Applied Biosystems) and a QuantiTect® One-step qRT-PCR kit (Qiagen). The 20 µl reaction volume contained 5 µl of extracted RNA. The following thermal profile was used: a single cycle of reverse transcription for 30 min at 50ºC, 15 min at 95ºC for reverse transcriptase inactivation and DNA polymerase activation, followed by 45 amplification cycles of 15 s at 94ºC, and 1 min at 60ºC each (combined annealing-extension step). Samples were run in triplicate. The Gene Expression Assay Mix (Applied Biosystems) employed for the study is shown in **Table 6.** As a housekeeping gene to normalize the data, we used beta actin (ACTB). The results were analyzed using the integrated 7500 System SDS Software version 1.3.1. The relative quantities of mRNA were therefore calculated using a DDCt method with efficiency correction.

Gene symbol	Gene name	Assay no.
ACTB	ß-actin	4352931E
NGFR	Nerve growth factor receptor (TNFR superfamily, member 16)	Rn 00561634_m1
TUBB3	Tubulin, beta 3	Rn 01431594_m1
MAP2	Microtubule-associated protein 2	Rn 00565046_m1
NTRK3	Neurotrophic tyrosine kinase receptor type 3	Rn 00570389_m1
NCAM	Neural cell adhesion molecule 1	Rn 00580526_m1
GFAP	Glial fibrillary acidic protein	Rn 00566603_m1
INSR	Insulin receptor	Rn 01637243_m1
NES	Nestin	Rn 00564394_m1
NTRK1	Neurotrophic tyrosine kinase receptor type 1	Rn 00572130_m1
CSPG4 (NG2)	Chondroitin sulfate proteoglycan 4	Rn 00578849_m1
TGFBR1	Transforming growth factor beta receptor 1	Rn 00562811_m1

Table 6. List of assays used for PCR

3.2. EXPERIMENTAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

3.2.1. Animal model of ALS

As an animal model of familial amyotrophic lateral sclerosis, we used hemizygous transgenic male rats (NTac: SD-Tg SOD1 (G93A) L26H) that overexpress human SOD1, carrying the Gly 93-Ala mutation. Rats used in the experiments with rat bmMSC were obtained from Taconic (Hudson, NY, USA). The same strain of rats as used in the other ALS experiments were bred in our animal facility from breeding couples kindly provided by Dr. Ludo Van Den Bosch (Dept. of Neurobiology, Vesalius Research Center, K.U. Leuven and VIB Leuven, Belgium). All animals were housed under standard laboratory conditions: a 12:12 h dark:light cycle, room temperature of 23ºC, 2 rats in one cage, with food and water supply *ad libitum.*

3.2.2. Criteria used to determine the beginning and the end stage of ALS in SOD1 (G93A) rats.

The time of transplantation for individual rats was established when two out of three tested parameters started to decline: the BBB score dropped from 21 to 17 – 16, grip strength decreased by more than 100 g compared with the individual baseline established for each animal prior to disease onset, or the animal started to lose body weight. In general, the first symptoms were observed using the grip strength test and the BBB locomotor test. The end stage of the disease was determined in as ethical a way as possible, to minimize the animals' suffering, and was diagnosed when the animals met at least two out of the following three criteria in any combination: the animals were unable to right themselves when placed on their side for 30s and/or had a decline in motor activity of 75% (BBB score from 21 to 5), a decline in grip strength of 75% (this generally corresponded to a grip strength decrease from 2050 g to 500 g) or a decline in body weight of 35% (on average, from 366 g to 237 g).

3.2.3. Isolation and preparation of bone marrow MSC

3.2.3.1. Isolation and preparation of BMSC from rats

GFP^+ MSC were obtained from transgenic Sprague Dawley rats (SD-Tg (CAG0EGFP) CZ-004Osb). The bone marrow was taken from the femurs and tibias of 16-day-old male animals that were euthanatized using CO_2. After cutting the epiphysis, the bone marrow was washed from the bones using a 2ml syringe with a 21 gauge needle, filled with Dullbecco's modified Eagle's medium with high glucose (DMEM), Glutamax 15µl/ml, 10% Fetal Calf Serum (FCS) and Primocin 2µl/ml (complete medium). The bone marrow was gently dissociated and then plated on petri dishes. The media was changed after 24h. When cells reached 75%–90% confluence, they were detached by trypsin/EDTA treatment and transferred into 75 cm^2 cell culture flasks. MSC from passage 4 were used for

implantation. Prior to implantation, the cultures were checked for the cells' ability to differentiate into adipogenic, osteogenic and chondrogenic phenotypes (Figure 10)

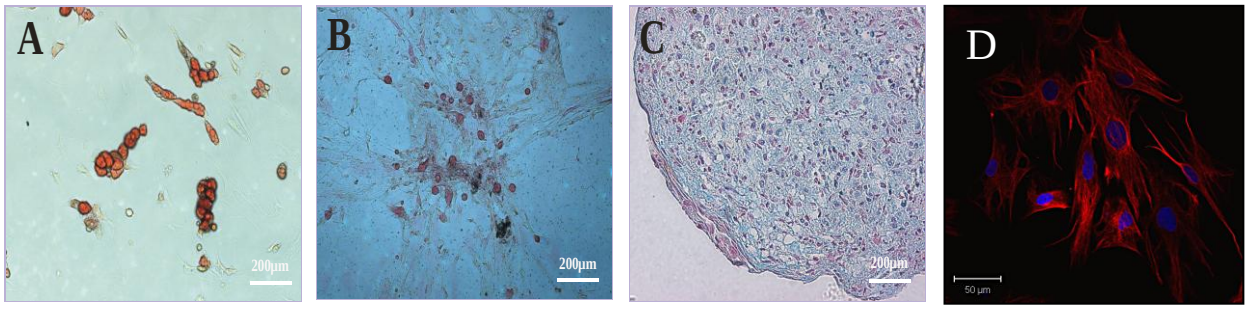

Figure 10. Mesenchymal properties of the transplanted cells were confirmed by their ability to differentiate towards adipogenic (A), osteogenic (B) and chondrogenic (C) phenotypes. In addition, MSC attached to the bottom of a plastic culture dish and expressed vimentin (D)

3.2.3.2. Isolation of human bone marrow MSC

Human bone marrow MSC (hBMSC) that were used in our experiments were provided by BioInova, s.r.o., a subsidiary of the Institute of Experimental Medicine. The cells were prepared according to a protocol that was described earlier by our group (Turnovcova et al., 2009). Briefly, after consent was obtained bone marrow samples were obtained by aspiration of approximately 30-50ml from the iliac crest of healthy patients. The mononuclear fraction containing hMSC was collected using Ficoll density-gradient centrifugation and then was transferred into a sterile tube. (Ficoll-Paque Plus; GE Healthcare Bio-Sciences AB, Uppsalla, Sweden). The cell suspension was then seeded on 10-cm Petri dishes at different seeding densities. The hMSC were isolated according to their selective adherence to the plastic surface and then expanded in α-minimal essential medium (α-MEM, Gibco-Invitrogen, Carlsbad, CA, USA) with 2.5% FBS containing 100U/ml penicillin and 100µg/ml streptomycin. Cell isolation was done in the same media as expansion. Non-adherent cells were washed out after 3 days, and subsequent cell culture changes were performed twice per week. Cultures were grown in a gassed

incubator at 37ºC and 5% CO_2. MSC from passage 3 were used for experiments; their multipotent properties were checked in the same manner as with rBMSC.

3.2.4. Implantation of cells

3.2.4.1. Combined intraspinal and intravenous application

Cell transplantation was carried out after the disease onset according to an earlier described procedure. Cell viability was assessed by the Trypan Blue method. Cells for intraspinal implantation were suspended at a concentration of $5x10^4$ cells per 1 µl of PBS. All rats were anesthetized by isofluorane (3%) vapor inhalation in air. Each animal received two intraspinal grafts at the Th10 level on the left and one on the right side (diagonally) with a distance between the injection sites of 3 mm. The cells were injected at a depth of 1 mm from the dorsal surface, 1mm laterally from the midline. The intravenous grafting of cells was performed through the femoral vein (v. femoralis). After the skin was cut in the inner thigh region, the femoral vein was carefully isolated and exposed from the femoral artery and saphenous nerve, and then the rat received an intravenous injection of $2x10^6$ MSC in 0.5ml of PBS using an Omnican 50 insulin syringe (B Braun, Melsungen AG OPM, Germany). After controlling hemostasis, the wound was closed in anatomical layers.

3.2.4.2. Intrathecal application

Following the onset of anesthesia, the rat was placed into a stereotaxic apparatus. The incision was made after shaving and cleaning the skin above the base of the skull. The muscles were retracted so that the dura overlaying the cistern magna was exposed (Figure 11). To incise the dura a 25 gauge needle was used. The return of the CSF signaled intrathecal access. To maintain visibility, the area was sparingly blotted, taking care not to remove the CSF. Intrathecal cannulation via the cistern magna followed by the application of hMSC/vehicle was performed by means of a

fine catheter (ALZET, cat. No 0007741). In order to minimize possible nerve damage, a catheter connected to a 50µl Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) via a gauge and filled with a cell suspension/vehicle was gently inserted into the aperture and positioned at the C8-Th1 level of the spinal cord. Subsequently, the cell suspension/vehicle (total volume 50µl) was injected into the subarachnoid space at a speed of 25 µl/minute using a Nano-Injector (Stoelting Co.).

3. 2.4.3. Postoperative care

To avoid the possibility of graft versus host disease, animals grafted with human bmMSC were immunosuppressed by a combination of drugs as described earlier; Sandimmun (Novartis Pharama AG, Basel, Switzerland) 10 mg/kg intraperitoneally, Immuran (GlaxoSmithKline, USA) 4 mg/kg intraperitoneally and Solu-Medrol (Pfizer, Puurs, Belgium) 2 mg/kg intramuscularly (Kozubenko et al.) were administered each day. Those animals that received a graft of rat BMSC were immunosuppressed with a daily injection of Sandimmun, 10 mg/kg intraperitoneally.

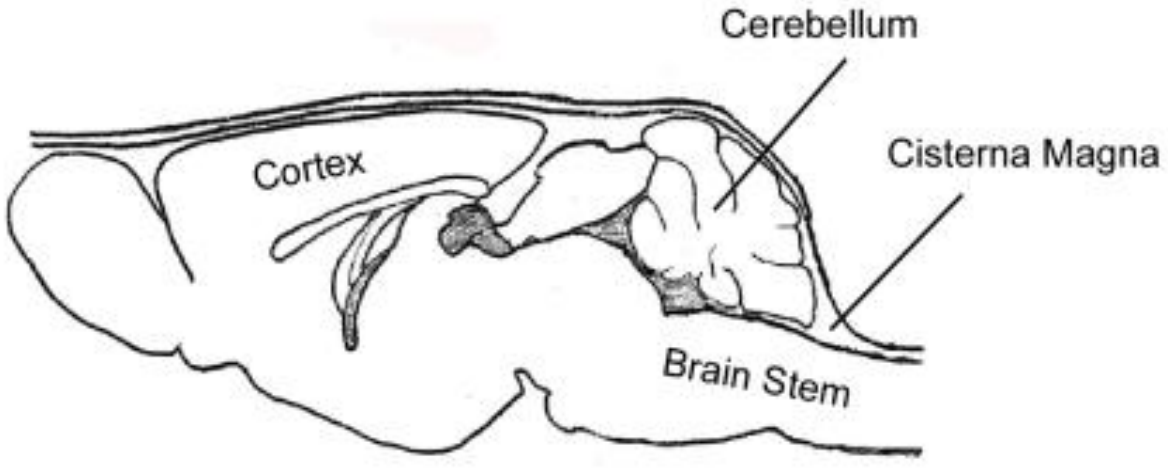

Figure 11. Schematic representation of the Cisterna Magna

3.2.5. Behavioral testing of motor functions.

3.2.5.1. BBB test

Currently, there are several methods that are used to evaluate an animal's motor activity after SCI. In our experiments we used one of the most popular scales, first developed by Basso, Beattie and Bresnahan (Basso et al., 1995). The animal's locomotion is evaluated using a 21 point scale: rats with a full range of movement receive a score of 21, while those with no observable hind limb movement receive a score of 0 (table 7). Using the BBB scale, the animal's recovery can be divided into 3 phases: early phase (0-7), middle phase (8-13) and late phase (14-21). The examiner assesses trunk stability, forelimb-hind limb co-ordination during gait and limb advancement, paw placement etc. in an open field using the BBB scale. We also used the grip strength test in combination with the BBB test to evaluate motor activity in SOD1 (G93A) rats in order to determine the onset and end-stage of ALS, as well as to evaluate the disease course. In order to avoid possible mistakes caused by the stress of the testing procedure itself, all rats were tested at the same time of the day and on the same day of the week (unless more frequent testing was required) by the same person.

3.2.5.2. Thirty seconds test

In order to determine the end stage of ALS in as ethical a way as possible, we used the "30 seconds" test in addition to the BBB test, grip strength test and body weight measurements. The animals were placed on their side and the time spent to right themselves was measured. Once rats were unable to right themselves within 30 seconds, it was regarded as the end stage of the disease, and the animals were sacrificed.

3.2.5.3. Grip strength test

The grip strength test allows for the study of neuromuscular function in rats by determining the maximum force generated by an animal. In this context, changes in grip strength are interpreted as evidence of motor neurotoxicity and dysfunction, as observed during ALS. In general, the grip strength meter (Grip Strength Meter BSGT2S, Harvard Apparatus, Holliston, MA, USA) was positioned horizontally, and a rat was held by its tail and lowered towards the grid (Figure 12).

Table 7: BBB test scale (Basso et al., 1995)

Score	Characteristics
0	No observable hind limb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL-HL coordination
11	Frequent to consistent weight-supported plantar steps and FL-HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL-HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL-HL coordination
14	Consistent weight-supported plantar steps, consistent FL-HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping
15	Consistent weight-supported plantar stepping, consistent FL-HL coordination, and no toe clearance or occasional toe clearance during forward limb advancement;

	predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel to the body at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
18	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

The animal was allowed to grasp the metal grid and then was pulled backwards in the horizontal plane. The force applied to the grid or to the bar just before the animal lost its grip was recorded as the peak tension, measured in grams. We used this test to diagnose the onset and end-stage of ALS, as well as to study the disease course in SOD1 (G93A) transgenic animals. Rats were considered for the transplantation of MSC when their grip strength decreased by more than 100 grams compared to the individual baseline established for each animal prior to disease onset. The end-stage was established when a rat‘s grip strength declined by 75% (this generally corresponded to a grip strength decrease from 2050 grams to 500 grams).

3.2.6. Transcardial perfusion of the animals

After the intraperitonial injection of a high dose of xylazine (Rometar 2%, 6 mg/kg, Spofa) and ketamine (Narketan 10%, 50 mg/kg, Vetoquinol), a thoracothomy was made on the left and right sides, the diaphragm was transected and the heart was

exposed. The right auricle of the right atrium was cut, and a cannula was introduced via the heart apex into the left ventricle. In order to wash out the blood from the cardiovascular system, heparinized PBS and cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 were infused transcardially by means of a peristaltic pump for 5-10 minutes. The tissue of interest was removed and additionally fixed in 4% PFA for 24 hours at +4ºC before further histological processing.

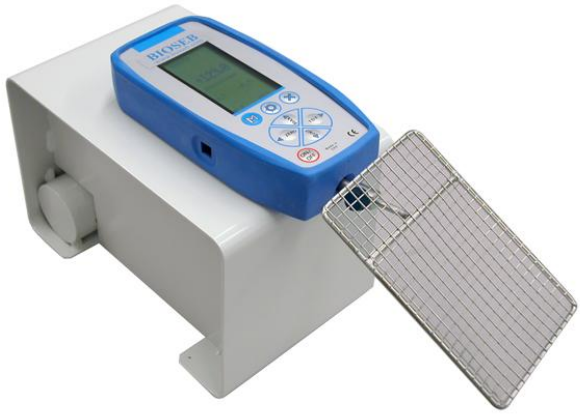

Figure 12. Grip strength meter

3.2.7. Immunohistochemical analyses

Spinal cords were cut into three parts: one for cutting sections in the longitudinal plane (2.5 cm long with the injection site in the center) and two for transverse sections (1 cm long cranially and caudally from the edges of the longitudinal sections). Longitudinal sections were used to check the distribution, properties and migration of the transplanted cells in the spinal cord. Transversal sections of the spinal cord were used for the quantitative evaluation of MN numbers, calculating the surface area and circumference of MN somas, analyzing plasticity in the cuneate nucleus, as well as the terminal deoxynucleotidyltransferase (TdT)-mediated dUTPbiotin nick-end labeling (TUNEL) assay. Spinal cords were dissected and post-fixed in 4% paraformaldehyde solution at 4°C overnight, then placed into a gradient of sucrose ranging from 10-30% sucrose in 0.2 M PB and

allowed to sink for cryoprotection. The immunohistochemical staining procedure was as follows: tissue sections attached to PLL-coated cover slips were incubated in 3% goat or donkey serum with 3% bovine serum albumin (BSA) in Tris buffer solution (TBS) with 0.2% Triton-X100 (Sigma-Aldrich) at 4ºC for two hours, followed by overnight incubation with the primary antibody and subsequent incubation with the appropriate species/subclass-specific secondary antibody for two hours. All primary and secondary antibodies used in experiments, their dilutions and manufacturers are listed in **Table 8**. To visualise the cell nuclei, some sections after washing with PBS were stained with 4 ′ 6-diamidino-2-phenylindole dichlorhydrate (DAPI) and mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA). The sections were evaluated using a Zeiss LSM 5 DUO (Zeiss, Oberkochen, Germany) confocal microscope equipped with an Ar/HeNe laser.

Table 8. Primary and secondary antibodies used for immunohistochemistry

Antibodies	Supplier	Species of origin	Dilution
NEURONAL			
a-NF160	Sigma-Aldrich, Gillingham, UK	mouse	1:200
a-MAP2	Chemicon; Temecula, CA,USA	mouse	1:800
a-Nestin	Chemicon; Temecula, CA,USA	mouse	1:2500
a-NeuN	Millipore, Billerica, MA, USA	rabbit	1:200
a-NCAM	Chemicon; Temecula, CA,USA	rabbit	1:200
a-bIII-tubulin	Sigma-Aldrich St., Louis,MO, USA	mouse	1:300
a-p75/NGF	Abcam	mouse	1:500
GLIAL			
a-glial fibrillary acidic protein	Sigma-Aldrich, Gillingham, UK	mouse	1:200

a-NG2	Chemicon; Temecula, CA,USA	rabbit	1:200
a-O1	Chemicon; Temecula, CA,USA	mouse	1:200
a-S100	Sigma-Aldrich St., Louis,MO, USA	mouse	1:200
a-CD31	Abcam	mouse	1:100
a-GFAP conjugated with Cy3	Sigma-Aldrich, Gillingham, UK	mouse	1:200
PERINEURONAL NETS			
a-Crtl1	R&D Systems (Minneapolis, MN, USA)	goat	1:100
a-parvalbumin Swant	(Swant,Bellinzona,Switzerland)	rabbit	1:2000
a-aggrecan	Chemicon (Temecula, CA, USA)	rabbit	1:500
a-brevican	Dr C. Seidenbecher	rabbit	1:4000
a-versican	Chemicon(Temecula, CA, USA)	rabbit	1:500
a-phosphacan	DSHB (IowaCity,IA,USA)	mouse	1:100
a-cholera toxin B	List Biological Laboratories; Campbell, CA	goat	1:2000
SECONDARY ANTIBODIES			
Alexa Fluor 488 or 594 conjugated anti-mouse IgG	Molecular Probes, Eugene, OR, USA	goat	1:200
Alexa Fluor 488 or 594 conjugated anti-rabbit IgG	Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK	goat	1:400
Alexa Fluor 488 or 594 conjugated anti-goat IgG	Molecular Probes, Eugene, OR, USA	donkey	1:500
Cy3 conjugated anti-mouse IgM	Millipore, Billerica, MA, USA	Goat	1:200

3.2.8. TUNEL assay

The TUNEL assay (ApopTag Kit; Oncor, Gaithersburg, MD, USA) was used to compare the intensity of TUNEL fluorescence in the MNs of MSC-treated and vehicle-injected animals with ALS at the terminal stage of the disease. For the quantification of TUNEL fluorescent intensity, images from an optical field of

315×315 μm were taken using a Zeiss LSM 5 DUO confocal microscope and a 40x objective. The outline of the soma was drawn in 10 neurons from each ventral horn (i.e. 20 cells/slice) in at least eight slices from the thoracic and lumbar parts of the spinal cord. Then the optical density (grey-scale levels of the corresponding pixels of the pre-processed image) along with the surface area and the circumference of the MN were determined by means of Axio Vision 4 software (Figure 13). The background optical density, calculated from a spinal cord section processed without the addition of a primary antibody, was subtracted. The values were calculated from the thoracic and lumbar levels as the mean of at least 160 cells from each animal.

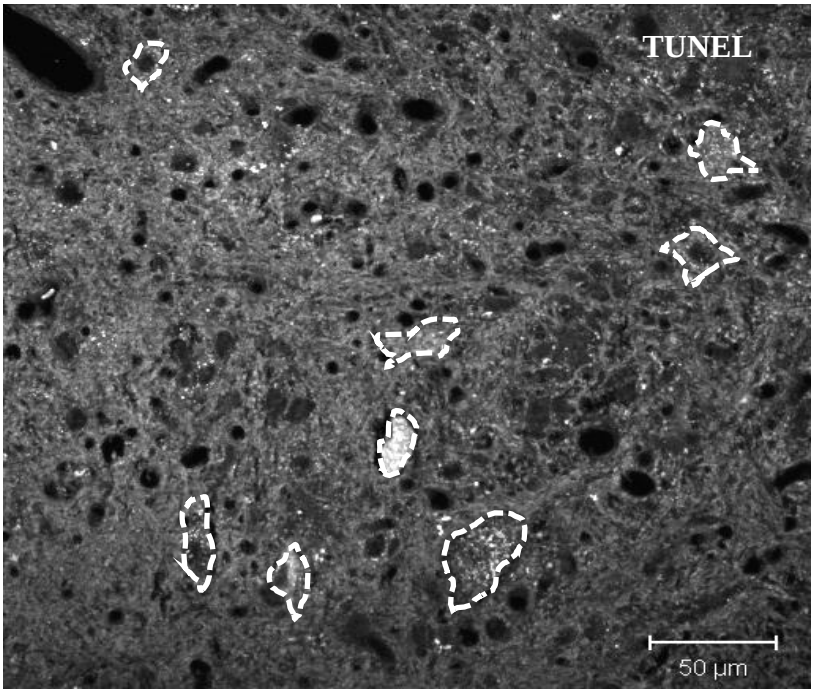

Figure 13. Quantification of TUNEL fluorescent intensity, circumference and surface area of spinal motoneurons

3.2.9. Quantitative analyses of motoneuron numbers in the ventral horns of the spinal cord

Four μm thick sections of the spinal cord were stained with the Luxor/Blue-Nissl method, then the number of motor neurons was determined (Figure 14). For the study of MN numbers, the optical density (greyscale levels of the corresponding

pixels of the pre-processed image) of the left and right ventral horns was recorded by means of a Zeiss Axio Observer microscope with a 20x objective and Axio Vision4 software (Carl Zeiss Vision GmbH, Germany). We counted the cells in a minimum of 10 slices from the thoracic and lumbar levels with an interval between slices of 100μm, in order to avoid double counting of the same neuron (i.e., 20 slices/animal). The number of MNs was counted by means of an unbiased stereological method on serial sections in both ventral horns at the thoracic (1cm segment) and lumbar (1cm segment) levels of the spinal cord (Bjugn and Gundersen, 1993, Mouton, 2002).

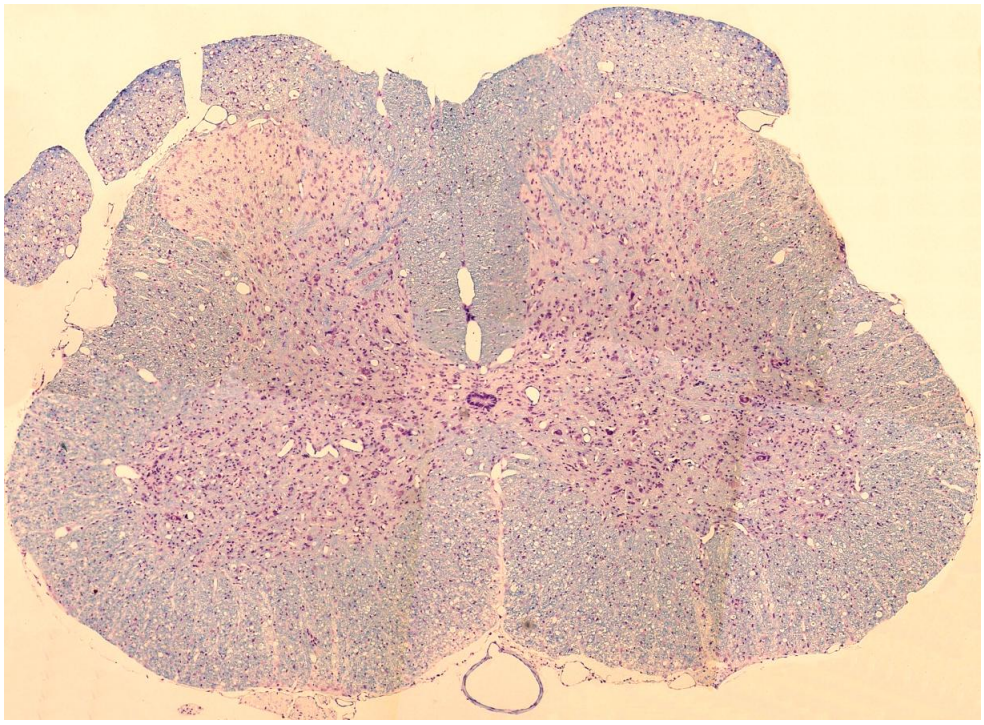

Figure 14. Crossection of the spinal cord at the lumbar level stained with Luxor/Blue-Nissl to visualize MN

An optical dissector and the Cavalieri method, that is, a two-stage method, were used to evaluate the number of MN in the reference space by means of the formula:

$$N = NV \times Vref,$$

where NV is the numerical density and Vref is the theoretically unbiased estimate of the reference volume. Vref was determined using the Cavalieri formula (Mouton, 2002):

$$Vref = \Sigma P \times a(p) \times T,$$

where: Vref = total volume of the reference space (spinal cord); ΣP = sum of points (MN) hitting the reference space; a(p) = area per point (ventral horns' area) (μm2) and T = distance between sections (μm). The numerical density (NV) was determined by the equation:

$$NV = \Sigma Q^{-} / (\text{number of dissectors} \times \text{volume of one dissector}),$$

where: ΣQ- = sum of objects (MN) counted; the number of dissectors is the number of dissectors counted throughout the reference space, and the volume of one dissector (μm3) = a(frame) × section height.

3.2.10. Plasticity in the cuneate nucleus

Considering that the normal forepaw primary afferents in the rodent cuneate nuclei are distributed in a somatotopic arrangement (Figure 15 A), Massey suggested that this could be used as a suitable model to assess collateral sprouting in the cuneate nuclei after the transection of the dorsal columns between the C6 and C7 root entry zones, leading to the denervation of all primary afferent input from digits 4 and 5 plus most of the input from digit 3 (Figure 15 B) (Massey et al., 2006). We used age-matched adult male (2-4 months old) link protein 1 knockout mice or CD1 mice (Charles River Laboratories) and made a unilateral dorsal spinal hemisection on the right side between the dorsal roots of C6 and C7. One week after injury, in order to visualize the forepaw innervation of the cuneate nucleus, we used cholera toxin B subunit

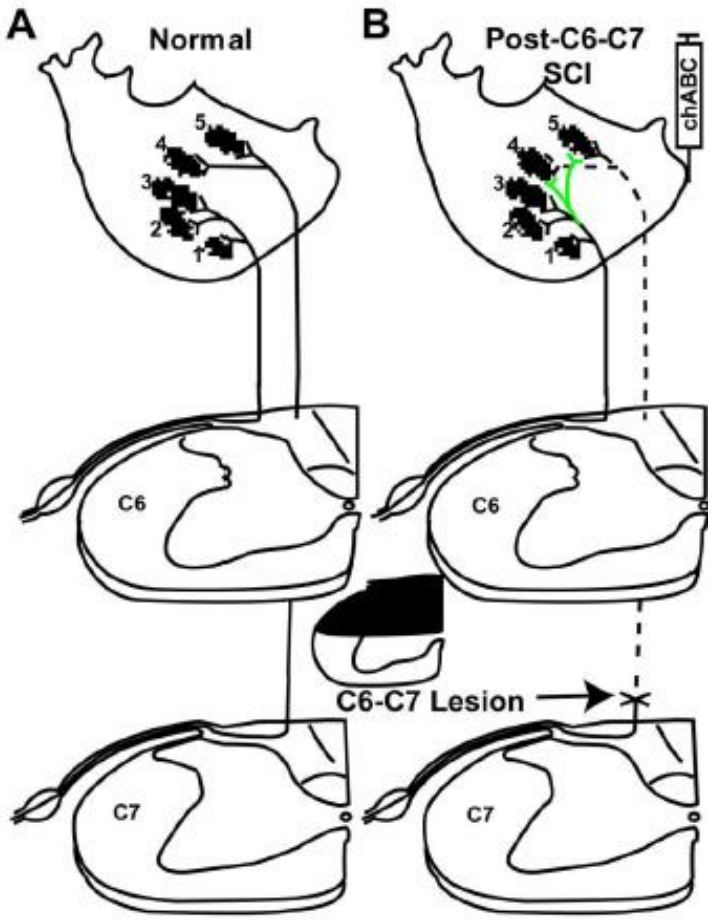

Figure 15. Somatotopic arrangement of the rodent cuneate nuclei under normal conditions (A) and collateral sprouting by spared forepaw primary afferents within the cuneate nucleus partially denervated by a C6–C7 dorsal column transection (B)

(1%, List Biological Laboratories; Campbell, CA) as a retrograde tracer. We injected1–2 µl of cholera toxin B subunit subcutaneously into the palmer side of each digit and into the glaborous foot pad of the right forepaw. Two weeks post-injury and one week post-tracing, the animals were killed. Twenty-micrometer thick cryostat sections were immunostained as described above. Primary antibodies were anti-cholera toxin B (1:2000, goat, List Biological Laboratories; Campbell, CA) and anti-glial fibrillary acidic protein (1:400, mouse, Sigma). Quantitation of the sprouting in the right cuneate nucleus was performed in each animal by

examining six sections (160 mm apart) throughout the length of the nucleus and measuring the area of cholera toxin B labelling. A perimeter was drawn around the cholera toxin B-traced afferents and measurements were taken in a square micrometer. The average areas of cholera toxin B tracing from each animal were combined to calculate group averages (either link protein 1 knockouts or CD1 controls), which were analysed using one-way ANOVA.

3.2.11. Statistical Analysis

All data were assessed with Student's t-test (Sigma-Plot 9.0); $p<0.05$ was considered significant and $p<0.01$ was considered very significant. Group diferences are presented as mean ± standard error of the mean (SEM), and the Kaplan-Meier method (Origin 7.3) was used to determine the difference in survival rate between the groups of MSC-treated and PBS-treated animals.

4. RESULTS

I. Paper 1. Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury

Authors: Arboleda D, Forostyak S, Jendelova P, Marekova D, Amemori T, Pivonkova H, Masinova K, Sykova E. Cell Mol Neurobiol. 2011 Oct;31(7):1113-22. Epub 2011 Jun 1.

4.1. Characteristics of predifferentiated adipose-derived stromal cells *in vitro* and after transplantation into the spinal cord of animals with SCI

In the set of *in vitro* experiments, we observed that AMSC harvested in the induction medium started to form spherical clusters already within 24 hours after plating. Five days later, 90% of the cells cultured in monolayer started to form different sizes of spheres (Figure 16 A). Sphere formation is the first step in testing the AMSC's neural differentiation capabilities and to induce differentiation toward neural precursors and more mature cellular stages. Immunocytological analysis revealed the strong expression of NCAM on the surface of the spheres. After the dissociated of the spheres cells expressed markers of early glial progenitor or Schwann cells such as NG2, S100, and p75 (Figure 16 B and C). Neural differentiation was promoted by replacing EGF with NGF and decreasing the concentration of bFGF in the medium. Two days later, most of the cells were NCAM-positive (Figure 16 D), while some of them started to express GFAP marker. We also observed some cells positively stained for neuronal marker □III-tubulin, but these cells did not have a typical neuronal morphology (Figure 16 D).

After transplanting AMSC and pAMSC into the rostral, central and caudal parts of the lesion site following a balloon-induced compression lesion of the spinal cord, both types of transplanted cells were detected by GFP fluorescence in the host spinal cord tissue 8 weeks post-transplantation. The two types of the grafted cells differed morphologically between the two groups. The AMSC within the injected

area appeared as large cell bodies floating in the tissue reminding the morphology observed during the *in vitro* experiments. After injection into the host SC transplanted cells did not attempt to establish close contact with the host cells (Figure 16 E). On the other hand, pAMSC implants were more robust than those of the AMSC and showed extensive migration from the injection. These cells seemed to configure hollow ducts along the neurofilaments, reminding the Schwann cells or OEG (Figure 16 F).

By visualizing host axons with NF160 staining we revealed that neurites were tightly wrapped by the implanted GFP^+ pAMSC (Figure 16 G). Transplanted cells were negative for O1, an oligodendrocytic marker (Figure 16 H); however, they were positive for the marker of oligodendrocyte precursor cells NG2 (Figure 16 I). By transmission electron microscopy we showed that pAMSC stimulated the inward migration of endogenous NG2 precursors to the lesion site, and were in a close contact with Schwann cells which migrated to the lesion from the spinal roots, and with myelinated as well as unmyelinated axons. (Figure 16 J and K). We did not observe any shift toward neuronal differentiation induced by the *in vivo* environment (i.e., we did not observe any positivity for □III-tubulin, NCAM, Nestin, or MAP2). We also found some transplanted AMSC as well as pAMSC positive for the endothelial marker CD31 (Figure 16 L and M).

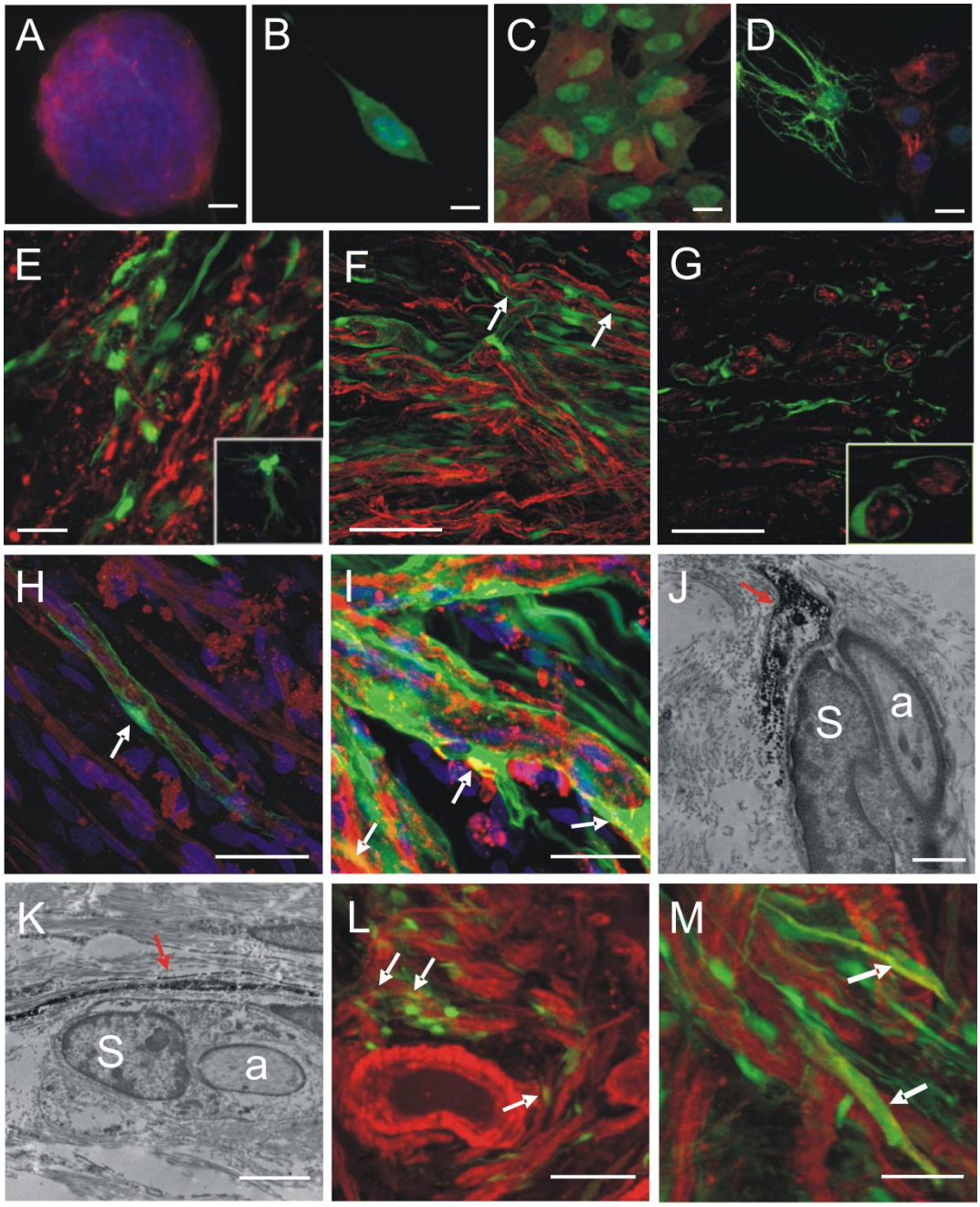

Figure 16. In vitro and in vivo characteristics of predifferentiated adipose-derived stromal cells. Spheres of pAMSC were all positive for NCAM (red)(A). Dissociated spheres plated on laminin-coated coverslips (B, C) and fixed after 6 days in neural medium (D). S100 (green) NG2 (red) (B); staining for Schwann cell (p75, red), GFP (green) (C); NCAM (red), □III-tubulin (green) (D). GFP^+ AMSC (green) 8 weeks after transplantation into a SCI did not interact with host axons (staining for NF160; red) (E); inset is at higher magnification and shows the lack of interaction between an AMSC and the host tissue. In contrast, GFP^+ pAMSC (green) formed hollow fibers and closely wrapped (white arrows) host axons (F, G; staining for NF160, red) as well as oligodendrocytes (H; staining for O1, red). Inset at higher magnification shows the close interaction of GFP^+ cells with neurofilaments. Some pAMSC

were positive for NG2 (I, white arrows) and formed guiding strands along the residual host tissue, attracting host NG2-positive progenitors (red) to the lesion site. TEM image showing a GFP$^+$ pAMSC (arrow) in close contact with a Schwann cell (S) and a myelinated axon (a) (J). GFP$^+$ process from a pAMCS (arrow) in the vicinity of a Schwann cell (S) and an unmyelinated axon (a) (K). Both AMSC (L) as well as pAMSC (M) were positive (arrows) for the endothelial marker CD31 (red). Cell nuclei are stained in blue (DAPI). Scale bars A–E, I—20 µm, F, G—50µm, j—1 µm, k—2.5 µm, H, L, M—25 µm.

4.2. Quantitative RT-PCR and electrophysiological properties of predifferentiated adipose-derived stromal cells in spheres and dissociated from the spheres

Data achieved from immunocytochemical analysis were confirmed by quantitative RT-PCR on spheres and on dissociated cells maintained in differentiation medium for 2 or 6 days. We observed the robust upregulation of p75 (40-fold) and NG2 (2.5-fold) in the spheres, which slowly decreased during the process of neural differentiation. The highest expression of early neural progenitor markers Nestin and NCAM, were found at the beginning of neural induction (a 5.7-fold increase for Nestin) or at the end (a 29-fold increase for NCAM), respectively (Figure 17 A). Other neural markers, such as GFAP, □III-tubulin, or MAP2, did not exceed a 0.5-fold increase in upregulation, (Figure 17 B). Regarding receptors, a low expression of the insulin and TGF□ receptors was observed during the whole culturing period, while the expression of the NGF receptor TrkA was upregulated at the end of NI. The expression of the NT-3 receptor TrkC decreased with time spent in culture (Figure 17 C).

The electrophysiological properties of pAMSC were measured from the cells 8 days after NI. By clamping the cell membrane and evoking membrane currents from a holding potential of -70 mV to values ranging from -160 to +20 mV for 50 ms at 10 mV intervals three types of membrane currents were registered. We did not reveal any activating Na^+ currents indicating cell differentiation into a neuronal lineage.

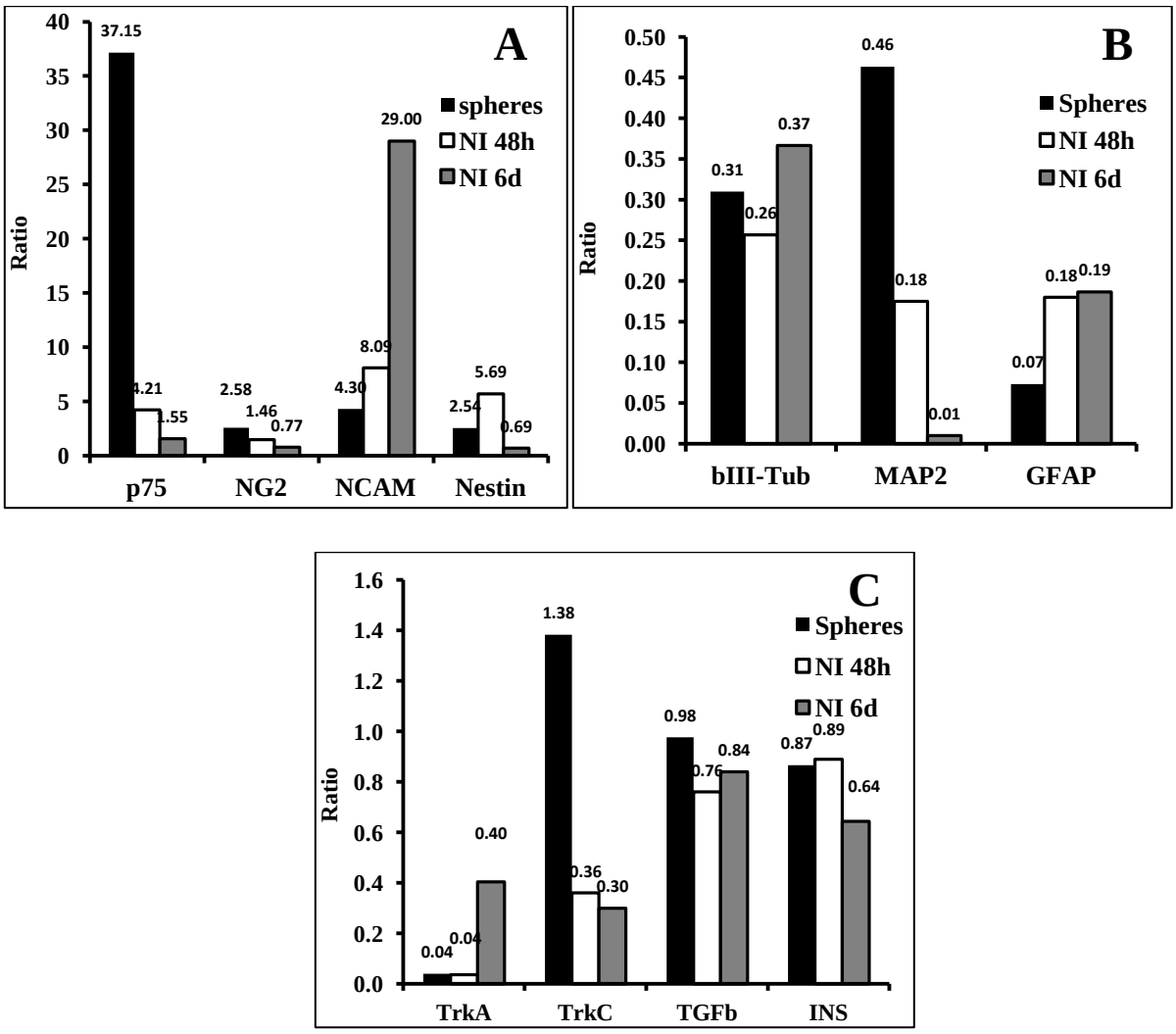

Figure 17. Quantitative RT-PCR analysis of spheres and dissociated cells from the spheres maintained in neural medium, 48 h and 6 days after neural induction (NI). The enhanced expression of glial markers and immature neural progenitor markers was found after exposure to differentiation medium.

4.3. Behavioral testing of rats after SCI

All the animals were tested prior the SCI and had normal motor function of their hind limbs that corresponded to 21 points according to the BBB scale. One week after SCI at the time of cell transplantation or vehicle injection, the animals were paraplegic with a BBB-score 1.41 ± 0.32. Over the course of 7 weeks, the control animals achieved BBB scores of 4.7 ± 0.34 (n= 12) with a slight improvement of their hind limb mobility but without any weight support. The cell-treated groups of

animals showed greater dynamism, vigilance, and frequent episodes of weight support as well as better muscle tone starting from 4 week after transplantation. The AMSC group achieved BBB scores of 7.0 ± 0.74 (n = 11) 7 weeks after transplantation; the pAMSC-treated group displayed motor improvement with BBB scores of 7.2 ± 0.46 (n = 12) at the same time point. The transplanted animals improved their gait and the ability to support their body weight. There were no significant differences in functional improvement between the AMSC- and pAMSC-treated animals (Figure 18).

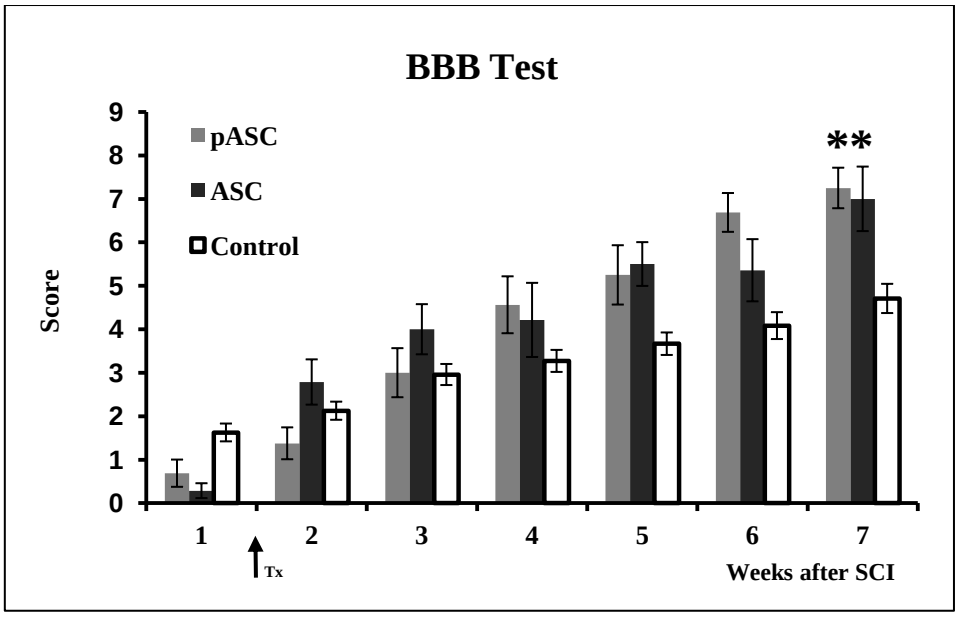

Figure 18. BBB open-field locomotor test. Hind limb gait was first assessed 1 week after SCI, i.e., before transplantation (Tx), and evaluated weekly thereafter for 7 weeks. Both the AMSC- (BBB = 7.0 ± 0.74; n = 11) and the pAMSC- (BBB = 7.2 ± 0.46; n = 12) treated groups displayed motor improvement compared with controls (BBB = 4.7 ± 0.34; n = 12). There was no significant difference between the transplanted groups

II. Paper 2. Animals lacking link protein have attenuated perineuronal nets and persistent plasticity

Authors: Carulli D, Pizzorusso T, Kwok JC, Putignano E, Poli A, Forostyak S, Andrews MR, Deepa SS, Glant TT, Fawcett JW. Brain. 2010 Aug; 133 (Pt 8): 2331-47. Epub 2010 Jun 20

4.4. Composition of PNN in the adult central nervous system

The composition of PNN in the rat cerebellum and spinal cord has been characterized earlier (Dityatev et al., 2007, Bruckner et al., 2008). PNN in the mice visual cortex have the same structure and are found primarily around neurons that express parvalbumin (PV) and Kv3.1b, markers of the fast-spiking GABAergic interneurons implicated in ocular dominance control. Eighty percent of PV-positive neurons are surrounded by PNN that contain Crtl1, neurocan, aggrecan, phosphacan, brevican and neurocan (Figure 19 and Figure 20).

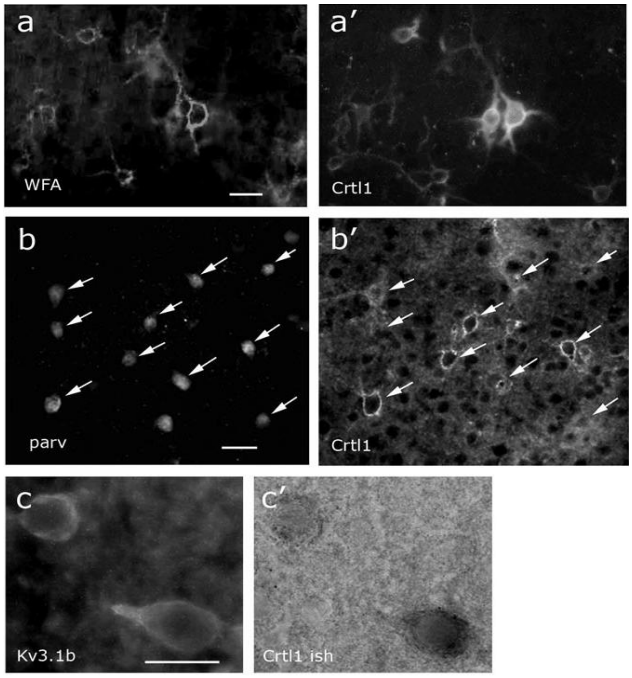

Figure 19. Perineuronal nets, demonstrated in (a) with WFA also contain Crtl1 link protein (a'). The great majority of Crtl1-containing perineuronal nets are around parvalbumin positive neurons (b, b').The proportion of parvalbumin-positive neurons with PNN is quantified in figure 20. Link protein mRNA visualized by in situ hybridization is seen in neurons which are immunopositive for Kv3.1b, a marker for inhibitory interneurons (c, c'). Bars =20um, 15um (c, c')

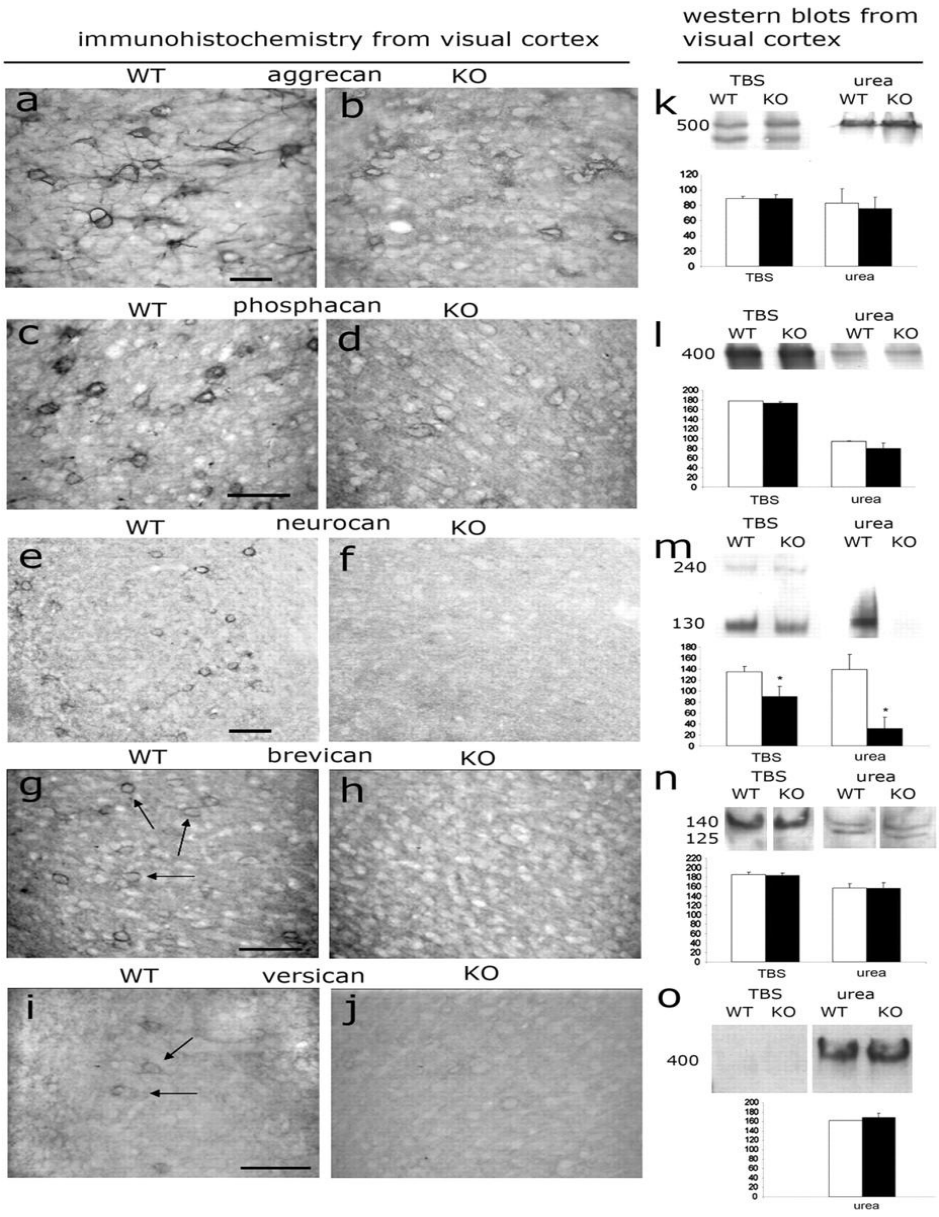

Figure 20. The lack of link protein affects the distribution but not the overall quantity of several CSPG that are normally enriched in PNN. The pictures show layers 2/3 of visual cortex. In control (WT) animals, the PNN contain the same CSPG as in rats (a, c, e, and i). In Crtl1 knockout animals vestigial PNN are still seen, with attenuated aggrecan and phosphacan staining localized just around the cell soma (b, d). Neurocan staining is barely visible (f), and brevican and versican staining are absent (h, j). However there is diffuse staining for all the CSPG in knockouts. The western blots from cortical tissue (k, l, m, n, o) show that all the CSPG are present in the brains of Crtl1 knockouts in normal overall amounts, except for neurocan-N, which is 30% decreased in the Tris buffered saline (TBS) extract and is no longer seen in the stable matrix compartment that can only be extracted in 6M urea. The protein quantifications are taken from three independent western blots. Bars = 30 mm. WT= wild-type; KO= knockout.

4.5. Developmental expression of PNN components in the CNS

It has been shown that many PNN components are already present in the immature CNS before the actual formation of PNN themselves (Carulli et al., 2007, Galtrey et al., 2008). As the structures form the molecule that triggers the construction of PNN should be upregulated. The formation of PNN begins in both rat and mice visual cortex at P14 (Figure 21 A-D). The visual cortex was examined during development using *in situ* hybridization and immunohistochemistry to discover which mRNAs and proteins are upregulated at this time (Figure 21 M). Of the mRNAs for PNN components expressed by PV positive cells, those for hyaluronan synthase (HAS), aggrecan, neurocan and tenascin-R were already present by P3, long before PNN formation. Brevican, phosphacan and versican, CSPG that are produced by glial cells, were also present from birth. At P7, before PNN formation, immunohistochemistry showed that all 12 of these molecules had a diffuse localization in the visual cortex ECM, similar to their distribution in the immature spinal cord and cerebellum (Geisler et al., 1991, Rhodes and Fawcett, 2004, Carulli et al., 2006, Galtrey et al., 2008). Only two PNN components were found whose mRNAs were upregulated at the time of PNN formation. These were the link proteins Crtl1 and Bral2, with Crtl1 showing a peak of expression at P14, coinciding with the onset on PNN formation, and Bral2 expressed from P21 onwards (Figure 19 E-M). The pattern of Crtl1 expression was the same in mice (Carulli et al., 2010b).

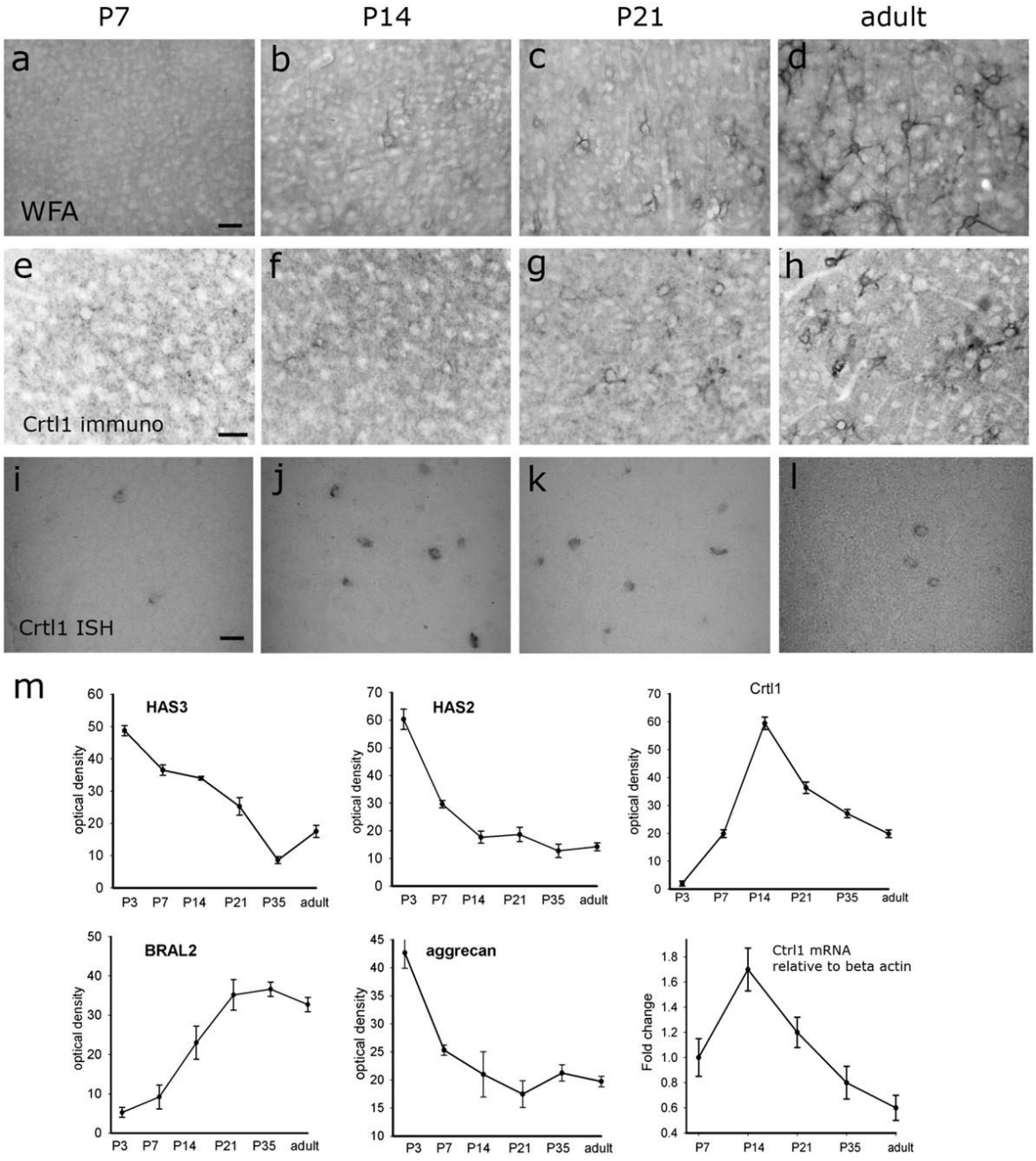

Figure 21. Developmental changes in the expression of PNN components in layers 2/3 of the rat visual cortex. (A-D) show staining with wisteria floribunda agglutinin (WFA), which reveals most PNN. The first signs of PNN formation are seen at postnatal day 14. (E-H) show immunohistochemistry for link protein Crtl1, showing that it is first detectable from P14 and is present in PNN. (I-L) show in situ hybridization for Crtl1, showing a peak of neuronal expression as PNN are forming at P14. (M) shows quantification of mRNA levels of PNN components by in situ hybridization, measuring the optical density of selected areas of interest around cortical neurons. The bottom right graph shows Crtl1 mRNA levels in mice measured by quantitative PCR relative to actin, showing that the time course is the same as in rats. Bars =20um.

4.6. Effect of dark rearing on PNN components in visual cortex

It was reported earlier that PNN formation in the visual cortex can be postponed by rearing animals in darkness, and than triggered by light exposure for less than one week (Pizzorusso et al., 2002). This model helped us to find an answer to the question "which PNN components are downregulated in rats as a result of dark rearing and upregulated when they are exposed to light"? The largest effect of dark rearing on mRNA levels was seen on Ctrl1, where the number of strongly labeled neurons was remarkably reduced (80-90% decrease) in dark reared animals, with the greatest change in cortical layers 2, 3 and 4 (Figure 22). On exposure to light, the number of neurons expressing high levels of Crtl1 mRNA reached normal levels after 2 days, at which time the formation of PNN, visualized with link protein or wisteria floribunda agglutinin (WFA) staining, was still incomplete (Figure 22 A-H), suggesting that upregulation of Crtl1 mRNA occurs at the onset of PNN formation. We saw similar but smaller changes in the mRNA levels of Bral2 and HAS2 in layers 2-3 and Has3 in layers 5 and 6, all of which showed a 30- 40% decrease in the number of labeled neurons after dark rearing with a reversion of HAS2 and HAS3 to normal levels after 2-3 days of light exposure.

4.7. Composition of perineuronal nets in the CNS of animals lacking Crtl1

The above observations suggest the hypothesis that PNN formation is triggered by the upregulation of the link protein Crtl1 in PV positive interneurons. As in cartilage, the link protein might act by stabilizing the binding of the various CSPG to hyaluronan (Morgelin et al., 1994, Watanabe et al., 1998). Hyaluronan is present around neurons that have PNN because they all express hyaluronan synthase from an early stage (Geisler et al., 1991, Rhodes and Fawcett, 2004, Carulli et al., 2006, Galtrey et al., 2008).

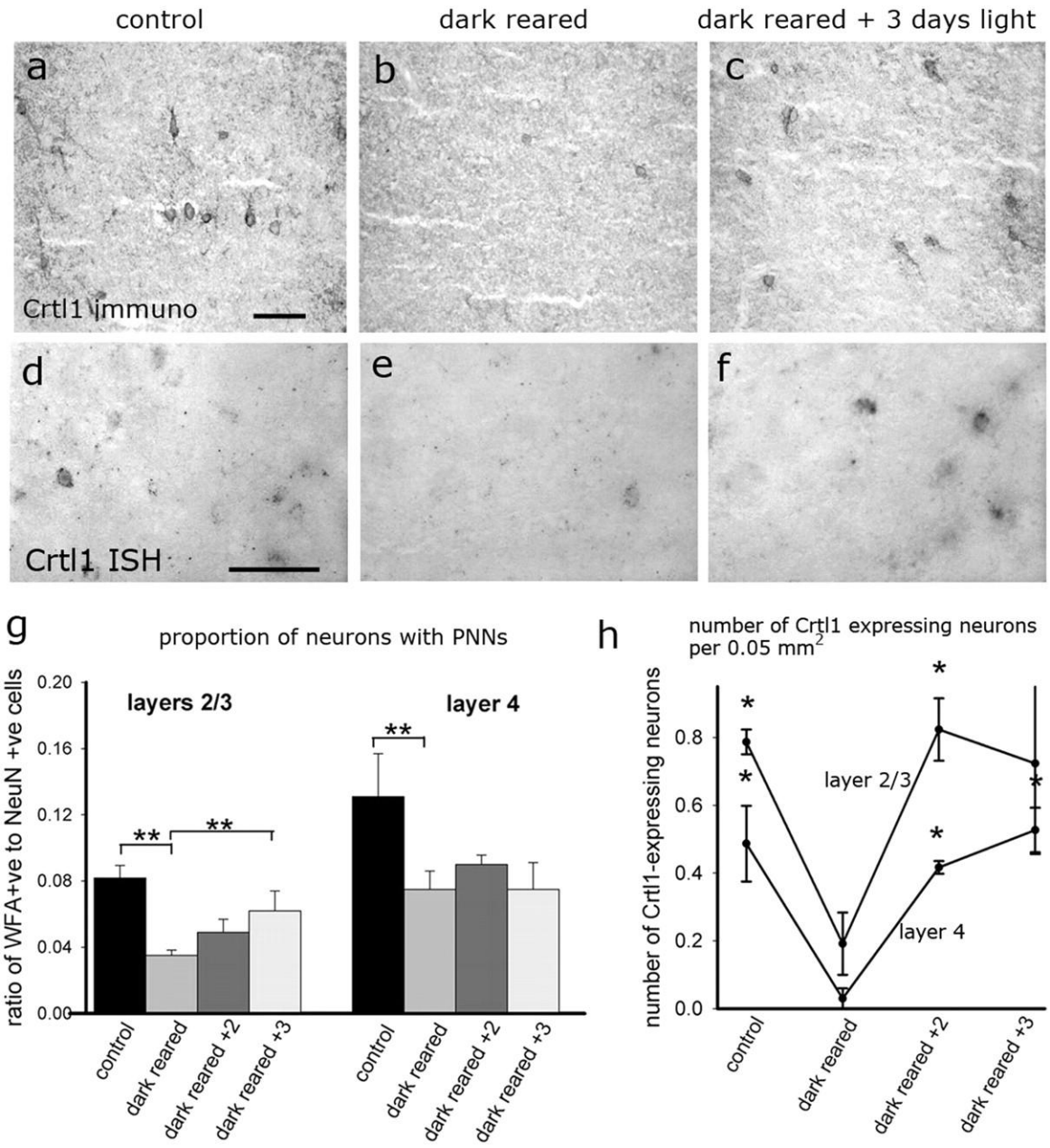

Figure 22. The effects of dark rearing and subsequent light exposure on PNN and Crtl1 expression in layers 2/3 of the rat visual cortex. Expression of Crtl1protein 1 in the normal adult cortex (a), but much reduced levels after dark rearing (b). 3 days of exposure to light leads to the beginnings of the appearance of Crtl1 in PNN (c). The proportion of neurons with PNN is quantified in (g) for layer 2/3 and layer 4. (d-e) shows 28 equivalent pictures of cortical layers 2/3 processed for in situ hybridization for Crtl1 mRNA. The mRNA is present in the normal cortex (d), downregulated after dark rearing (e) and upregulated again after light exposure (f). These observations are quantified in (h) for cortical layers 2/3 (above) and layer 4 (below). Bars =50um. *=p<0.05 relative to control (g) and relative to dark reared (h).

The presence of the link protein could therefore allow the capture of CSPG into the hyaluronan pericellular coat around PV neurons. This idea was tested in rescue transgenic mice lacking Crtl1 in the adult CNS. Immunohistochemistry for the PNN markers and components and for hyaluronan binding protein revealed that PNN in the Crtl1 knockout animals were very attenuated (Figures 24). In the normal CNS PNN surround the neuronal soma and dendrites, but in knockout animals WFA and aggrecan staining around the dendrites was absent, although there was some attenuated staining around the somata. The main change was a large reduction in the area of the W. floribunda agglutinin positive profiles due to the absence of staining around dendrites, and in the intensity of WFA staining around PV positive neurons (Figure 24 G-H). The same picture was observed in the cuneate nucleus (Figure 24 I-J). In addition we examined the levels of Nogo receptor in normal and Crtl1 KO animals because these molecules also affect ocular dominance plasticity (McGee et al., 2005). We found no differences (Figure 23).

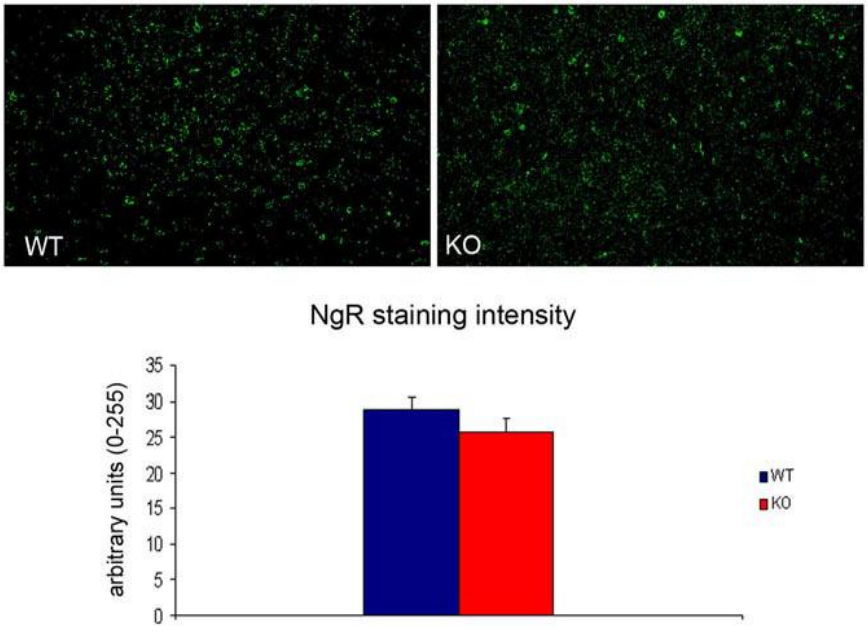

Figure 23. Nogo receptor immunostaining from layer 2/3 of the Crtl1 KO and wild type mouse visual cortex. Nogo receptor is present, mainly in neuronal structures. There is no difference between the two types of mouse.

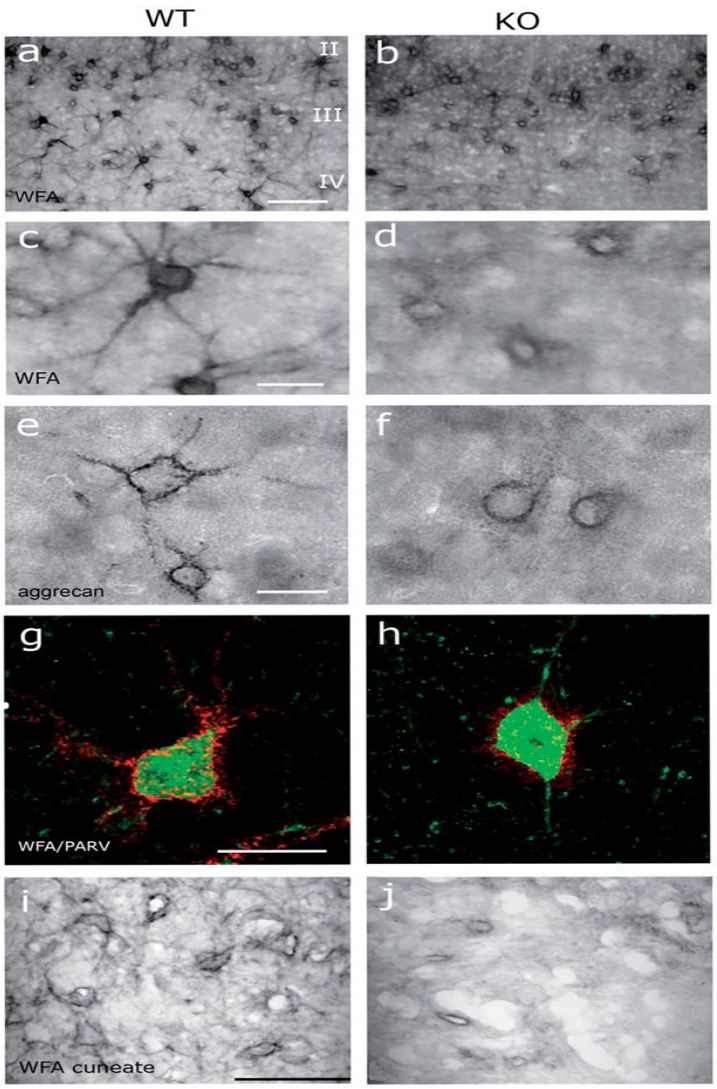

Figure 24. The appearance of PNN in the visual cortex and cuneate nucleus of wild-type and Crtl1 knockout mice. (A-D) show WFA staining of the visual cortex layer 3, comparing PNN at low and high power, and showing the absence of PNN around dendrites in Crtl1 KOs. A similar result is seen with aggrecan staining (E-F). The absence of PNN around parvalbumin-positive neuronal dendrites in knockouts is not due to the absence of dendrites in knockout animals; (G-H) show parvalbumin stained neurons in normal and KO animals with normal dendritic morphology, but with less WFA-stained PNN in KO animals (h). (I, J) WFA staining of the cuneate nucleus in normal and KO animals. Bars = 70um (A, B), 15um (C-H), 50um (I, J).

4.8. Plasticity in the cuneate nucleus in Crtl1 knockout animals

To check whether the absence of PNN might promote the anatomical sprouting of the preserved ascending axons into denervated regions of the cuneate nucleus, as happens after ChABC treatment, we used the experimental model developed by

Massey et al. (Massey et al., 2006). One week after unilateral dorsal hemisection of the spinal cord of control and Crtl1 knockout mice (between levels C6 and C7), an injection of cholera toxin B subunit, which labels the ascending sensory axons by transganglionic transport, was made into the forepaw of the animals. In control animals, sensory axon innervation of the cuneate nucleus was restricted to the region normally innervated by the lateral digits with no apparent sprouting into the denervated regions. In Crtl1 knockout animals, we saw labeled axons innervating the regions previously innervated by the medial digits, indicating sprouting of the preserved axons (Figure 25 A–C). Quantification of this sprouting by drawing a perimeter around all the labeled axons showed a significantly larger area occupied by axons in the Crtl1 knockout animals (Figure 25 D).

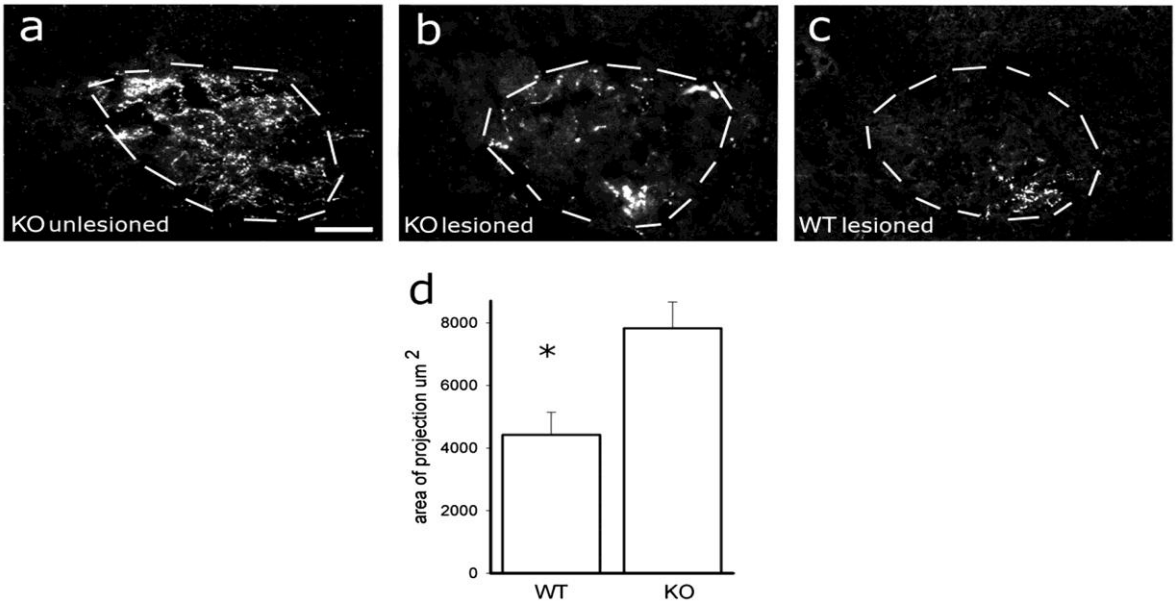

Figure 25. Cholera toxin B injections into the forepaw label sensory axons occupying the entire cross-section of the cuneate nucleus. Axons traced from the forepaw are shown in a KO animal, with the outer extent of the nucleus traced (A). The appearance is the same in WT mice. After a dorsal column hemisection lesion between C6 and C7, innervation of the cuneate nucleus from the forepaw is reduced to the area occupied by the two lateral digits. In Crtl1 KO (B; n=7), but not in control animals (C; n=12), these unlesioned connections sprout to re-innervate the regions normally innervated by the medial two digits. (D) Quantification of this sprouting was performed by tracing around the outline of the stained terminals, then measuring the area of innervation (p<0.05 by T test). Bar = 100µm.

III. Paper 3. Mesenchymal stromal cells prolong lifespan in a rat model of amyotrophic lateral sclerosis

Authors: Forostyak S, Jendelova P, Kapcalova M, Arboleda D, Sykova E. Cytotherapy. 2011 Oct;13(9):1036-46. Epub 2011 Jul 8

4.9. Effect of the combined intraspinal and intravenous implantation of rat BMSC on the disease course and overall survival of SOD1 (G93A) rats

To perform a clinically relevant experiment, we transplanted rat BMSC into symptomatic animals. The onset and progression of the disease were studied by monitoring motor activity, grip strength and body weight. When the rats achieved the highest test scores, then maintained a plateau of all the tested parameters followed by a gradual decline in performance, it was considered as the onset of the disease, and the animals were selected in a random fashion for cell implantation or vehicle injection accordingly. The end stage of the disease was determined as described earlier in the Methods.

All rats started to exhibit the first symptoms at almost the same age. To clarify disease onset, the rats were observed for 3–4 days after the first signs of disease appeared. There was no significant difference between the two groups at the time of rBMSC (114.3±1.85 days) or vehicle (113.75±1.73 days) administration (Figure 26 A, E). Starting from the 20th week of age, the rats treated with cells started to show significantly better motor function in all four limbs, as assessed by the grip strength test, and motor activity, as tested by the BBB test, compared with the control rats. This trend remained until the end stage of the disease, reaching statistical significance at a number of time-points after rBMSC grafting (Figure 26 A, B), suggesting that the transplanted cells slowed down the decline in motor performance. Rats treated with rBMSC lost body weight more slowly than the control animals, but the difference between the two groups was not statistically significant (Figure 26 C). The lifespan of the animals in the group treated with

BMSC was longer on average by 11 days, compared with the control group of animals (Figure 26 D, F; $P \leq 0.05$).

Most of the rats from the treated group were diagnosed as having reached the end stage of ALS mostly due to weight lose and a decrease in grip strength, while their motor activity (BBB) still did not reach the critical level and only 22% could not right themselves within 30 seconds after being placed on their side. In contrast, rats from the control group were diagnosed as having reached the end stage once they demonstrated impaired motor activity and grip strength and reached the critical level of body weight loss, and most of the animals (87.5%) could not right themselves from their side within 30 seconds (**table 9**). Thus, the animals that received rBMSC displayed a less severe impairment of their motor system before they were sacrificed compared to the control animals.

BMSC Group				**Control Group**			
Rat	**BBB/30'**	**GrStrT**	**Weight**	**Rat**	**BBB/30'**	**GrStrT**	**Weight**
1	+/-	-	+	**1**	+/+	+	-
2	+/+	-	+	**2**	+/+	+	+
3	+/-	-	+	**3**	+/+	+	-
4	-/-	+	+	**4**	+/-	-	+
5	-/-	+	+	**5**	+/+	+	+
6	+/+	+	+	**6**	+/+	-	+
7	+/-	-	+	**7**	+/+	+	+
8	-/-	+	+	**8**	+/+	+	-
9	+/-	-	+				
10	**Not measured**						
11	**Not measured**						

Table 9. Criteria used to determine the end stage of ALS in individual rats. The end stage of the disease was established according to the criteria shown in the table and described in the text. Once a rat reached two or more thresholds during behavioral testing, it was regarded as being at the end stage of the disease.

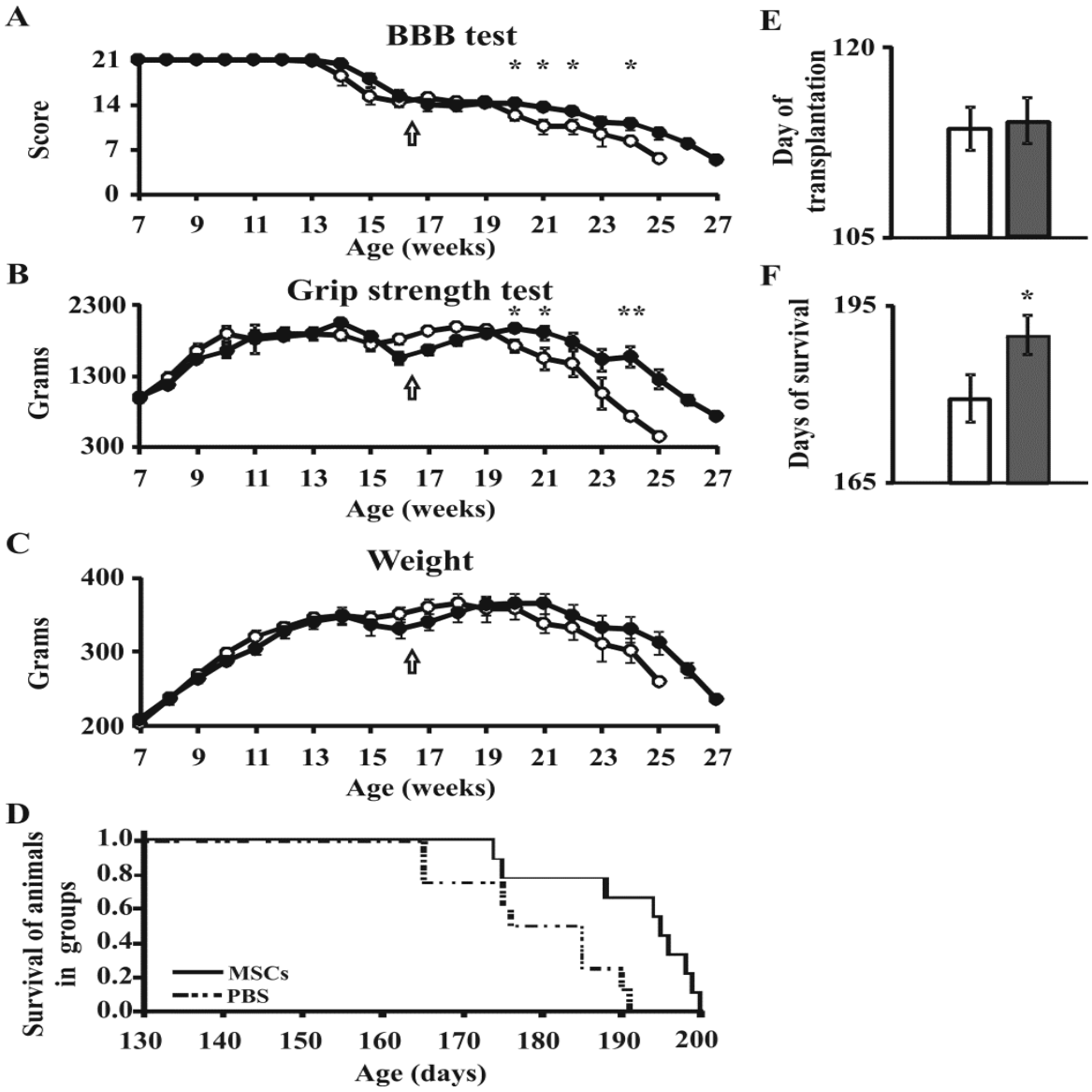

Figure 26. Characteristics of disease progression in groups of SOD1 (G93A) rats treated with rBMSC (filled circles) or sham-treated (empty circles). Motor activity (A) and grip strength (B) did not show any significant differences between the groups until week 19. From week 20 onwards, the rats treated with BMSC showed significantly higher scores on the BBB (A) and grip strength (B) tests. MSC treatment did not significantly delay the loss of body weight but showed a tendency to slow it down (C). There were no differences between the mean time of BMSC (114±5.5 days) transplantation and PBS (113.75±5 days) injection in the two groups of animals (E). The lifespan of animals was significantly prolonged by MSC treatment by 11 days as group means (P≤0.05). Compared with an injection of PBS, BMSC transplantation significantly increased overall survival from 179±3.6 days to 190±3.3 days, respectively (D, F). BMSC-treated animals are shown as continuous lines (n=9), control animals as interrupted lines (n=8).

4.10. Effect of grafted rat BMSC on the survival and characteristics of host motoneurons.

To confirm the behavioral improvement and extended lifespan, we performed MN counts. At the end stage of the disease, the average motor neuron counts were significantly higher at the thoracic and lumbar levels of the spinal cords of the BMSC-treated group compared to controls. The overall number of MN counted by a stereological method in serially sectioned spinal cords in both ventral horns at the thoracic level (Figure 28 G) in the BMSC-treated group was 5834±1391 per 1cm of spinal cord, while in the control group the number was 3773±689 ($p \leq 0.01$). At the lumbar level of the spinal cord (Figure 28 H) the BMSC-treated group had 12004±1896 MN per 1cm of spinal cord, while in the sham-treated group there were 8763±2531 MN ($p \leq 0.01$). There were no significant differences in the measured surface area of the ventral horns at either the thoracic or the lumbar levels of the spinal cord.

Several studies have reported a possible role for apoptosis in both the human disease and in rodent models of ALS (Kostic et al., 1997, Li et al., 2000). We therefore used the TUNEL assay to study the extent of DNA fragmentation in animals at the early stage and the end stage of the disease. TUNEL-positive cells revealed DNA fragmentation; however, when the first signs of the disease appeared, the TUNEL fluorescence was predominantly located in the cytoplasm of the motor neurons (due to effects on mitochondrial DNA) (Figure 28 A, B), whereas at the end stage of the disease the most intense fluorescence was in the nucleus (Figure 28 C, D). We measured the surface area (Figure 28 A, B) and circumference (Figure 28 C, D) of the MN somas (**table 10**). A significantly larger surface area of the somas of MN was found in rats that had been grafted with BMSC compared with vehicle-injected animals at different levels of the spinal cord (Figure 28 A, B). The average circumference of MN somas at the thoracic level (Figure 28 C) as well as at the lumbar level (Figure 28 D) of the spinal cord in the

cell-treated animals was significantly larger compared to animals injected with PBS. The intensity of TUNEL staining in the somas of MN at the thoracic (Figure 28 E) level, where the cells were implanted, was significantly lower in cell-treated animals than in animals injected with PBS, whereas at the lumbar level of the spinal cord there were no significant differences in the intensity of fluorescence between the treated and control groups (Figure 28 F).

For TUNEL analysis we used 9 and 8 rats at the late stage of the disease from the treated and vehicle-injected groups, respectively. We did not make a quantitative TUNEL analysis at the early stage of the disease as we had only 2 rats available for such analysis from the BMSC-treated group, which died or were sacrificed 11 days after the BMSC were grafted, and no animals from the sham-treated group. These 2 rats were used for comparison and are presented in **Figure 29**. The above results show that the implanted BMSC had a positive effect on the survival of MN and that such MN were of larger size than those in vehicle-injected rats. Our results could possibly be explained by the activation of BCL2 genes by the MSC or by the inhibition of caspases (especially 1 and 3), which have been shown to be active in spinal motor neurons in the ALS animal model, thus influencing disease progression, but more experiments should be done to confirm this speculation.

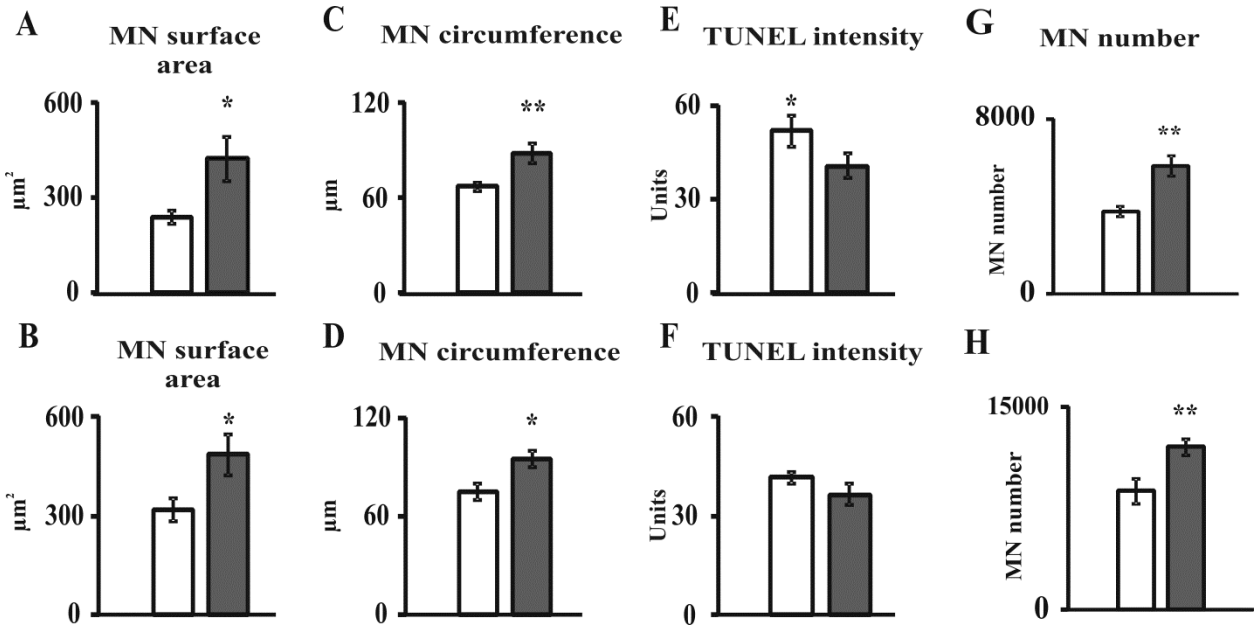

Figure 28. Quantitative analyses of the surface area, circumference, TUNEL staining intensity and number of motor neurons. To determine the surface area and the densitometric mean of fluorescence due to apoptosis, perimeters were drawn around the neuronal somas in the upper thoracic and lumbar levels of the spinal cord as shown in Figure 21 A. The mean surface area of neuronal cell bodies from the MSC-treated group was significantly larger at the thoracic (A) and lumbar (B) levels relative to the sham treated group, $p<0.05$. The somas of motor neurons from the cell-treated group had a larger circumference at the thoracic (C, $p\leq0.01$) and lumbar (D, $p\leq0.05$) levels than those from rats after PBS injection. Despite the larger neuronal somas in the cell-treated group of animals, the intensity of TUNEL staining was significantly less at the thoracic (E, $p\leq0.05$) level and showed a tendency towards a lower intensity at the lumbar (F, $p>0.05$) level of the spinal cord, compared to sham treated animals. MSC significantly increased the number of MN in the upper thoracic (G) and lumbar (H) levels of the spinal cord in the treated group of animals ($P\leq0.01$ and $P\leq0.01$, respectively) compared with controls. The numbers of MN were counted using an unbiased stereological method in serially sectioned spinal cords from both the left and right ventral horns. Error bars indicate SEM. MSC-treated animals are shown in grey columns (n=9), control animals in empty white columns (n=8).

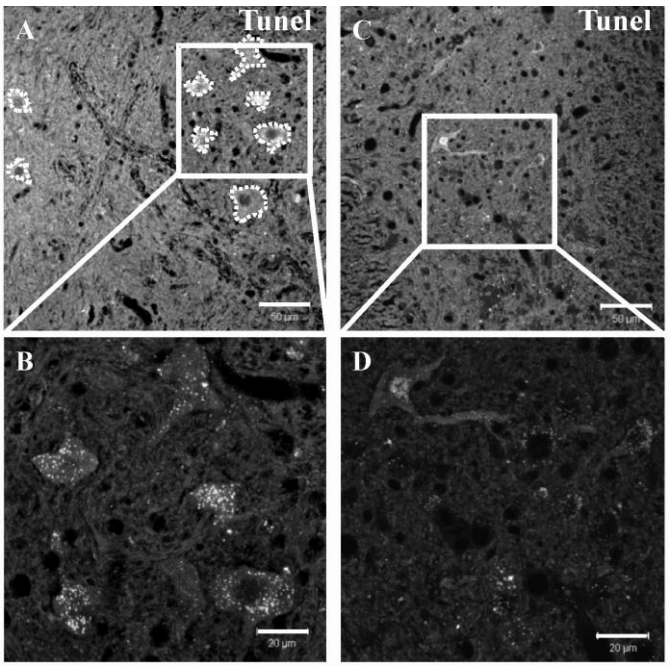

Figure 29. DNA fragmentation revealed by the TUNEL assay in the ventral horns of SOD1 rats at the early and end stages of ALS. TUNEL-positive cells reveal DNA fragmentation in the cytoplasm (most likely mitochondria DNA) of most α-motoneurons 2 weeks after the first symptoms of the disease appeared, 11 days after MSC transplantation (A, B), whereas the nuclear DNA remains TUNEL-negative. The number of MN in the ventral horns at the end stage of the disease in 9 rats, analyzed using a comparative method, was lower compared to rats 11 days after the onset of the ALS (n=2). At the end stage of the disease (10-11 weeks after transplantation) the nuclear DNA of motoneurons begins to show signs of apoptosis (C, D). The dashed circumference around the cell bodies in part A shows the circumference of MNs that were used for measuring the circumference, MN surface area and TUNEL staining intensity. Scale bars 50μm (A, C), 20μm (B, D).

	rBMSC	PBS	p-value
	MN surface area, μm^2		
Thoracic	422.6 ± 70.3	240.2 ± 21.1	0.045
Lumbar	485.9 ± 63.5	317.1 ± 35.8	0.041
	MN circumference, μm		
Thoracic	88.4 ± 6.2	67.5 ± 2.6	0.003
Lumbar	95 ± 4.9	75 ± 5.4	0.014
	Intensity of TUNEL staining, units		
Thoracic	40.6 ± 3.9	51.8 ± 5.1	0.049
Lumbar	36.6 ± 3.3	41.8±1.8	0.087

Table 10. Quantitative analyses of MN surface area, soma circumference and intensity of TUNEL staining. The values of MN surface area, soma circumference and the intensity of TUNEL staining are presented as group mean ± s.e.m.

4.11. The fate of rat BMSC after intraspinal transplantation into symptomatic SOD1-rats.

MSC-treated rats received an intraspinal graft of GFP^+ BMSC at a concentration of $5x10^4$ cells per 1 µl of PBS and an intravenous injection of BMSC at a concentration of $2x10^6$ per 0.5ml of PBS. We found that many GFP^+ BMSC survived in the spinal cords of animals immunosuppressed with cyclosporine until the end of the experiment (65 days after transplantation) and that the cells were able to migrate along the rostra-caudal axis in the white matter and on the surface of the spinal cord in the subarachnoid space (Figure 30 A, B, C, D and E). An attempt was made to determine the number of surviving GFP^+ BMSC in the spinal cords at the end of the experiment. We found that the total number of cells that survived after intraspinal injection until the end stage of the disease was about 60±15% of the initial number that was grafted intraspinally. However, it was very difficult to determine this number more precisely as the number of surviving GFP^+ BMSC at the site of transplantation was quite high, thus making it very difficult to identify (and count) individual cells in the tissue. Transplanted GFP^+ BMSC integrated into the host spinal cord but did not express either the astrocytic marker (GFAP) or the neuronal markers NF-160, MAP2, β-III-tubulin or Nestin (Figure 30 and 31). Unfortunately, we have no evidence for the fate of the GFP-negative BMSC that were implanted intravenously as they were not labeled by any marker. We observed stronger expression of the neurofilament marker NF160 in the cell-treated animals compared to control rats, which could be explained by the paracrine properties of BMSC having neuroprotective and trophic effects on the host neurons (Figure 31 A-H).

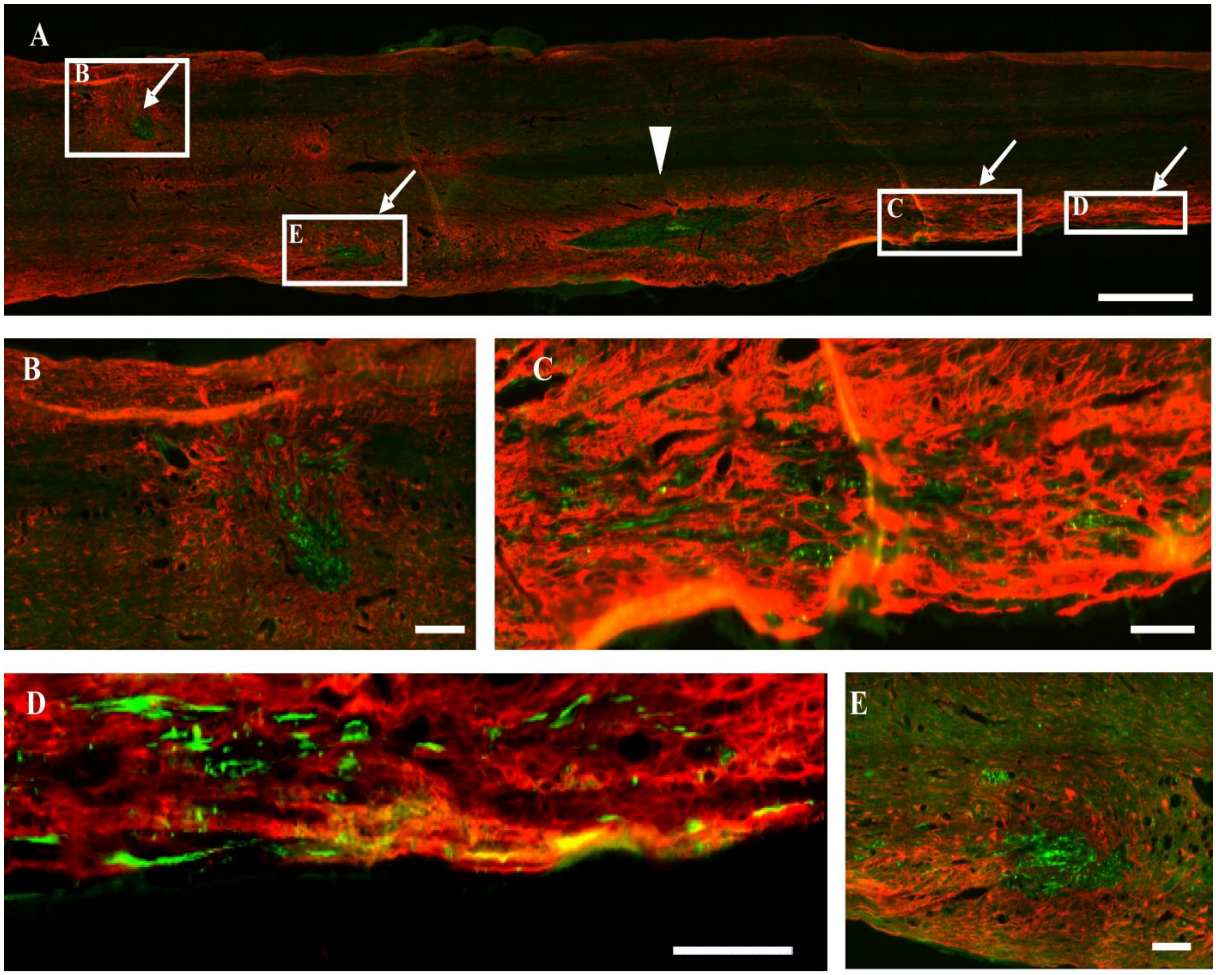

Figure 30. Integration and migration of rat BMSC after intraspinal transplantation. A. Two months after the transplantation of GFP$^+$ BMSC into the spinal cord of SOD1(G93A) rats, BMSC are located mostly in the white matter of the spinal cord and have migrated (arrows) in both the rostral and caudal directions up to 5 mm (scale bars 1000µm). Grafted cells are negative for the astrocytic marker GFAP. Higher magnification views of the migration of the cells within the spinal cord are presented in parts B, C, D and E (scale bars 200µm). The arrowhead shows the injection site. GFP$^+$ BMSC are in green, astrocytes (GFAP$^+$) in red color.

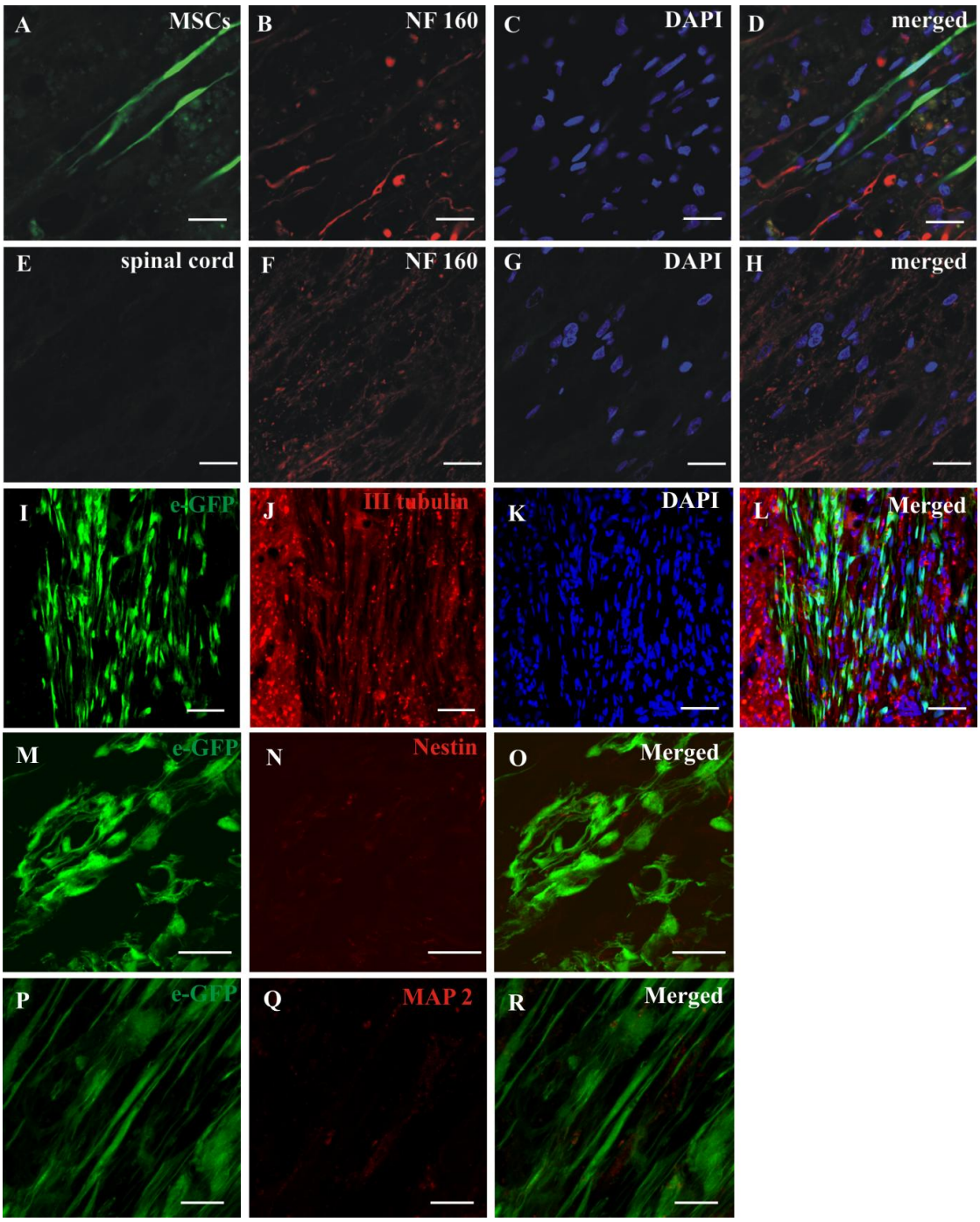

Figure 31. Transplanted rat BMSC did not express neuronal markers in the recipient spinal cord. Neurofilament staining (NF 160) of SOD1 (G93A) rat spinal cords at the terminal stage of the disease in a control animal (E-H) or in a treated animal 65 days after cell implantation (A-D). DAPI was used to stain the cell nuclei (blue). MSC are oriented in the same direction as the host axons (A, B, D). The BMSC-treated animal (A-D) shows the increased expression of NF-160 compared to the sham treated rat, demonstrating the neuroprotective influence of BMSC (E-H). BMSC do not express neuronal markers such as NF-160, MAP2, β- III-tubulin or Nestin (I-R). Scale bars 20μm.

4.12. Effect of the intrathecal implantation of human BMSC on the disease course and overall survival of symptomatic SOD1 (G93A) rats

Mutant rats started to show the first symptoms of ALS at the age of approximately 170-180 days and were considered for transplantation a few days later (Figure 32 A). An intrathecal (via cistern magna) injection of 500 000 human BMSC suspended in 50µl of DMEM significantly extended the lifespan of the cell-treated animals (209.3 days) when compared to the vehicle-injected rats (195.7 days) (Figure 32 B, C). By testing grip strength (Figure 33 A) and locomotor functions (Figure 33 B), we established that the cell-treated animals showed significantly better motility and strength when compared to the rats injected with DMEM. The difference in motor activity started to appear shortly after the delivery of the cells, whereas there was no difference in the dynamics of the body weight loss between the two groups (Figure 33 C).

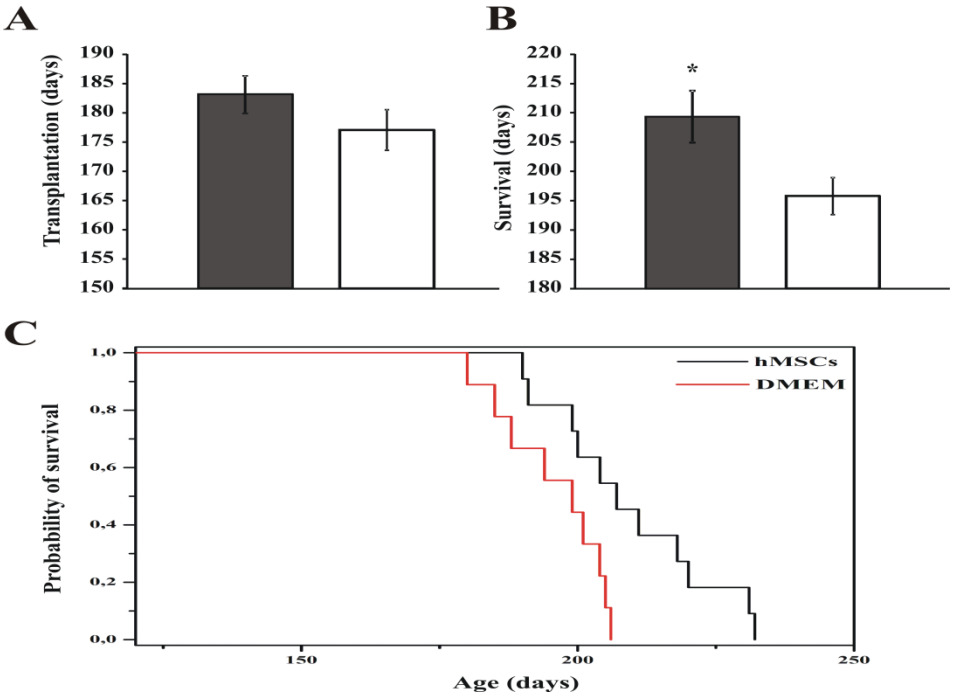

Figure 32. Animal survival after an intrathecal injection of human BMSC into SOD1 (G93A) rats. Animals from both groups were treated with hBMSC (black columns) or vehicle-injected (white columns) at approximately the same period of their life (A). Overall survival in the hBMSC-treated group was significantly longer (p=0.027) comparing with the sham-treated group, by 13.6 days (B). hBMSC-treated animals are shown in black color (n=11), control animals in red color (n=9). Error bars indicate SEM. (Unpublished data)

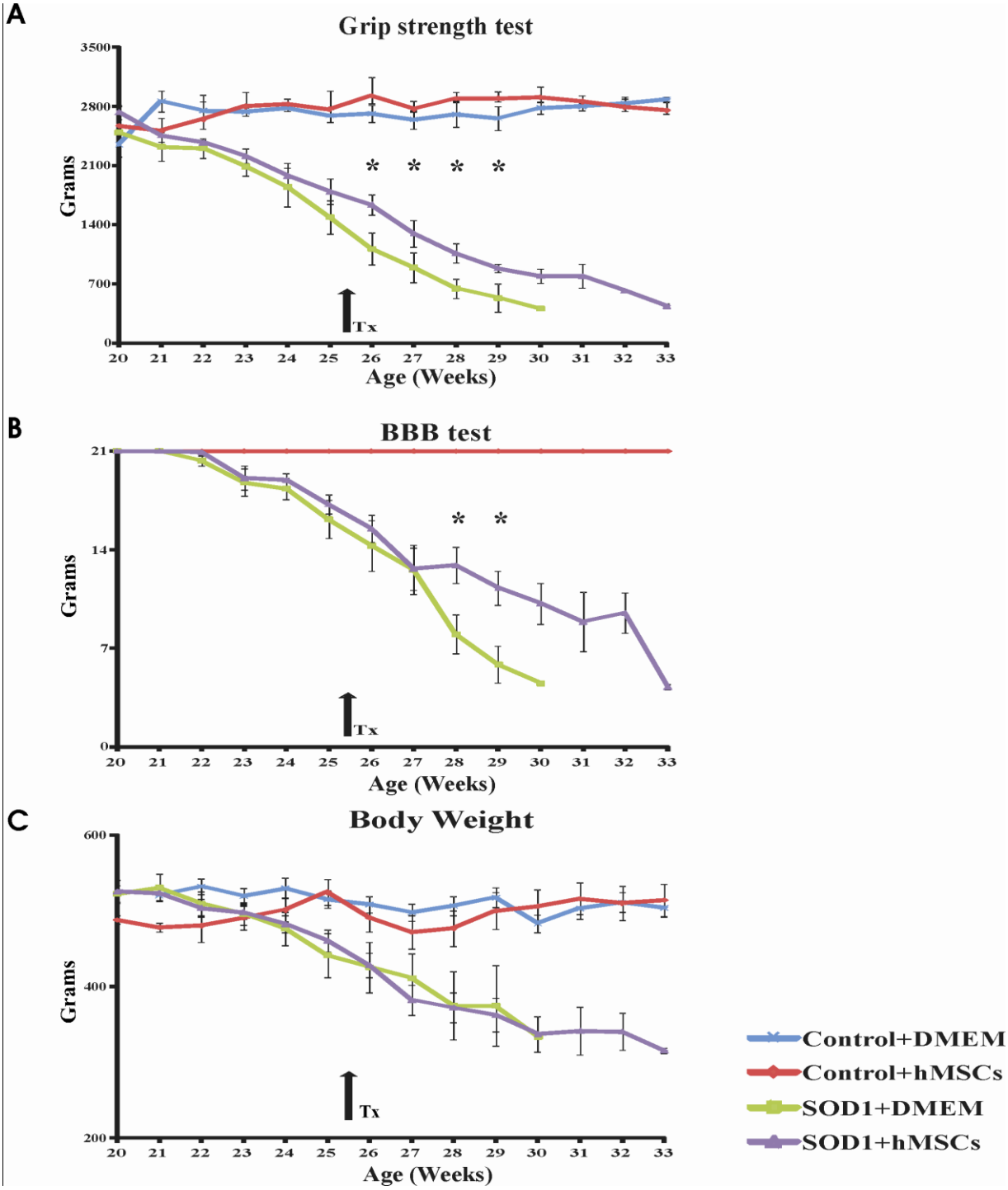

Figure 33. Characteristics of disease progression in groups of SOD1 (G93A) rats treated with hBMSC and DMEM. Shortly after the intrathecal application of hBMSC, the decrease in motor function was slowed down when compared to the sham-treated group of animals. Animals from the cell-treated group showed significantly higher scores on the grip strength (A) and BBB (B) tests. The application of hBMSC did not delay the body weight loss (C). Number of rats in all groups: SOD1-group grafted with hBMSC (n=11), vehicle-injected SOD1-group (n=9), non-SOD1control group injected with hBMSC (n=5) and non-SOD1 rats injected with DMEM (n=5). Significance evaluated at p≤0.05. (Unpublished data)

5. DISCUSSION

5.1. General approach to stem cell therapy in the treatment of neurological diseases.

Diseases of the central nervous system usually have a devastating character, are impossible to treat, and have a very pessimistic prognosis. Maladies such as SCI and ALS develop their symptoms due to dysfunctions in the spinal cord, and the only drugs that are currently used and have been shown to have minor positive effects on the clinical status of patients are methylprednisolon and Riluzole, respectively. Unfortunately, the results of such treatment are still unsatisfactory.

Recent advances in stem cell biology have shown that stem cells might provide an inexhaustible source of neurons and glia and have opened an avenue for therapeutic strategies aimed at neuroprotection or cell replacement in disorders affecting the brain and spinal cord (Lindvall and Kokaia, 2006).

It is expected that after the application of stem cells into the pathological environment resulting from SCI or neurodegenerative diseases, the cells will be able: i) to release neurotrophic factors that will promote and facilitate axonal sprouting; ii) to regenerate damaged nerve tissue through differentiation or transdifferentiation into mature neural cells (such as motoneurons and oligodendrocytes), thus promoting the remyelination of the surviving axons and the restoration of specific functions; and iii) to fill pseudocystic cavities, thus acting as a scaffold that will support axonal outgrowth between the rostral and caudal stumps and to stimulate the revascularization of the damaged nervous tissue (Hejcl et al., Akiyama et al., 2002, Ohta et al., 2004, Nistor et al., 2005, Deshpande et al., 2006, Sykova et al., 2006b, Lee et al., 2007). The first experimental studies aimed at treating neurodegeneration or traumatic injury of the CNS used fetal or embryonic tissue in order to isolate various types of stem or progenitor cells, including ESC,

NSC, spinal precursor cells, glia-restricted precursor cells etc. (McDonald et al., 1999, Hofstetter et al., 2002, Ogawa et al., 2002, Nistor et al., 2005, Xu et al., 2006, Lee et al., 2007). Some of these cells resulted in tumor formation during testing in various animal models, while others resulted in significant functional, behavioral and morphological improvement of the animals' behavior and lead to clinical trials in human patients. For instance, Geron Corporation has recently announced the enrollment of patients with complete, subacute thoracic SCI into clinical trials involving the administration of human ESC-derived oligodendrocyte progenitor cells. This event prompted some concern among the scientific community regarding the lack of any evidence for the replication of the preclinical results in independent laboratories and also in regards to the choice of patients (subacute vs. chronic) and the clinical indication (SCI vs. neurodegenerative diseases) (Bretzner et al., 2011). Another work published by Perrin et al. reported an antipathic effect after an intraspinal graft of either naïve human embryonic NPC or the same cells engineered to express Neurogenin 2; grafted with naïve NPC, rats displayed significantly worse gross motor recovery compared with sham-operated rats, whereas animals treated with Neurogenin 2-engineered NPC show faster recovery as early as 7-10 days after implantation (Perrin et al., 2010). On the other hand, it is also necessary to keep in mind that the transplantation of human embryonic stem cell-derived neural progenitors (at both early and later stages of differentiation) might cause hyperproliferation and the formation of teratomas (Seminatore et al., 2010). We can conclude that the results achieved from the use of embryonic or fetal stem cells are at the cutting-edge of current research but still ambiguous for routine clinical application in patients. Current concerns are: a high risk of tumorigenesis, limited access to human material, as well as logistical, immunological and ethical issues (Widner et al., 1988, Vaquero and Zurita, 2011).

A solution to these issues might be provided by stem cells generated from the adult organism. This alternative to embryonic and fetal cells, if isolated from affected individuals, could be used either as an *in vitro* model of different diseases, helping us to understand the mechanisms underlying the pathological processes, or could be used as therapeutic agents after preliminary cultivation, thus avoiding the risk of GVHD if the donor and recipient are the same individual. Different cell types (e.g., MSC, AMSC, OEG, iPS etc.) possess multipotent properties and could be obtained autologously or generated from healthy living donors or *post mortem* tissue. The cutting-edge of modern cell biology is the generation of functional induced motoneurones from the patient's own fibroblasts, which after transplantation could replace the dying MN of ALS patients (Son et al., 2011). Considering that neurodegenerative diseases most often develop in elderly patients, there are some concerns related to the "quality" of stem cells if generated from an aged organism and whether these cells might be used for cell replacement, neuroprotection and neuregeneration. Dimos et al. showed using the example of iPS cells generated from an 82-year-old woman with a familial form of ALS, that these cells possessed the same properties as do embryonic stem cells and that they could successfully differentiate into motoneurons (Dimos et al., 2008). However, in the case of iPS cells, which are generated using a cocktail of overexpressed transcriptional factors transferred to skin fibroblasts or other somatic cells by transfection of viral vector infection, it is necessary to note that the time is not yet ripe for their use in humans as the risk of genetic modification of both donor and host cells is too great (Wichterle and Przedborski, 2010).

In the current work we show that AMSC and BMSC have a neuroregenerative and neuroprotective effect after their administration into SCI and also in the treatment of ALS. Grafted cells incorporated into the recipients' spinal cords and significantly improved the motor function of paraplegic rats and

positively changed the disease progression and general survival of rats with ALS. Furthermore, several reports have shown that the transplantation of MSC into acute SCI leads to the formation of bundles that bridge the lesion, reduce cavity formation and enhance axonal sprouting (Hofstetter et al., 2002, Wu et al., 2003). Therefore, growing interest in cell therapy approaches utilizing MSC has made these cells among the leading candidates for human application.

5.2. Effect of mesenchymal stromal cells in the treatment of SCI.

It is increasingly clear that MSC present a very attractive source of cells for reparative therapy (Rice and Scolding, 2008). The MSC population, isolated from either bone marrow or adipose tissue, has been shown in *in vitro* studies to express a large variety of neuronal genes, transcription factors with potential neural involvement suggesting a wide differentiation potential (Blondheim et al., 2006, Zhu et al., 2008). Our results obtained from *in vitro* experiments revealed that predifferentiated adipose-derived MSC express NCAM, NG2, S100 and p75 and the neural precursor markers NCAM and Nestin; however, these cells did not show the expected neuronal electrophysiological properties. The *in vitro* characteristics of BMSC were already published earlier. For example, our results are in agreement with those reported by Tropel et al., who showed the *in vitro* differentiation of MSC along the neuronal pathway toward a functional phenotype; however, these cells were not tested *in vivo*, thus it is hard to evaluate their regenerative possibilities (Tropel et al., 2006).

We had this idea in mind when designing experiments to explore the use of AMSC and pAMSC as an alternative source of MSC that could be derived from human adipose tissue and could potentially be used for the treatment of patients with SCI. To our knowledge, we were the first to report that the implantation of naïve and pAMSC into paraplegic rats after a balloon-induced compression lesion

promotes the recovery of motor function without causing any side effects. Another work recently published by Ra et al. also reported the lack of any tumorigenicity after the intravenous injection of human AMSC into immunodeficient mice; moreover, the intravenous injection of autologous AMSC into patients with SCI did not show any adverse effects connected with the procedure (Ra et al., 2011). These results are also in agreement with previous publications from our group, suggesting that AMSC could be used as an alternative to BMSC for the cellular therapy of patients with SCI (Amemori et al., Sykova et al., 2006a, Sykova et al., 2006b, Urdzikova et al., 2006). Indeed, some studies, after comparing the *in vitro* properties of AMSC and BMSC, have indicated that AMSC produce a significantly larger amount of cytokines and growth factors compared to BMSC, thus they could have a broader therapeutic range (Banas et al., 2008).

The therapeutic effect of MSC transplantation could be explained by a number of their features. Among other, MSC have unique immunologic properties: they are not immunogenic, do not stimulate alloreactivity, escape lysis by cytotoxic T-cells and natural killer (NK)-cells, and can be transplanted across MHC barriers and between human leukocyte Ag (HLA)-mismatched individuals (Le Blanc, 2003). It was also reported that the intravenous delivery of BMSC enhances remyelination throughout a focal demyelinated spinal cord lesion (Akiyama et al., 2002). Intraspinal grafting of BMSC into the injured spinal cord was shown to promote axonal regrowth and to reduce the lesion volume (Gu et al., 2010). Our results also support the idea that adult MSC fulfill the requirements for a potential therapeutic agent for regenerative purposes: we have shown that the intraspinal or systemic (intravenous) administration of MSC isolated from either bone marrow (rat or human) or adipose tissue is a relatively simple and safe procedure and that the recipient animals tolerated the grafting procedure very well and did not show any side effects connected with the surgery itself or with any uncontrolled cell

proliferation in the postoperative period (Turnovcova et al., 2009, Arboleda et al., 2011, Forostyak et al., 2011). It is also important to note that after acute SCI, there is a therapeutic time window within which the application of stem cells can ameliorate the consequences of secondary injury by preserving rather than replacing the host nervous tissue. Cellular therapy during the chronic phase of SCI aims to reconstruct the spinal cord via cellular replacement, glial scar modification, axonal guidance and the filling of formed syringomyelia, thus leading to functional regeneration (Hejcl et al., Zurita and Vaquero, 2004, Zurita et al., 2008).

All of the above properties of MSC, along with long experience with the transplantation of BMSC in the treatment of haematological malignancies, lead to the first preclinical and clinical trials, initially to treat myocardial infarction and later to treat stroke, ALS, PD and other diseases of the CNS (Bang et al., 2005, Schachinger et al., 2006). These and other trials utilizing different methods of BMSC application showed that the grafting of such cells is a safe procedure that can bring benefits for patients (Sykova et al., 2006a, Urdzikova et al., 2006, Mazzini et al., 2011, Vaquero and Zurita, 2011).

Experimental studies suggest that the therapeutic effect of grafted cells starts before the establishment of a tissue bridge suitable for the passage of axons, therefore the recovery of neurological functions at the early post-transplantation stage could be explained by the activation of different regenerative processes, mainly the release of neurotrophic factors (Zurita and Vaquero, 2004). Based on preclinical experiments in rats with SCI that showed significant improvement in behavioral scores (BBB test and plantar test) after the intravenous implantation of BMSC labeled with iron oxide nanoparticles 7-21 days post-injury, followed by *in vivo* magnetic resonance imaging (MRI), a nonrandomized phase I/II clinical study was started in August 2003 in patients at the Motol faculty hospital in Prague (Jendelova et al., 2004, Sykova and Jendelova, 2005, 2006). In this study

autologous BMSC were grafted intraarterially via *arteria vertebralis* or intravenously into 20 patients with SCI at the cervical or thoracic level, and the effect of the treatment was evaluated by the ASIA protocol, the Frenkel score system and electrophysiological measurements of motor and somatosensory evoked potentials (MEPs and SEPs) 3, 6 and 12 months after cell administration (Sykova et al., 2006a). The results of the trial showed that the transplantation of the cells is a safe procedure. The most significant regenerative effect was observed in a few patients who received cells during a therapeutic window of 3-4 weeks after SCI, whereas those transplanted during the chronic phase of SCI did not show significant improvement of locomotor or sensory function. These results also correlate well with those reported from clinical trials performed by Park et al. and Cristante et al., in which stem cells were used in the treatment of complete SCI followed by neurologic evaluation in 6 and 39 patients, respectively (Park et al., 2005, Cristante et al., 2009). Autologous MSC transplantation also has been shown to have a positive effect and to be a safe procedure in patients with a severe cerebral infarct (Bang et al., 2005). Even though some small series of experiments involving patients showed an improvement of motor and sensory functions after the administration of BMSC, significant hurdles remain before these findings can be responsibly translated to novel therapies. In particular, we need to better understand the mechanisms of action and the behavior of stem cells in the pathological environment after transplantation; other clinical trials with larger and more homogenous groups of patients are needed, so as to enable better comparison with control treatments (Lindvall and Kokaia).

5.3. Effect of mesenchymal stromal cells in the treatment of ALS.

Studies examining the effect of stem cells on asymptomatic animal models of ALS have shown a positive effect on motor activity and survival after the intravenous

transplantation of human umbilical cord blood (hUCB) and mouse and rat neural stem cells, the intraspinal grafting of mouse BM cells and the intrathecal delivery of hMSC (Kim et al., Mazzini et al., 2004, Garbuzova-Davis et al., 2008, Vercelli et al., 2008, Mitrecic et al., 2010). However, these studies cannot have any direct relevance for the treatment of human patients as there are no specific markers for diagnosing ALS in advance of the first symptoms (Turner et al., 2009). We have shown that, even after symptoms have appeared, the combined (intraspinal and intravenous) transplantation of rat BMSC as well as the intrathecal transplantation of hMSC can result in a neuroprotective effect that increases motor activity and extends the lifespan of ALS rats. Our results confirm those of Boucherie et al., who used symptomatic ALS rats for the intrathecal delivery of hMSC, with a similar outcome (Boucherie et al., 2009). The very rare (< 1%) expression of neuron-like markers such as nestin and MAP2 in MSC has been reported after intraspinal transplantation into SOD1 mice, while others have reported massive differentiation into astrocytes (Vercelli et al., 2008, Boucherie et al., 2009). In our study we did not observe NF160-, MAP2-, βIII-tubulin- or nestin-positive cells among the transplanted GFP^+ MSC. However, there was a higher expression of neurofilaments in the cell-treated animals compared with the rats injected with PBS. This suggests that the implanted cells did not transdifferentiate but were nonetheless able to protect the host environment. Even more, we have shown using the TUNEL assay that the engraftment of BMSC into symptomatic animals influenced the extent of apoptosis in MN and supported the survival of larger neurons. Our results could be explained by the activation of BCL2 genes by MSC or by the inhibition of caspases (especially 1 and 3), which have been shown to be active in spinal MN in the ALS animal model (Kostic et al., 1997, Li et al., 2000).

We also have to consider the fact that the effect of MSC is dose- and passage- dependent. It has been established earlier by our group that BMSC of

early passages (up to the fifth) are the most suitable for inducing a neuroprotective effect on the post-traumatic spinal cord. The work by Choi et al. has shown that MSC from earlier passages are more suitable for stem cell therapy because of their stability, anti-inflammatory and neuroprotective effects (Choi et al., 2010). In our experiments involving the intraspinal and intravenous application of passage four rBMSC (10^5 and $2x10^6$ cells, respectively), we have shown that this combination is effective in providing a neuroprotective effect and prolonging the survival of symptomatic SOD1 (G93A) rats. We also performed experiments involving the intrathecal delivery of hBMSC. Using this approach, we have seen that injecting $5x10^5$ hBMSC intrathecally is sufficient to cause a significant difference in the motor activity and survival of rats at the early stage of ALS when compared with a vehicle-injected group of animals. Our results are more relevant to the real clinical situation and are in accordance with previously published works describing the positive effect on asymptomatic SOD1 animals induced by the application of 10^6 cells, while 10^5 cells failed to extend the lifespan or to increase the MN count in the same animal model (Kim et al., Habisch et al., 2007). Another published work employing the intrathecal delivery of hMSC into symptomatic ALS rats, co-localized a decreased motoneuronal loss in the lumbar spinal cord, preserving motor function and extending the survival of SOD1(G93A) rats with decreased inflammation, the attenuated proliferation of microglial cells and the reduced expression of COX-2 and NOX-2 (Boucherie et al., 2009).

To deliver a sufficient number of cells intraspinally into patients would require a rather invasive approach, including a laminectomy and opening of the meninges of patients who are already affected by a devastating disease. In the literature there are indications that the blood-brain barrier is affected in SOD1 transgenic animals, thus allowing transplanted cells to pass through the barrier following intravenous administration and exerting a local neuroprotective influence

on the host cells (Garbuzova-Davis et al., 2007). Recently, it has been reported that 7 days after the intravenous injection of neural stem cells into the tail vein, up to 6% of the cells were found in the central nervous system of pre-symptomatic ALS rats, up to 13% in symptomatic ALS rats, while only up to 0.3% in wild-type rats (Mitrecic et al., 2010).

The successful application of MSC in rodent models of FALS established a platform for clinical studies in human patients (Vercelli et al., 2008). The outcome after nearly 9 years of monitoring 19 ALS patients, enrolled in two phase I clinical trials, showed no clear clinical benefits in these patients. However, the collected data show support for the implantation of autologous bone marrow MSC into the dorsal spinal cord, as no structural changes (including tumor formation) or deterioration in psychosocial status were found, and all patients coped well with the procedure (Mazzini et al., 2003, Mazzini et al., 2010, Mazzini et al., 2011). Another clinical study used the transplantation of mononuclear CD133(+) autologous stem cells from the peripheral blood into the frontal motor cortex of ALS patients (Martinez et al., 2009). This method of cell application significantly prolonged the survival of the treated patients and the maintenance of their lifestyle compared with untreated control patients. Deda et al. reported the results of a one year follow-up after the implantation of bone marrow-derived hematopoietic progenitor stem cells into the anterior part of the spinal cord of thirteen patients with a bulbar form of SALS: nine patients became much better compared with their pre-operative status; one patient was stable without any decline or improvement in his status; and three patients died 1.5, 2 and 9 months, respectively, after stem cell therapy as a result of lung infection and myocardial infarction (Deda et al., 2009). Taking together the current state of research, we can conclude that the recruitment and selection of appropriate patients into larger trials will be needed to test the efficacy of MSC treatment.

5.4. PNN and plasticity in the adult CNS.

5.4.1. Modulation of neuroplasticity with chondroitinase.

The last decades have brought new evidence of the considerable capacity of the CNS for some degree of recovery following injury, which can be further enhanced by influencing plasticity through the digestion of PNN with chondroitinase ABC (Fawcett et al., 2007, Kwok et al., 2008, Bartus et al., 2011). Barritt et al. were the first to specifically demonstrate that a bacterial enzyme isolated from Proteus vulgaris called chaseABC can induce plasticity in both injured and intact spinal pathways within the spinal cord following injury (Barritt et al., 2006). Both acute and delayed treatment with chaseABC has been shown to promote the significant rescue of injured rubrospinal projection neurons, restoring cell area in the injured red nucleus up to 80% and 70%, respectively (Carter et al.).

Chondroitinase ABC acts through the digestion of the condensed compartment of the ECM around neurons in the form of PNN. The persistence of CSPG-containing PNN throughout life is thought to be crucial for the maintenance of the structural integrity of synaptic contacts (Bartus et al., 2011). However, under pathologic conditions such as SCI or TBI, the adult mammalian CNS is hindered from spontaneous recovery. As already mentioned, the condensed ECM of PNN contains several CSPG together with long chains of hyaluronan (HA), tenascin-C and tenascin-R with the addition of one or more link proteins (Crtl1/Hapln1 and Bral2/Hapln4) and larger amounts of tenascin-R; all of these molecules form a dense cartilage-like structure. Interestingly, despite our knowledge about the composition of PNN, until now it has been a mystery which molecule produced by PNN-enveloped neurons triggers their formation.

We have shown for the first time that the production of link protein is the major event that triggers the formation of PNN in the extracellular matrix and eventually limits plasticity in adult CNS structures such as the cuneate nucleus and

visual cortex (Carulli et al., 2010a). Anatomical plasticity was evaluated using an elegant model previously used by Massey, in which terminal sprouting into denervated territory has been shown to be enhanced by chaseABC treatment (Massey et al., 2006). They reported no growth of the preserved terminals into the denervated regions, but after treatment with chaseABC, the residual sensory inputs sprouted into the denervated regions. We found that Crtl1 KO animals responded to partial denervation of the cuneate nucleus similarly as did chaseABC-treated animals, with sprouting of the residual axons into denervated regions of the cuneate nucleus, but this did not occur in control animals. Our results suggest that in adult animals, the presence of PNN is the key factor in the extracellular matrix-related restriction of plasticity. Evidence for this also comes from an earlier publication by Apostolova et al., who reported that animals lacking tenascin-R, have abnormal PNN and retain some plasticity in adulthood (Apostolova et al., 2006). Our results, together with previously published findings, provide direct evidence that PNN control plasticity in the CNS and suggest that digestion of these structures is how ChABC reactivates CNS plasticity.

5.4.2. Modulation of plasticity and regeneration by a combination of chondroitinase and stem cells.

Approximately 70–80 percent of spinal neurons are encapsulated by PNN, and these are particularly abundant around projection and large motoneurons (Murakami et al., 1995). For this reason, we can also hypothesize that the role of PNN is not limited to the restriction of plasticity, but that they could also be involved in the mechanisms underlying the development of CNS pathologies or serve as a possible biological marker to diagnose motoneuron diseases. In our experiments we observed attenuated PNN around the spinal MN of SOD1-rats at the terminal stage of ALS (data not shown). Collins and Bowser reported a

decreased level of tenascin-R protein in the CSF as well as a striking loss of normal tenascin-R immunoreactivity around the cell bodies and processes of MN and also the aberrant morphology of PNN in the spinal cord of ALS patients. These authors suggested that the changes in the extracellular matrix are implicated in the pathogenesis of ALS (Collins M. A., 2011). Considering these findings, we can speculate that the extensive migration of intraspinally grafted MSC, found in our experiments involving SOD1 rats, might be explained by the lower density of the ECM caused by attenuated PNN, as reported in studies combining chaseABC with stem cell implantation.

The ability of chaseABC to promote plasticity and functional recovery after acute and chronic injury of the mammalian spinal cord is extremely encouraging in translational medicine (Tester and Howland, 2008, Garcia-Alias et al., 2009). However, considering the complex pathways and interactions within the spinal cord and in the CNS generally, a combination of chaseABC with other strategies might bring even better results. The combination of chaseABC with NPCs promoted the engraftment and migration of the transplanted cells and induced the outgrowth of a greater number of growth-associated protein-43-positive fibers at the lesion epicenter, compared with NPC transplantation alone (Ikegami et al., 2005). Similar effects were achieved after the intraspinal transplantation of olfactory mucosa progenitor cells in combination with chaseABC (Huang et al.). Karimi-Abdolrezaee used a combination of chaseABC, NPC and growth factors to enhance neuroanatomical plasticity in the chronically injured spinal cord; this strategy significantly improved neurobehavioral recovery and axonal integrity, promoted the plasticity of the corticospinal tract, enhanced the plasticity of descending serotonergic pathways, and was accompanied by the better integration, extensive migration and differentiation of NPC into oligodendrocytes within the recipient spinal cord (Karimi-Abdolrezaee et al., 2010). Interestingly, apart from the

improvement of motor and sensory functions, combined cell implantation and chaseABC delivery can be beneficial for bladder function after complete SCI (Fouad et al., 2009). A recent publication has shown that implanting an autologous peripheral nerve graft into the animals that underwent a C2-hemisection combined with chaseABC treatment, led to an increase in the number of serotonergic fibers, promoted local sprouting, and enhanced graft entry and exit of axons, which in turn promoted the functional regeneration of respiratory pathways in the spinal cord (Alilain et al., 2011).

Encouragingly, as outlined above, new strategies offer interesting perspectives for neuroanatomical and functional recovery in spinal cord pathologies. However, the truth is that before human clinical application can become routine, many significant challenges still remain, most notably in assessing the safety, routes and patterns of administration and biodistribution, as well as a better understanding of the molecular mechanisms underlying the beneficial effects of different therapeutic agents. Further studies in non-human primates will also be keenly anticipated in the future and might bring us closer to the treatment of human patients.

6. CONCLUSIONS

The main findings of the thesis are summarized below:

1. We have optimized protocols for cell delivery (intraspinal, intravenous and intrathecal) and have shown that the transplantation of MSC by any of the above protocols is a safe procedure.
2. An *in vitro* analysis of pAMSC revealed the enhanced expression of neurotrophic factors and neural markers as well as better survival in the host tissue after implantation into SCI if compared with naïve AMSC. Both pAMSC and naïve AMSC interacted closely with the host tissue, wrapping host axons and oligodendrocytes, and rarely expressed NG2 or CD31, but not neuronal markers.
3. We established that the organization of CSPG into perineuronal nets is triggered by the synthesis of Crtl1by neurons and therefore that this is a key event in diminishing plasticity in the adult CNS.
4. Animals lacking the link protein Ctrl1 (Hapln1) responded to partial denervation of the cuneate nucleus similarly as did chaseABC-treated animals, with the sprouting of the residual axons into the denervated regions.
5. The combined grafting of naïve rBMSC into symptomatic rats decelerates the loss of motor functions and increases the general survival of SOD1 (G93A) transgenic rats. Treated rats had a larger number of MNs, and these MN were of a larger size and less affected by apoptosis.
6. Intraspinally grafted GFP^+ rBMSC survived until the end stage of familial ALS, migrated in the white matter both rostrally and caudally from the injection site and did not differentiate into either neuronal or glial phenotypes.
7. The intrathecal delivery of hBMSC into symptomatic rats significantly prolongs motor function and the overall survival of the animals when compared to a vehicle-injected group of animals.

7. SUMMARY

Adipose-derived MSC could be used as an alternative for bone marrow MSC in the treatment of acute SCI. We used the intraspinal grafting of rat adipose-derived naïve and predifferentiated MSC to improve motor function after a balloon-induced compression lesion of the spinal cord. Grafted cells survived for seven weeks after transplantation, improved motor activity and integrated into the host tissue. They expressed the oligodenrocyte precursor marker NG2 and, occasionally, the astrocytic marker GFAP, but did not transdifferentiate into a neuronal phenotype.

Bone marrow MSC may change the disease course and extend lifespan in a rat model of ALS. Combined intraspinal and intravenous transplantation of rat BMSC was performed in symptomatic rats overexpressing the SOD1 G93A gene. Cell-treated animals lived longer compared with sham-treated rats and displayed significantly improved motor activity and grip strength. Rat BMSC survived until the end stage of the disease and were migrating along the white matter of the spinal cord. Grafted cells increased the number of host cells displaying positive staining for neurofilaments and significantly increased the number and also the size of the remaining spinal motoneurons 10-11 weeks after delivery, compared with vehicle-injection. The defragmentation of DNA, a sign of apoptosis, was less pronounced after combined cell therapy.

The effect of intrathecal (cistern magna) application of human BMSC on the motor function and survival of SOD1 G93A rats was evaluated after confirming the disease onset. The injection of hBMSC into the cerebrospinal fluid of symptomatic rats resulted in a slower decline of motor function and prolonged survival compared to vehicle-injected rats.

Perineuronal networks are found in the extracellular matrix around neurons; the digestion of these structures with chaseABC reactivates CNS plasticity. The

molecule that triggers PNN formation was identified and also direct evidence that PNN control plasticity in the adult CNS was found: mice lacking Crtl1 sprouted residual axons into the partially denervated cuneate nucleus after dorsal hemisection, between levels C6 and C7, in a similar manner as did chaseABC-treated animals.

Mesenchymal stromal cells are multipotent cells that currently are one of the best candidates for the treatment of neurological disorders such as spinal cord injury and amyotrophic lateral sclerosis. The results described in the thesis show that the transplantation of MSC, isolated either from fat tissue or bone marrow, is a safe and effective procedure that enhances regeneration/neuroprotection after SCI and in ALS. A combination of MSC with chaseABC in the treatment of SCI could facilitate regeneration and thus could be an attractive approach for translational bench-to-bedside studies.

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9. LIST OF AUTHOR'S PUBLICATIONS

1. **Forostyak S**, Jendelova P, Kapcalova M, Arboleda D, Sykova E. Mesenchymal stromal cells prolong lifespan in a rat model of amyotrophic lateral sclerosis. Cytotherapy. 2011 Oct; 13(9):1036-46. Epub 2011 Jul 8. **(IF 2.925)**
2. Arboleda D, **Forostyak S**, Jendelova P, Marekova D, Amemori T, Pivonkova H, Masinova K, Sykova E. Transplantation of Predifferentiated Adipose-Derived Stromal Cells for the Treatment of Spinal Cord Injury. Cell Mol Neurobiol. 2011 Oct; 31(7):1113-22. Epub 2011 Jun 1.**(IF 2.423)**
3. Carulli D, Pizzorusso T, Kwok JC, Putignano E, Poli A, Forostyak S, Andrews MR, Deepa SS, Glant TT, Fawcett JW. Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. Brain. 2010 Aug; 133(Pt 8):2331-47. Epub 2010 Jun 20. **(IF 9.490)**

Overall IF of all publications: 14.838

10. MEETING ABSTRACTS

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