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L'essentiel c'est la santé.

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«Quand on ne sait pas, on ne se pose pas trop de questions, mais quand on commence à disposer d'un début d'explication, on veut à tout prix tout savoir, tout comprendre.» (Bernard Werber).

Il serait injuste de ma part de commencer mes remerciements sans commencer par tous mes professeurs de biologie. Je n'ai pas la prétention de dire que ma passion pour les sciences du vivant m'est apparue comme par magie, c'est eux qui m'ont passionné. Cette phrase de Weber résume parfaitement cet état d'esprit dans lequel j'étais durant mes études : comprendre la complexité du vivant depuis une vision simpliste des premiers cours de SVT jusqu'à l'infiniment complexe, la recherche. Voilà qu'elle était la clef de ma motivation. Sans aucune surprise, la recherche scientifique s'est imposée à moi comme une évidence, mais le chemin était déjà tortueux.

Lorsque que j'ai entrepris de trouver un doctorat, j'étais déjà fasciné par les cellules souches et les espoirs qu'elles portaient. Malheureusement, à cette époque, les thèses sur ce sujet n'étaient pas légion. Un peu têtu, j'ai préféré l'expatriation plutôt que de me détourner de mon sujet de prédilection. C'est à ce point que je veux remercier, du fond du cœur, toute ma famille. Il n'est pas facile de se séparer d'un fils, d'un frère ou d'un tonton. Pourtant, ils m'ont tous soutenu, sans exception. Leurs encouragements ont été fondamentaux dans la réussite de cette thèse, je ne pourrai jamais assez les en remercier.

« Apprenez à penser et vous découvrirez tout ce que vous êtes capable de comprendre. » (Oswald Wirth).

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« ***Qui pense peu, se trompe beaucoup.*** » (Léonard de Vinci).

Les découvertes découlent d'expériences qui elles-mêmes sont entreprises parce que, à un moment ou un autre, une idée surgit. Il faut bien se rendre compte de la nécessité d'émettre des hypothèses, de les partager et de se remettre en question quand on vous apporte des critiques constructives.

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« *L'envie de savoir est le plus puissant moteur humain.* » (Bernard Weber).

On reproche souvent aux scientifiques de se concentrer un peu trop sur leurs éprouvettes et en perdre la perception du monde qui les entoure. La recherche scientifique et les avancées médicales ne concernent et n'intéressent pas seulement les scientifiques eux-mêmes mais toute la population. J'espère ne pas être tombé dans ce travers. Les rares occasions qui m'ont permis d'expliquer mon travail au grand public m'ont confirmé qu'il était à la fois intéressé et émerveillé par notre métier. Mes travaux n'ont pu être possibles que grâce à leur générosité à travers l'opération TELEVIE. Ils veulent que l'on progresse. Merci à tous ces anonymes.

« *Dans les sciences, le chemin est plus important que le but. Les sciences n'ont pas de fin.* » (Chargaff).

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Cédric

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Summary

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal, degenerative disorder of the CNS. The hallmark of this disease is the premature and selective death of upper and lower motor neurons (MNs) in the brain and spinal cord, leading to fatal paralysis.

Although the archetypal vision of neurotoxicity in neurodegenerative diseases is based on the idea that a specific neuronal population is particularly vulnerable to a cumulative toxic event (protein aggregation, mitochondria dysfunction, compromised axonal transport etc...), experimental evidence illustrate that ALS possibly does not arise strictly from damage within MNs. There is now convincing data supporting a non-cell autonomous mechanism in which neurodegeneration is influenced by the toxicity of non-neuronal cells in the vicinity of neurons such as astrocytes and microglia. Considering the accumulation of data implicating astrocytes in the pathogenesis of ALS (loss of GLT-1, secretion of toxic factor, enhanced inflammation, etc...), approaches aiming at replacing astrocytes at site of lesions constitute promising therapeutic strategies.

Rapid progresses in the characterization of adult stem cell biology have generated considerable enthusiasm for the development of therapeutic strategies for CNS insults. Several observations support the hypothesis that stem cells may display a valuable influence on

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diseased host tissues by exerting a protective “chaperone” effect to neurons after differentiation in glial cells.

Hence, we decided to study the neuroprotective potential of adult mesenchymal stem cells (MSCs) in ALS. In contrast to neural stem cells (NSCs) which localization in the central nervous system complicates their isolation, MSCs are easily isolated from the bone marrow. The relevance of using on MSCs in stem cell therapies of neurodegenerative disorders is also justified by their capacity to (trans)differentiate into neural cells. For this purpose, we exposed MSCs to growth factors involved in the astroglial differentiation of NSCs.

The differentiation of MSCs was characterized by the acquisition of astrocyte morphology in addition to an increased expression of gene related to NSCs (nestin) and astrocytes (glutamine synthetase). The astroglial differentiation of MSCs is associated with the acquisition of a glial-like specific regulation of the production of GDNF, a potent neurotrophic factor for neurons. Then, we characterized the glutamate uptake in differentiated MSCs, a critical function of astrocytes. Our data demonstrate that the differentiation of MSCs is associated with an increased expression of the high affinity glutamate transporter, GLT-1. Thus, our *in vitro* results confirm the astrocytic differentiation potential of MSCs and we decided to use then in stem cell therapy of ALS.

Indeed, we demonstrated that mechanism of stem cell recruitment is present in the spinal cord during the development of the disease by the secretion of stem cell factor (SCF). We injected MSCs derived from healthy animals into the cerebrospinal fluid of a transgenic rat model of familial ALS (expressing a mutated form of the human superoxide dismutase-1, hSOD1^{G93A}) at disease onset. MSCs were found to infiltrate the nervous parenchyma and migrate substantially into the ventral grey matter by interacting with the SCF. At the site of lesion, MSCs differentiated massively into astrocytes around MNs. The intrathecal delivery of MSCs preserved motor functions and extended the survival of hSOD1^{G93A} rats. Investigation of the lumbar spinal cord 35 days after graft demonstrated that the generation of healthy astrocytes from MSCs decreased motor neuron loss. However, this beneficial effect is not related to a decreased excitotoxicity by the rescue of GLT-1 expression but rather a decreased inflammation around MNs.

Together, the data presented in this thesis highlight the protective capacity of adult MSC-derived astrocytes in the treatment of ALS.

List of abbreviations

AD: Alzheimer's disease	ELISA: enzyme linked immunosorbent assay
ALS: amyotrophic lateral sclerosis	ERK: extracellular signal regulated kinase
AMPA: 1-4 the α - amino-3 hydroxy-5 methylisoazol-4 propionate	ESCs: embryonic stem cells
ANG: angiogenin	FACS: fluorescent activated cell sorting
ATP: adenosine tri-phosphate	FDA: food and drugs administration
BBB: blood-brain-barrier	FBS: fetal bovine serum
BCL-2: B-cell lymphoma-2	FGF: fibroblast growth factor
BDNF: brain derived neurotrophic factor	G-CSF: granulocyte-colony stimulating factor
BrdU: 5-bromo-2-deoxyuridine	GDNF: glial derived neurotrophic factor
BSA: bovine serum albumin	GFAP: glial fibrillary acidic protein
CHAPS: 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate	GLAST: glutamate aspartate transporter
CNS: central nervous system	GLT-1: glutamate transporter-1
CNTF: ciliary neurotrophic factor	GluR2: glutamate receptor subunit-2
COX-2: cyclooxygenase-2	GS: glutamine synthetase
CSF: cerebrospinal fluid	HD: Huntington's disease
DAPI: 4',6-diamidino-2-phenylindole dihydrochloride hydrate	HGF: hepatocyte growth factor
DHK: dihydrokainate	HSCs: hematopoietic stem cells
DNA: deoxyribonucleic acid	HSP: heat shock protein
DTT: dithiothreitol	IARS-2: isoleucine-tRNA synthetase-2
EAAT: excitatory amino acid transporter	IGF-1: insulin-like growth factor-1
EAE: experimental autoimmune encephalomyelitis	IGHMBP-2: immunoglobulin mu binding protein-2
EGF: epidermal growth factor	

List of abbreviations

IL: interleukin	Rac-1: ras related C3 botulinum toxin substrate-1
iNOS: inducible nitric oxide syntase	RNA: ribonucleic acid
iPS cells: induced pluripotent stem cells	ROS: reactive oxygen species
kDa: kilo Dalton	SCF: stem cell factor
Kir channel: inwardly rectifying potassium channel	SDF-1: stromal derived factor-1
LNGF: low-affinity nerve growth factor	SMN: survival motor neuron
LPS: lipopolysaccharide	SOD1: Cu/Zn superoxide dismutase 1
MAP-2: microtubule associated protein-2	SSEA: stage-specific embryonic antigen
MAPC: multipotent adult progenitor cell	TGF-β: tumor growth factor- β
MAPK: mitogen activated protein kinase	TNF- α: tumor necrosis factor- α
mCOX-1: mitochondrial cytochrome C oxydase subunit-1	t-PDC: L- <i>trans</i> -pyrrolidine-2, 4-di-carboxylic acid
MN: motor neuron	UCBCs : umbilical cord blood cells
MSCs: mesenchymal stem cells	VAMP : vesicle-associated membrane protein
NADPH: nicotinamide adenine dinucleotide phosphate	VEGF: vascular endothelial growth factor
NO: nitric oxide	
NT-3: neurotrophin-3	
PBS: phosphate buffered solution	
PCR: polymerase chain reaction	
PD: Parkinson's disease	
PGE-2: prostaglandin E-2	
PMSF: phenylmethylsulfonyl fluoride	

Introduction

1. The concept of stem cells

A stem cell is characterized by three fundamental properties that distinguish them from other cell types. First, a stem cell has the ability of long lasting self-renewal. Second, a stem cell is unspecialised and finally it is able to acquire the characteristics of a range of specialised cell types under physiological (during development and regeneration) and experimental conditions.

1.1. Embryonic stem cells

1.1.1. What is an embryonic stem cell?

The very first stem cell in the life of an organism is the zygote. Once sperm fertilizes the egg cell, it gives rise to a new cell called the zygote. This cell is termed “*totipotent*” as it has the property to engender all the cells that compose an embryo and support its development (placenta and umbilical cord) *in utero*. Early stages of embryonic development give rise to the blastocyst, which is composed of three different structures: the trophoblast, which is the layer of cells that surround the blastocyst; the blastocoel, which is the cavity of the blastocyst and the inner cell mass, a cluster of cells (~ 30 cells) containing embryonic stem cells (ESCs) (Fig. 1).

ESCs are “*pluripotent*”, because they are responsible for the generation of all the cells of an embryo. They give rise during

development to all derivatives of the three germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into every of the ~200 cell types of the adult organism (Fig. 2).

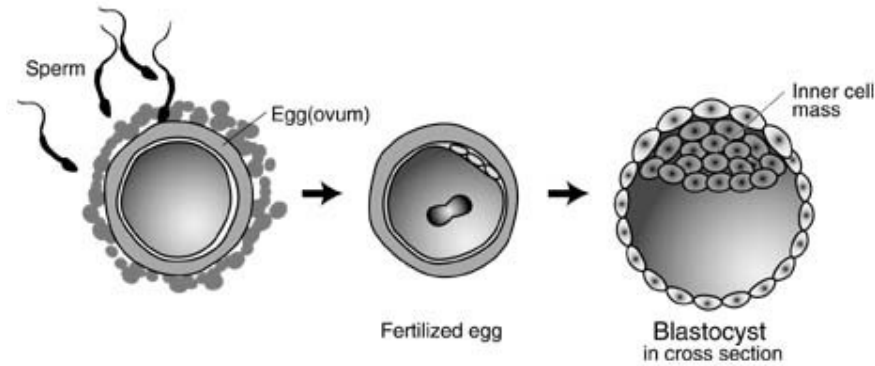


Figure 1: The blastocyst formation.

1.1.2. Evidence for pluripotency

To prove the pluripotency of cells derived from the inner cell mass, ESCs have been isolated and characterised (Table 1). ESCs were first derived from mouse embryo in 1981 by two independent groups (Evans and Kaufman, 1981; Martin, 1981) and then from human embryo in 1998 (Thomson et al., 1998). They possess a potentially unlimited capacity for self-renewing and a limited number of cells from the inner mass can produce millions of ESCs. Later, the injection of ESCs from one blastocyst into the cavity of another blastocyst was shown to give rise to chimeras, a mixture of tissues and organs with cells deriving from both donor ESCs and the recipient blastocyst, proving that ESCs can really participate in the formation of the three

germ layers. Then, their plasticity was tested after injection in adult animals where they developed into benign tumors called teratomas. These tumors contain cell types from all three primary germ layers of the embryo: endoderm, mesoderm and ectoderm. Finally, pluripotency of ESCs can be observed *in vitro*. ESCs aggregate and form so called “embryonic bodies” which resemble teratomas, a disorganised array of differentiated cell types derived from endoderm, mesoderm and ectoderm.

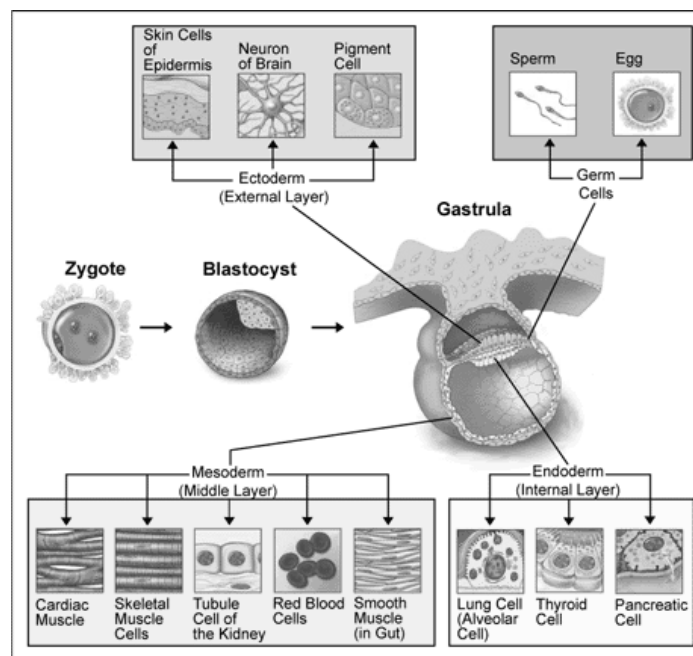


Figure 2: The three germ layers and their derivatives
(www.ncbi.nlm.gov)

Introduction

ESCs represent a potent therapeutic tool. The possibility to control mechanisms which orchestrate their differentiation into specific cells of the whole organism holds promises for regenerative medicine (Murry and Keller, 2008) (Fig. 3).

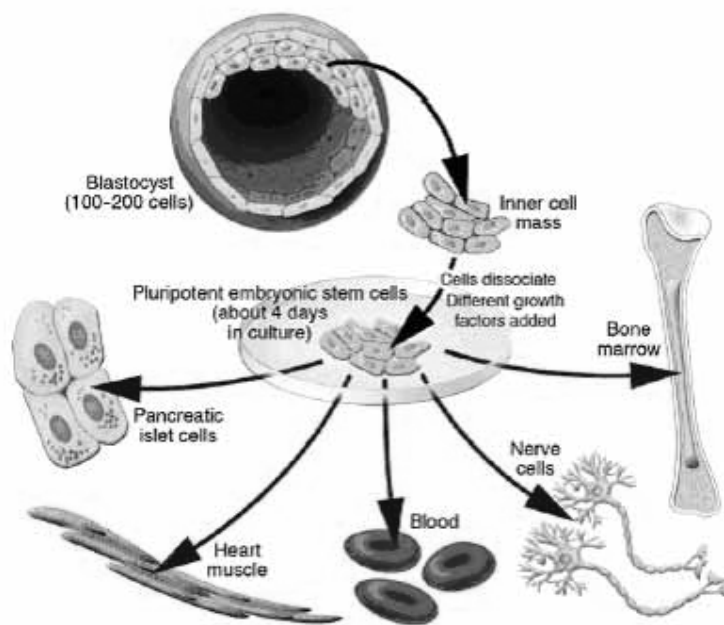


Figure 3: Pluripotency of embryonic stem cells derived from the inner cell mass. (www.innovitaresearch.org)

1.2. Adult stem cells

1.2.1. What is an adult stem cell?

For many years, researchers have been seeking to understand the body's ability to repair and replace cells and tissues of certain organs and several lines of evidence implicated the presence of stem cells in adult tissues (Table 1). Adult stem cells are viewed as undifferentiated cells in a differentiated tissue. They can renew themselves and yield all the specialized cell types from which they originated. The first observation of tissue regeneration came from the study of the bone-marrow. Discovery of blood forming stem cells came from the rescue of lethally irradiated mice receiving bone-marrow and in early 1960, Till and McCulloch identified cells able to renew themselves and to give rise to blood cells (Till and McCulloch, 1961). Hematopoietic stem cells (HSCs) illustrate the difficulty to isolate adult stem cells as about 1 out of 10.000 or 15.000 bone marrow cells is thought to be a stem cell. Today, adult stem cells were identified in many tissues such as bone marrow, cornea and retina of the eye, the dental pulp of the tooth, liver, skin, gastrointestinal tract, pancreas, brain, spinal cord, muscles, and also within the blood stream.

1.2.2. Adult stem cell plasticity

Adult stem cells were considered “*multipotent*” but tissue-restricted for a long time as *in vivo* these cells seemed to be quiescent, proliferation and differentiation appearing only to maintain the homeostasis of the tissue where they reside.

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It was not until recently that anyone seriously considered the possibility that stem cells in adult tissues could generate the specialised cell types of another type of tissue from which they normally reside, either a tissue derived from the same embryonic germ layer or from a different germ layer. The presumed ability of tissue specific stem cells to adopt the fate of cell types different from the tissue of origin has been termed “plasticity” (Brazelton et al., 2000; Krause et al., 2001) or transdifferentiation (Lagasse et al., 2000; Anderson et al., 2001). For example, HSCs may differentiate into a tissue that, during normal embryonic development, would arise from a different germ layer such as neural tissue (Brazelton et al., 2000; Mezey et al., 2000) (derived from ectoderm). Reciprocally, stem cells isolated from the brain may differentiate to form hematopoietic cells in the adult (Bjornson et al., 1999) or into many different cell types when introduced in chimeric embryo (Clarke et al., 2000).

The differentiated cell types that result from plasticity are usually reported to possess the morphological characteristics of the differentiated cells and to exhibit their characteristic markers. In reports evidencing that transplanted adult stem cells show plasticity *in vivo*, the stem cells typically were proven to integrate into a mature host tissue and assumed at least some of its characteristics (Ferrari et al., 1998; Brazelton et al., 2000; Mezey et al., 2000; Orlic et al., 2001). Many experiments focusing on plasticity involve injury of a particular tissue, which is intended to model a particular human disease or injury (Lagasse et al., 2000; Orlic et al., 2001). However,

the generation of mature, fully functional cells remains partially documented.

Collectively, studies on plasticity suggest that stem cells can be detected in the vast majority of adult tissues and are not fixed entities, which means that exposure to a new environment may give rise to a variety of cell types that may be unrelated to their origin (Fig 4).

1.3. Generation of pluripotent stem cells from somatic cells: the next step?

ESCs have the capacity to proliferate extensively while maintaining pluripotency, in other words they can generate every single cell type of the body. Hence, they are a promising donor source for cell regenerative therapies. However, their derivation and clinical use in human is limited by technical (restricted number of embryo, difficulty to isolate human ESCs, teratoma) and ethical (use of human embryo) issues. On the other hand, adult stem cells can be easily isolated from a patient, avoiding ethical problems. Although multipotency of adult stem cells is a well accepted dogma, their plasticity appears more restricted.

A crucial issue in the field of stem cell research is the generation of fully pluripotent stem cells which utilisation should be less problematic. One possible mean to avoid these issues is by reprogramming the nuclei of differentiated cells to pluripotent ESCs. Somatic cells have been shown to be reprogrammed as early as 1952 in pioneer studies by Briggs and King (Briggs and King, 1952).

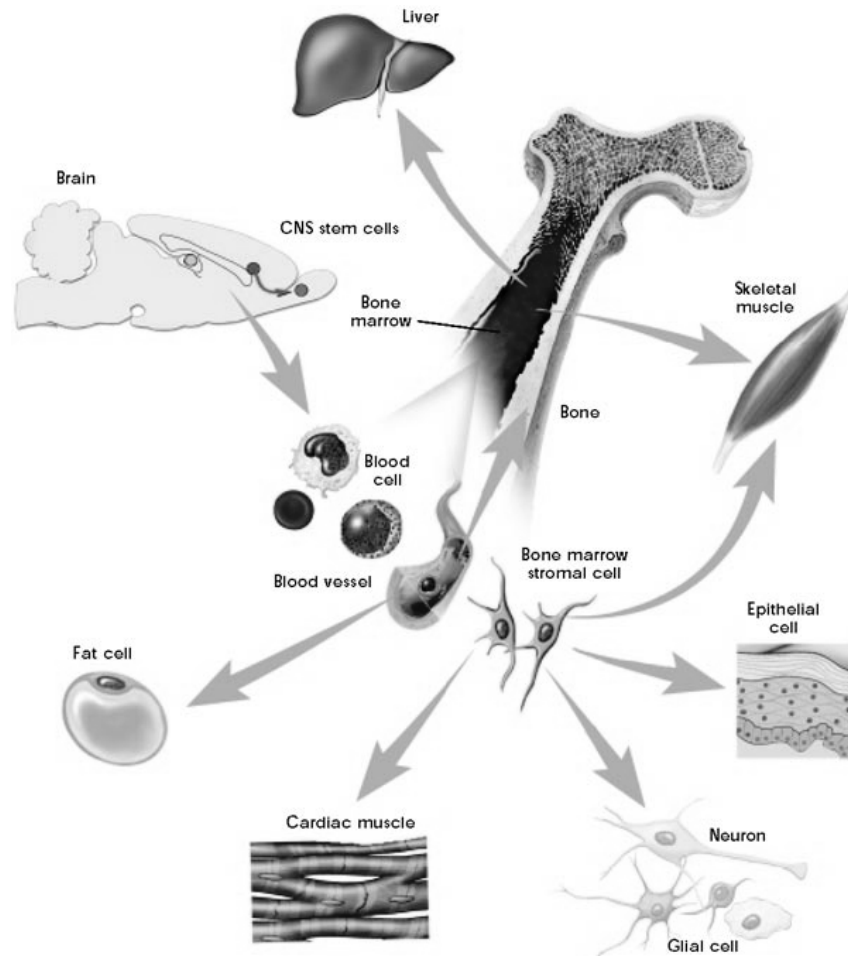


Figure 4: Adult stem cell plasticity (www.stemcells.nih.gov)

Nevertheless, a breakthrough came in 1996 with the production of the sheep “Dolly”, as the nuclear content derived from follicle cells introduced into oocytes was effective in giving rise to a new animal (Wilmut et al., 1997). Subsequently, somatic cloning was successfully performed in other species such as cow, mouse, goat, pig, cat and rabbit (Gurdon and Byrne, 2003). Another possibility of reprogramming somatic cells is the fusion with ESCs (Tada et al., 2001; Cowan et al., 2005). Even though these two methods always require the use of embryonic material, they have demonstrated that unfertilized eggs and ESCs contain factors that can confer totipotency or pluripotency to somatic cells. This mechanism remains unclear but in some of these experiments, cells acquired epigenetic configuration (histone acetylation and methylation) reminiscent of ESCs for genes implicated in pluripotency such as oct4 (Kimura et al., 2004). Based on these results, Takahashi and colleagues examined 24 different factors for their ability to induce pluripotency in somatic cells. This analysis demonstrated that the introduction of four transcription factors (oct4, Sox2, c-Myc and KLF4) via retrovirus in adult mouse fibroblasts resulted in the generation of induced pluripotent stem cells (iPS cells), which are similar to ESCs in morphology, proliferation and teratoma formation (Urushitani et al., 2006). Nevertheless, these iPS cells significantly differed from ESCs in gene expression, methylation patterns and their transplantation into blastocyst gave rise to chimeric embryos but not to adult chimeras. Recent improvements have been obtained by using a more stringent selection of the reprogrammed somatic cells, giving rise to iPS cells indistinguishable

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from ESCs in global gene expression, DNA methylation, histone modification and having the capacity to generate adult chimeras (Takahashi et al., 2007; Wernig et al., 2007).

In conclusion, expression of selected transcription factors proved to be a robust method to induce reprogramming of somatic cells to a pluripotent state in order to produce potent tools for regenerative therapy. Dopaminergic neurons and hematopoietic progenitors have already been generated from iPS cells and used to improve symptoms in a rat model of Parkinson's disease (PD) (Wernig et al., 2008) and to cure anemia in mice (Hanna et al., 2007) respectively.

However, the genome integration of retrovirus-transduced oncogenes represents a serious limitation to the eventual use of iPS cells for therapeutic application in human. The discovery that virally transduced proteins are only needed for a short period (Brambrink et al., 2008) allowed the generation of iPS cells without viral integration (Stadtfeld et al., 2008).

In addition to clinical purpose, this technique appears relevant to understand the pathological mechanisms implicated in human diseases and drug development through the generation of disease-specific stem cell lines. Up to now, a range of iPS cells carrying human genetic defects have been generated from patients suffering from Lesh-Nyhan syndrome, Huntington's disease (HD), Swachman-Bodian-diamond syndrome, juvenile diabetes, PD, Down syndrome, Becker muscular atrophy, Duchenne muscular dystrophy, Gaucher disease, adenosine deaminase deficiency-related severe combined immunodeficiency and

amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008; Park et al., 2008).

	Embryonic stem cells	Adult stem cells	Induced pluripotent stem cells
Localization	Embryo (Blastocyst)	Adult tissues	Adult tissues
Self-renewal <i>in vitro</i>	Unlimited	Senescence	Unlimited
Self-renewal <i>in vivo</i>	-	Senescence	-
Plasticity	Pluripotency	Multipotency	Pluripotency
Specific markers	Nanog Oct3/4 Sox2 Klf4 C-myc SSEA1-4 Alkaline phosphatase	Different sets of markers according to the localization, ex: NSCs: Sox2 ⁺ , Nestin ⁺ , Mushashi ⁺ MSCs: CD90 ⁺ , CD45 ⁻ , CD34 ⁻ HSCs: CD45 ⁺ , CD34 ⁺	Nanog Oct3/4 Sox2 Klf4 C-myc SSEA1-4 Alkaline phosphatase
Advantages	Pluripotency Unlimited self-renewal	Adult material Autologous cell lines	Pluripotency Unlimited self-renewal Adult material Autologous cell lines
Disadvantages	Use of embryos Tumors (teratomas) Different plasticity between cell lines	Only multipotent (compared to ESCs) Heterogenous populations	Tumors (teratomas)

Table 1: Principal characteristics of ESCs, adult stem cells and iPS cells.

2. Adult stem cells for neurological disorders: benefit beyond neuronal replacement?

The hallmark of several degenerative disorders in the central nervous system (CNS) such as ALS, PD, HD and Alzheimer's disease (AD) is the massive loss of one or several types of neurons. Spontaneous neurogenesis involving endogenous neural stem cells (NSCs) has been evidenced both in humans and in experimental animal models of neurodegenerative diseases (Curtis et al., 2003; Jin et al., 2004; Tattersfield et al., 2004; de Hemptinne et al., 2006; Shan et al., 2006) and brain or spinal cord injuries (Yagita et al., 2001; Rice et al., 2003; Jin et al., 2006). However, this putative endogenous repair process appears insufficient to compensate neuronal loss and to ensure functional recovery. These observations have raised interest for the use of exogenous embryonic and adult stem cells in substitution therapies with the hope that these cells could generate new neurons after being grafted in lesioned nervous tissues (for review (Lindvall et al., 2004)). Although functional neuronal substitution should appear as a complex and ambitious outcome from stem cell transplantation, this perspective has received predominant attention (Zhang et al., 2001; Deshpande et al., 2006; Roy et al., 2006) and has eclipsed a variety of other benefits potentially offered by stem cells. Nevertheless, accumulating evidence suggests that in most experimental studies, the benefit orchestrated by stem cells is not related to neuron replacement. Thus, the identification of the multifaceted behaviour of stem cells has

led to the emerging concept that when introduced in the lesioned CNS, non-differentiated stem cells could achieve beneficial effects through intrinsic neuroprotective capacities. In addition, multipotent stem cells could also differentiate into a variety of glial phenotypes, and thereby contribute to non-neuronal cellular replacement. This constitutes a major issue as several recent studies suggest that non-neuronal cells may play a pivotal role during the progression of several neurodegenerative disorders such as ALS (Beers et al., 2006; Boillee et al., 2006b; Nagai et al., 2007; Van Damme et al., 2007), spinocerebellar ataxia (Garden et al., 2002; Custer et al., 2006), multiple system atrophy (Yazawa et al., 2005) and prion disease (Raeber et al., 1997). Therefore, adult stem cells do not operate along a unidirectional path (i.e. generating new neurons) but likely exert a variety of biological activities that would efficiently face various harmful mechanisms of degenerative disorders.

Stem cells are defined as precursor cells that have the ability to self-renew and to generate various mature cell types. Pluripotent populations deriving from the inner mass of blastocysts, the embryonic stem cells (ESCs), are capable of long-lasting *in vitro* propagation while retaining the capacity to generate all somatic lineages, including neural cells (Zhang et al., 2001; Reubinoff et al., 2001). Nevertheless, even if promising results have been obtained with ESCs in experimental models of nervous insults (Kerr et al., 2003; Deshpande et al., 2006; Corti et al., 2006), the risk to evolve into teratomas after transplantation (Bjorklund et al., 2002) combined

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with ethical issues inevitably raises limitations regarding the use of ESCs in cellular therapies.

More restricted stem cells can also be obtained from a variety of tissues at different stages of development, including mature tissues from adult organisms. NSCs exist within multiple regions of the CNS (subventricular zone, hippocampus etc...) and it is possible to isolate and expand these cells to give rise to progenitor cells restricted to defined neural lineages (neuronal and glial) (Gage, 2000). For many years, the general belief was that adult tissue-derived multipotent stem cells were developmentally restricted to the tissue where they reside. However, more recent studies shed light on some population of adult stem cells endowed with the capacity to transdifferentiate into cells belonging to distinct germ layers, including neural cells (Bjornson et al., 1999). In addition to adult NSCs, this concept was largely described for two prototypic stem cell populations from the adult bone marrow: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). These two populations have remarkable advantages when considering exogenous stem cell based therapies, such as the lack of ethical issue concerning their source and the possibility of autologous transplants, avoiding any risk of rejection and abundant side effects related to immunosuppression. In addition, these stem cell populations express key cell surface receptors (i.e. CXCR4, c-Kit) (Wynn et al., 2004; Bantubungi et al., 2007) of which ligands are upregulated in CNS lesions (Bonavia et al., 2003; Sun et al., 2004; Miller et al., 2005; de Hemptinne et al., 2006) and which are strongly

implicated in cell recruitment. Indeed, both cell types were shown to efficiently migrate towards the site of injury within the CNS. Nevertheless, the transdifferentiation of adult stem cells from mesodermal origins into cells belonging to the ectodermal lineage remains matter of debate. In vitro, the putative transdifferentiation is usually supported by the detection of specific markers whereas the adoption of functional neuronal properties is not clearly established (for review (Phinney and Isakova, 2005)). In addition, those protocols enabling the rapid -within hours- transformation of adult stem cells into neural cells (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) showing typical neural morphologies and expressing selected protein are actively criticised. Paradoxically, increased expression of neural markers which was found to occur without changes in the corresponding mRNA was also observed when protein synthesis was inhibited (Lu et al., 2004). The apparent increase in immunolabeling for some neuronal markers after rapid chemical induction could be due to the cell shrinkage and concentration of antigens in the condensed cytoplasm, without transcriptional or translational changes. Indeed, several studies have evidenced that undifferentiated stem cells maintained in culture express specific neural proteins (Morrison and Weissman, 1995; Goolsby et al., 2003; Wislet-Gendebien et al., 2003; Tondreau et al., 2004; Deng et al., 2006). In addition, changes in the morphology of cultured cells occurring within hours is commonly associated with disruption of the actin cytoskeleton and retraction of the cell edges (Neuhuber et al., 2004) resulting in the condensation of cytosolic materials. *In vivo*, accumulating data have highlighted the

difficulty to distinguish authentic transdifferentiation of stem cells from their fusion with host cells. This phenomenon, even if considered as insignificant in healthy animals, is robustly increased by pathological processes in the CNS (such as inflammation) (Johansson et al., 2008) and the presence of heterocaryons in grafted stem cells should be systematically investigated before speculating on the possible cell transdifferentiation.

2.1. Adult stem cells: innate properties for the treatment of neurodegenerative disorders?

In several preclinical studies which aimed at improving the outcome of stem cell treatment in neurodegenerative diseases, some benefits could not be explained by cell differentiation but appeared more likely related to some therapeutic effects brought by undifferentiated cells. Several observations support the hypothesis that stem cells may display a valuable influence on diseased host tissues by exerting a protective “chaperone” effect.

2.1.1. Trophic support for dying neurons

Neurotrophic factors constitute a family of polypeptides supporting neuronal maturation and specification during development as well as modulation of axonal growth, plasticity and neurotransmission throughout adulthood. Indeed, efficient protection of suffering neurons was evidenced after administration of neurotrophic factors in models of neurodegenerative disorders [e.g. insulin growth factor-1 (IGF-1) in ALS, nmd and wobbler mice (Vergani et al., 1997; Gorio et

al., 1999; Kaspar et al., 2003) ; glial derived neurotrophic factor (GDNF) in Parkinson rats and ALS mice (Acsadi et al., 2002; Wang et al., 2002a; Wang et al., 2002b) ; vascular endothelial growth factor (VEGF) in ALS mice and rats (Azzouz et al., 2004; Zheng et al., 2004; Storkebaum et al., 2005), hepatocyte growth factor (HGF) in ALS mice (Sun et al., 2002) and ciliary neurotrophic factor (CNTF) in wobbler mice (Mitsumoto et al., 1994a; Mitsumoto et al., 1994b; Pun et al., 2006)]. Interacting with their cognate receptors, neurotrophic factors generate survival signals in neuronal cells. Besides, these factors may also directly interfere with cell mechanisms responsible for neuronal death, as for instance apoptosis which persists for several months in model of degeneration triggered by acute lesions (Chopp and Li, 1996). In accordance, neurotrophic factors secreted by stem cells were found to directly inhibit cellular death through the upregulation of antiapoptotic and anti-oxidative stress proteins (Yasuhara et al., 2006; Madhavan et al., 2008).

The distribution of these factors into the nervous parenchyma is limited as a consequence of their restricted diffusion across the blood brain barrier and their relative short half-life. In addition, systemic administration of these growth factors is likely to induce toxicity for healthy cells throughout the organism. Hence, several sophisticated approaches have been developed to ensure the production and/or delivery of these growth factors at the site of the lesions (infusion pump, adenoviruses, and cellular vectors). More recently, engineered stem cells have also been considered as valuable vectors for the

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production of a specific recombinant neurotrophic factors. Indeed, stem cells have been shown to be spontaneously recruited to the site of lesions in the nervous system after injection into the blood stream, the cerebrospinal fluid or directly into the brain or the spinal cord, supporting a local delivery of recombinant proteins. However, there is now accumulating evidence suggesting that non-modified stem cells could also provide benefit through their capacity to constitutively secrete a large number of growth factors. For example, NSCs injected into the spinal cord after traumatic injury were shown to promote axon sprouting by secreting nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), GDNF and neurotrophin-3 (NT-3) (Lu et al., 2003). In neurodegeneration models like PD, NSCs appeared to efficiently decrease Parkinson symptoms by rescuing dopaminergic neurons through production of SCF (Yasuhara et al., 2006) or GDNF (Ourednik et al., 2002). Likewise, transplantation of NSCs into the lumbar spinal cord of ALS rodents was shown to postpone the disease onset, to preserve the viability of motor neurons (MNs) and to prolong animal survival (Xu et al., 2006; Corti et al., 2007). In these studies, molecular and histological analyses of the spinal cord of grafted animals revealed a significant neuroprotection that correlated with increased levels of VEGF, IGF-1, GDNF and BDNF.

Mimicking NSCs, MSCs were also frequently reported to promote functional recovery after brain injury in several experimental models (Chen et al., 2001; Lu et al., 2001; Mahmood et al., 2001; Honma et al., 2006). Thus, soluble extracts from brain tissues that had been

subjected to ischemia were found to promote the secretion of growth factors such as basic FGF, VEGF, epidermal growth factor (EGF), transforming growth factor- β (TGF- β), NGF, IGF-1 and BDNF by cultured bone marrow stromal cells (Chen et al., 2002; Qu et al., 2007). Consistently, transplanted marrow stromal cells in a model of traumatic brain injury increased NGF, VEGF, HGF, BDNF and basic FGF levels, which correlated with improvement of neurological functions (Mahmood et al., 2004a). Together, these studies provide evidence for the capacity of adult stem cells to respond to signals produced by lesioned tissues, causing enhanced production of a variety of trophic factors. Worth mentioning, the production of growth factors was also documented for other adult stem cells. Thus, in *mdf* mice, grafts of HSCs were shown to reduce the progression of motor neuron degeneration and concomitantly improved motor function, both responses being attributed to the release of GDNF by grafted cells in the vicinity of MNs (Cabanès et al., 2007). The improvement of neuronal cell survival after grafts likely involves a synergy between exogenous stem cells and endogenous cells. Thus, the increased levels of trophic factors in the nervous parenchyma could arise either from the grafted stem cells responding to local signals associated with the degeneration or from endogenous cells which could be activated by chemicals released by the grafted cells or by direct cellular contacts (Mahmood et al., 2004a; Capone et al., 2007).

2.1.2. Interaction with inflammatory cells

During the last decade, inflammation has been identified as a key factor in the pathogenesis of degenerative disorders in the CNS. Microglia and infiltrated T-cells mediate effective innate immune responses to both infectious and traumatic challenges in the nervous parenchyma. However, microglia appear to exert a complex influence during nervous insult which is not restricted to neuroprotection, as upon activation these cells may also promote some facets of neurodegeneration. In experimental models of disorders where neuroinflammation is observed, there is accumulating evidence that grafted stem cells survive transplantation, and specifically migrate to the damaged area (Bulte et al., 2003). Indeed, stem cells also express essential cell-adhesion molecules, including integrins and chemokine receptors, required for their recruitment to the sites of inflammation (Pluchino et al., 2005). The immune-like function of NSCs was clearly exemplified in the study by Pluchino and coll. examining the benefit provided by undifferentiated neural progenitors administered into the blood stream and the cerebrospinal fluid of a mouse model of experimental autoimmune encephalomyelitis (EAE). These multipotent cells were found to enter the inflamed CNS and to decrease the infiltration of T-cell through enhanced apoptosis (Pluchino et al., 2005). In addition to their intrinsic immune properties, grafted NSCs likely interact with the tissue resident population of immune cells that participate in the inflammatory process. Within the CNS, microglial cells, originating from the

monocytic lineage at early stages of development, constitute the first line of immune defence. This cell population releases a variety of cytokines and growth factors and reacts quickly to tissue injury or infection in order to phagocytose debris and to present antigens to T-cells. Yet, activation of microglia is commonly associated with a decreased production of growth factors, reducing the trophic support of neuronal and other glial cells. In a model of spinal cord injury, grafted NSCs were shown to interact closely with endogenous activated microglial cells, promoting their production of neuroprotective factors and contributing to significant functional recovery (Ziv et al., 2006).

Alike NSCs in the central nervous system, MSCs are also endowed with potent immunoregulatory activities. In peripheral tissues, these cells were shown to alleviate severe acute graft-versus-host disease (Le Blanc et al., 2004; Ren et al., 2008), exhibit anti-inflammatory properties after lung injury (Ortiz et al., 2007) and can efficiently down-regulate the response of T-cells (Di Nicola et al., 2002; Bartholomew et al., 2002; Tse et al., 2003; Rasmusson et al., 2003; Meisel et al., 2004; Pierdomenico et al., 2005; Inoue et al., 2006; Krampera et al., 2007; Ren et al., 2008), B-cells (Corcione et al., 2006) and natural killer cells (Krampera et al., 2006; Spaggiari et al., 2006). It remains unresolved whether immunosuppression was induced by soluble factors or through direct contact between MSCs and immune cells. However, activation of MSCs by proinflammatory factors is needed to develop immunomodulatory properties,

highlighting the importance of cellular crosstalks between grafted cells and host immune cells (Ren et al., 2008). This latter study indicates that immunosuppression is not an innate property of MSCs, but is induced by local activating signals, illustrating again an adaptation of stem cells to the surrounding environment.

While MSCs have been frequently proposed to repress the activation of several immune cells, an essential issue that remains unsolved is whether MSCs-mediated immune modulation takes place in the CNS. One study demonstrated that intravenous injection of MSCs resulted in a reduced infiltration of blood-borne cells in the CNS of EAE mice correlating with an improved functional recovery (Zhang et al., 2005). The authors suggested a relation between the capacity of MSCs to down-regulate T-cells activity and the autoimmune nature of EAE. Nevertheless, although systemically injected stem cells are known to down regulate splenic inflammatory responses after brain injury (Lee et al., 2008), there is no clear evidence that MSCs can directly regulate immune cells within the CNS parenchyma.

2.1.3. Stimulation of endogenous repair processes

Evidence for enhanced endogenous neurogenesis has been reported in several models of CNS insults such as stroke (Arvidsson et al., 2002); (Felling and Levison, 2003), cerebral ischemia (Jin et al., 2001), epilepsy (Parent et al., 1997) and in neurodegenerative disorders such as HD (Curtis et al., 2003), AD (Jin et al., 2004) and ALS (Chi et al.,

2006; de Hemptinne et al., 2006). Though it is proposed that neurogenesis *per se* may contribute to nervous repair (Raber et al., 2004), the overall benefit that can be expected from endogenous stem cells appears extremely limited. Nevertheless, the existence of a constitutive capacity of repair opens attractive therapeutic perspectives regarding the possibility to promote such endogenous neurogenesis. Convincing data have been obtained using NSCs intrastratially grafted in a rat model of PD where behavioural recovery was associated with enhanced neurogenesis in the subventricular zone (Yasuhara et al., 2006). Likewise, in presymptomatic *nmd* mice, grafts of NSCs result in a decreased loss of MNs which is accompanied by an increased proliferation of endogenous precursors, through an up-regulation of IGF binding protein-5 (Corti et al., 2006), a molecule known to control proliferation and differentiation of neural progenitor (Ajo et al., 2003; Zhang et al., 2007b).

In the bone marrow, MSCs are known to play a crucial role in supporting the self-renewal and differentiation of HSCs (Dazzi et al., 2006) and experimental data indicate that MSCs could also influence or instruct endogenous neural progenitor cells during differentiation. For example, *in vivo* experiments suggest that MSCs can induce the proliferation of NSCs (Mahmood et al., 2004b) and their differentiation into oligodendrocytes (Rivera et al., 2006) or astrocytes (Wislet-Gendebien et al., 2004), raising hope for new therapeutic solutions in the treatment of CNS disorders that specifically involve impaired activities of these glial cells. Consistently, MSCs

transplanted in the mice hippocampus were shown to stimulate proliferation, migration and differentiation of endogenous NSCs into glial and neuronal cells, with increased secretion of neurotrophic factors such as VEGF, NGF, CNTF and basic FGF (Munoz et al., 2005). Additionally, when endogenous regenerative processes are activated, MSCs may form bundles and serve as guides for regenerating neuropil in spinal cord lesions (Hofstetter et al., 2002; Ankeny et al., 2004; Himes et al., 2006). Finally, since MSCs were shown to produce TGF- β , known to inhibit scar formation in skin (Cowin et al., 2001), TGF- β could enhance synaptogenesis through an impairment of astrocytes scarring by grafted MSCs in the traumatised CNS. Interestingly, these data highlighting the putative influence of exogenous adult stem cells on endogenous NSC fate could provide a rational explanation for the improvements observed in disease models after transplantation of a fairly small number of stem cells.

2.1.4. Stimulation of angiogenesis

Alteration in the local blood flow is believed to participate in the progression of neuronal death after stroke or in neurodegenerative disorders. Accumulating evidence suggests that after transplantation, NSCs migrate towards ischemic boundary regions in embolic stroke and that engrafted cells are associated with increased angiogenesis (Jiang et al., 2005). Similarly, cerebral neovascularisation, restoration of cerebral blood flow and reconstitution of the blood-brain-barrier in animal models of stroke is also obtained with HSCs (Shyu et al.,

2006) and MSCs through enhanced production of VEGF (Borlongan et al., 2004) and FGF-1 (Kasper et al., 2007).

2.2. Generation of glial cells: new concept of stem cell therapy

In most parts of the mammalian brain, neurons are largely outnumbered by glial cells (astrocytes, oligodendrocytes and microglia). Despite the key roles assumed by these different cell types in CNS development and adulthood, they have generally received little attention until recently. Nevertheless, several studies conducted with animal models of human diseases have clearly demonstrated the involvement of these cells in the pathogenesis of nervous disorders and in healing processes. As a consequence, the possible acquisition of glial functions by grafted stem cells is now considered as a relevant explanation for the benefit of stem cell-based therapies in CNS disorders.

2.2.1. Stem cell derived astrocytes

Astrocytes exert a wide variety of biological activities contributing to the viability of neurons in physiological conditions and their protection under potentially harmful circumstances. Thus, these glial cells provide nutrients and metabolic precursors, regulate acid-base balance via carbonic anhydrase activity and efficiently control oxidative metabolism in the nervous parenchyma. In close interaction with endothelial cells, astrocytes establish the blood-brain-barrier, preventing the contact of neurons with toxic chemicals. In addition,

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astrocytes are essential cellular partners in the neuronal circuitry as they actively modulate the concentration of excitatory and inhibitory transmitters. Lesions within the CNS lead to proliferation and hypertrophy of astrocytes that participate in the glial scar bordering the lesion. Reactive astrocytes ensure several essential functions for stabilising fragile nervous tissues after injury. Glial scars serve to restore the blood-brain-barrier, prevent spreading of potentially devastating inflammatory responses and limit cellular degeneration (Bush et al., 1999; Faulkner et al., 2004). Consistent with these chaperone roles of astrocytes, glial cells showing impaired functions are also clearly identified as key actors in the initiation or progression of several degenerative disorders (for review (Maragakis and Rothstein, 2006)). Therefore, it is generally recognised that the restoration or enhancement of typical astrocytic activities should provide efficient neuroprotection and attenuate the extension of several diseases (e.g excitatory amino acid uptake, release of neurotrophic factors, synthesis of glutathione).

In the CNS, NSCs are the natural precursors for neurons, astrocytes and oligodendrocytes. Accordingly, *in vitro* studies have largely illustrated the possibility to derive glial cells and in particular astrocytes, using neurospheres grown from NSCs isolated from human or rodent brain and spinal cord samples. Hence, differentiation into astrocytes has been frequently described as the default pathway for NSCs fate. Considering their secondary role in central nervous activities, the *in vivo* differentiation of NSCs into astrocytes was long

seen as an inevitable shortcoming in experimental attempts to derive functional neurons. Nevertheless, in several studies conducted with NSCs in animal models of CNS lesions or diseases, favourable outcomes such as signs of functional recovery have been reported despite preferential astroglial fate of transplanted cells. Thus, in a model of focal ischemia, reduction of sensorimotor deficits was obtained using NSCs grafts despite massive differentiation into astrocytes (Chu et al., 2004). Likewise, predominant differentiation of grafted NSCs into astrocytes associated with amelioration of functional performance was also demonstrated in a model of cerebral haemorrhage (Jeong et al., 2003). In a primate model of PD (methyl-phenyl-tetrahydropyridine injections), NSCs were found to protect projecting neurons of the nigrostriatal pathway from extensive degeneration. As only a minority of transplanted NSCs turned into cells expressing tyrosine hydroxylase and dopamine receptors, this protection was attributed to the generation of astrocytes producing GDNF along the nigrostriatal circuitry (Redmond, Jr. et al., 2007). In EAE mice, besides generating oligodendrocytes, a large proportion of NSCs were shown to evolve into astrocytes which are endowed with the capacity to reduce proliferation of inflammatory cells (Einstein et al., 2003). Similarly, in a rat model of HD (intraatrial injection of quinolinic acid), functional recovery observed in behavioural tests appeared to be significantly improved after transplantation of NSCs previously exposed to CNTF (McBride et al., 2004), known as an inducer of stem cell differentiation into astrocytes (Johe et al., 1996).

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Along with the unforeseen plasticity of MSCs that could give rise to cell types belonging to the neural lineages, several reports have clearly documented their capacity to adopt a glial fate. As mentioned for NSCs, early studies were focused on the differentiation of MSCs into neurons and the detection of astrocyte markers received only modest attention. Nevertheless, the acquisition of astrocytic characteristics by transplanted MSCs has been described several times. First indications of glial differentiation were obtained after direct injection of bone marrow derived MSCs into the rat brain as the cells subsequently developed an astroglial phenotype, revealed by a positive glial fibrillary acidic protein (GFAP) staining (Azizi et al., 1998; Kopen et al., 1999). Since then, these observations have been confirmed and the astroglial potential of MSCs was also evidenced *in vitro* (Jiang et al., 2002; Deng et al., 2005; Bossolasco et al., 2005; Wislet-Gendebien et al., 2005). Of importance, recruitment and predominant astrocytic differentiation of MSCs in models of nervous disorders or injuries have been associated with functional recovery (Zurita and Vaquero, 2004). However, considering the diversity of cell types arising from these transplanted cells, the benefit specifically resulting from astrocyte-like cells derived from MSCs remains to be established.

Worth mentioning, a major pitfall of these studies is related to the difficulty to undoubtedly validate the differentiation of NSCs or MSCs into astrocytes. In most cases, the astrocytic nature of differentiated cells is based on the positive detection of GFAP. Though this intermediate filament is widely considered as a typical

hallmark of mature astrocytes, additional markers should be characterized, as it is documented that some astrocyte subtypes do not express GFAP (Walz and Lang, 1998). More importantly, considering the arising hypothesis that the potential benefit is related to the neuroprotection brought by these cells, well-defined functional properties of astrocytes should also be investigated. As already mentioned, a positive outcome obtained with astrocytes derived from NSCs was associated with the release of growth factors (Redmond, Jr. et al., 2007). Similarly, the expression of the neuroepithelial stem cell intermediate filament (nestin) by differentiated MSCs grown in defined conditions correlated with the capacity of the cells to take up and metabolize extracellular glutamate (via glutamate transporters and glutamine synthase) and to release GDNF (de Hemptinne et al., 2004; Boucherie et al., 2008).

Concerning HSCs, astroglial transdifferentiation of cells from the hematopoietic lineage was initially illustrated in 1997 by Eglitis and colleagues, showing that exogenous HSCs detected in mice brain after bone marrow transplants stained positive for GFAP (Eglitis and Mezey, 1997). The differentiation of brain-infiltrated bone marrow into astrocytes and their long-term persistence in the nervous parenchyma was also demonstrated later in human after analysis of brain specimens from bone-marrow-transplanted patients (Cogle et al., 2004). Nevertheless, as approximately 1 in 100,000 cells in freshly isolated bone marrow is considered as a MSCs, participation of these non-HSCs in the generation of neural cells cannot be excluded.

Nevertheless, as shown with MSCs, more recent *in vitro* investigations confirmed the possibility to drive differentiation of HSCs into GFAP expressing cells when exposed to appropriate culture conditions (Reali et al., 2006).

2.2.2. Stem cell derived oligodendrocytes

Beside a trophic influence during development as well as in adult nervous tissues, the formation of an insulating myelin sheath wrapping nerve fibres constitutes the essential activity of oligodendrocytes in the central and peripheral nervous systems. This myelination process is critical for physiological neuronal communication as evidenced by the devastating consequences associated with demyelination, best characterised in multiple sclerosis and spinal cord injuries. In chronic demyelination diseases, spontaneous remyelination has been observed during transient relapse, but this process is largely overcome during acute phases, leading to permanent axonal damages. This spontaneous repair process has been characterised in EAE, the usual experimental model of multiple sclerosis, suggesting the existence of specific signals related to inflammation and demyelination that would favour the differentiation of endogenous stem cells toward an oligodendrocyte lineage (Picard-Riera et al., 2002). Consistently, several studies have considered the transplantation of *in vitro* expanded NSCs into models of EAE or in myelin-deficient mice (shiverer mice). In these models, signs of functional recovery were correlated with the differentiation of neural progenitors into oligodendrocytes endowed with evident myelinating capacities

(Yandava et al., 1999; Pluchino et al., 2003). Lesions resulting from mechanical traumatism (spinal cord injuries) also lead to extensive demyelination and the injured microenvironment was shown to promote NSCs recruitment and differentiation into oligodendrocytes. Hence, grafted cells were shown to produce myelin and substantially supported locomotor recovery (Akiyama et al., 2001; Karimi-Abdolrezaee et al., 2006). Worth mentioning, the subsequent planned elimination of grafted cells was followed by the progressive loss of acquired benefit, confirming their specific role in the healing process (Cummings et al., 2005).

Considering the biologic potential offered by MSCs, the possibility to use these cells as an alternative to NSCs in demyelinating disorders was examined both *in vitro* and *in vivo*. Convincingly, MSCs derived from cultured bone marrow and maintained in defined conditions were found to express some oligodendrocytes markers such as the low affinity NGF (LNGF) receptor, Krox 20 and CD104 (Keilhoff et al., 2006). Furthermore, when grafted at the vicinity of sciatic nerve lesions, these oligodendrocyte-like cells efficiently contributed to neuronal regeneration. Similarly, evidence for remyelination of spared white matter tracts and enhanced axonal growth by MSCs derived-oligodendrocytes was obtained in experimental models based on mechanical or chemical lesions of the spinal cord (Akiyama et al., 2002; Cizkova et al., 2006).

Experimental attempts to generate oligodendrocytes from HSCs have led to inconsistent results, reflecting the diversity of strategies used to

isolate subpopulations of these stem cells. Thus, no sign of oligodendrocytic differentiation was observed after transplantation of bone marrow cells (Yagi et al., 2004) or CD34⁺ cells (Sasaki et al., 2001) in adult rodent models of demyelination. In contrast, the transplantation of a non-purified bone marrow fraction in a model of demyelinated dorsal column was followed by efficient myelination that specifically involved the grafted cells (Sasaki et al., 2001). Nevertheless, the identity of myelinating cells should be addressed in order to confirm the involvement of HSCs which could be contaminated with bone marrow derived MSCs. Yet, the generation of cells expressing specific markers of oligodendrocytes from a enriched population of adult hematopoietic progenitor cells (CD117⁺) was observed after intracerebral transplantation into the neonatal mouse brain (Bonilla et al., 2002).

2.2.3. Stem cell derived microglia

Though early studies on the plasticity of adult stem cells have evidenced the potential of NSCs to differentiate into hematopoietic cells including macrophages (Bjornson et al., 1999), there is presently no clear-cut demonstration for a microglial identity of cells derived from NSCs or MSCs. In contrast, as microglia originate from blood cells penetrating the CNS during ontogenesis, it is not surprising that HSCs can give rise to microglia. Systemic transplantation of crude populations (Eglitis and Mezey, 1997; Corti et al., 2002; Cogle et al., 2004) or purified subpopulations (CD34⁺ b220⁺ cells) (Davoust et al., 2006) of bone marrow extract was followed by the migration of stem

cells in the CNS and their differentiation into microglia, expressing specific markers such as *iba1*, CD45 or CD11c. However, although the implication of microglia in the development of neurodegenerative diseases is well documented (Boillee et al., 2006b; Wu et al., 2007), the potential benefit given by the graft of healthy microglia remains poorly investigated. Worth mentioning, the substitution of the microglia surrounding MNs in a transgenic murine model of ALS by bone marrow cells from wild type littermates expanded their lifespan (Corti et al., 2004; Beers et al., 2006), encouraging the design of future therapies focusing on microglial precursors.

2.3. Conclusion

Rapid progresses in the characterization of adult stem cell biology have generated considerable enthusiasm for the development of therapeutic strategies for CNS insults. Evidence reviewed here challenges the vision that transplanted adult stem cells could provide therapeutic benefit entirely through neurons-replacement mechanisms. Considering the complex processes that are implicated in the differentiation of stem cells into a specific neuronal lineage and in the integration of these cells in the host neuronal network, other mechanisms should certainly be considered (Fig. 5). Moreover, the survival of functional neurons derived from grafted stem cells in the harmful environment generated by the disease is likely to be compromised. Though the acquisition of neuronal properties by adult stem cells has been evidenced both *in vivo* and *in vitro*, demonstrations for their contribution to functional recovery in models

of nervous disorders remain sparse and additional studies are needed to reinforce this therapeutic option. Besides, recent evidence for the existence of endogenous repair processes has raised interest for alternative therapies aiming at preserving residual neurons or promoting neurogenesis. There is accumulating reports clearly suggesting that adult stem cells may efficiently alter the neuronal environment through diverse activities commonly associated with glial or endothelial cells. Data summarized in this chapter indicating that both undifferentiated and differentiated stem cells may exert neuroprotective and neuroimmunomodulatory properties certainly highlight the potential offered by adult stem cells in the development of cell-supported therapies of diverse neurological disorders.

Although we mainly focused on MSCs, HSCs and NSCs, additional adult stem cell populations from the skin, umbilical cord blood or adipose tissue could be useful in such therapies. In addition, another key issue is related to the identification of the most effective cells in the heterogeneous stem cell populations isolated from adult tissues. The protocols for purification mainly rely on physical properties of the cells such as adhesion (MSCs), floating (HSCs and NSCs) and neurosphere formation (NSCs). Inevitably, these processes lead to variation in the purity of the cell population and the isolation of defined subsets of stem cells populations could greatly enhance the efficiency of cell-based therapies. Though further in vivo studies are required to confirm the long term protective benefit provided by the transplantation of adult stem cells in model of nervous disorders,

available experimental data certainly offer substantial promises for future clinical applications in regenerative medicine.

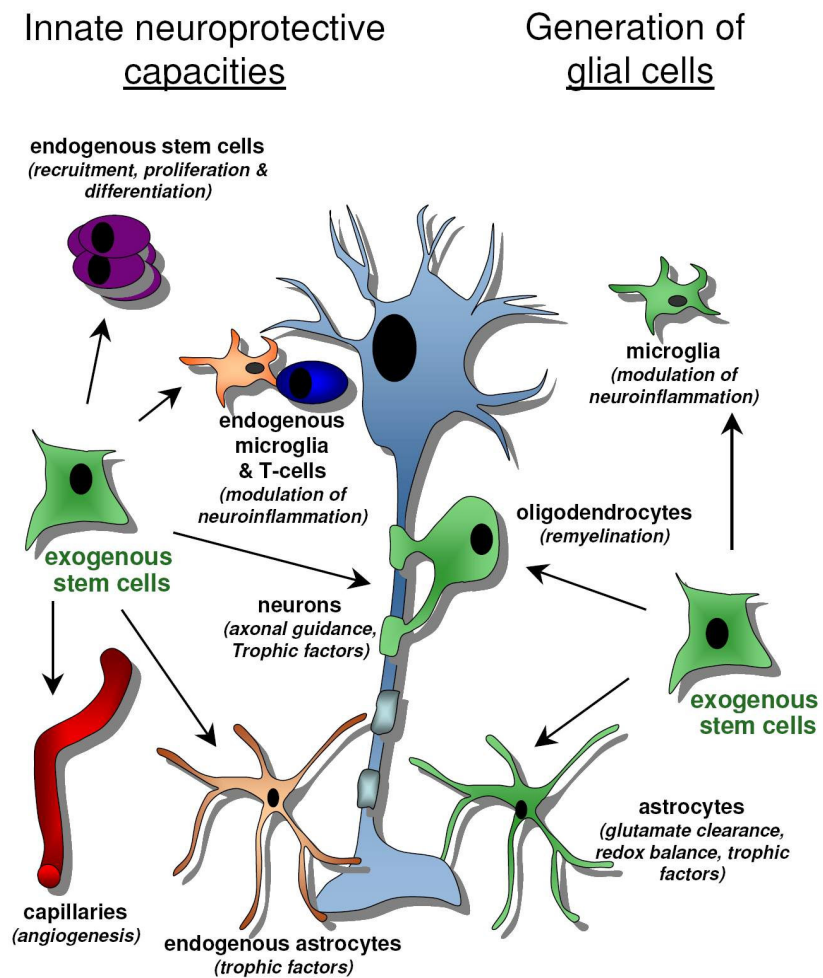


Figure 5: Protection of neurons by adult stem cells.

3. Mesenchymal stem cells: a tool for cell therapy

3.1. The bone marrow

The bone marrow, present in large bones consists of several cellular types which constitute a niche for hematopoiesis. Indeed, in addition to HSCs and hematopoietic progenitors, the bone marrow contains another stem cell population which is able to generate osteocytes, chondrocytes and adipocytes present in the bone marrow: the MSCs (Figure 6).

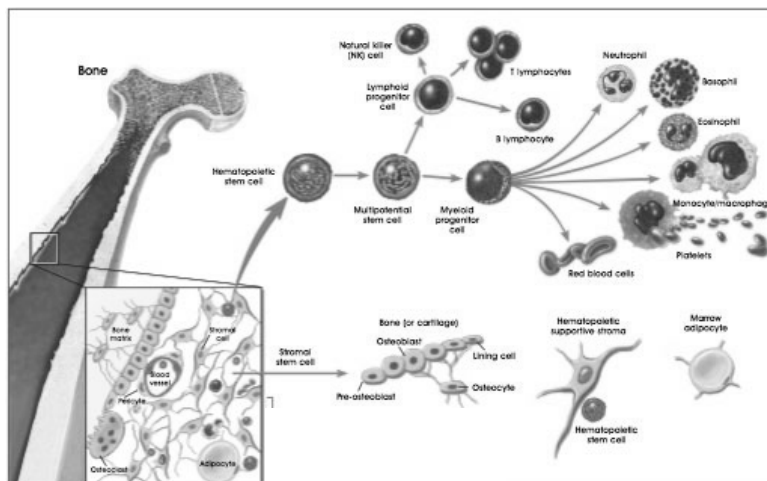


Figure 6: Cohabitation of hematopoietic stem cells and MSCs in the bone-marrow (www.stemcells.nih.gov)

3.2. What is a mesenchymal stem cell?

In early 60, Friedenstein and co-workers first demonstrated that whole bone marrow transplanted *in vivo* differentiated into osseous tissue (Petrakova et al., 1963; Friedenstein et al., 1966). Moreover, they showed that if the grafts were conducted in an open system, such as under the kidney capsule of mice, the resulting heterotopic ossicle was invaded by host-derived hematopoietic cells and functioned as a hematopoietic organ. These findings indicated that adult bone marrow contained the cellular constituents to regenerate *de novo* the hematopoietic niche of bone tissue. Subsequent studies demonstrating that marrow-derived osseous tissues exhibited the capacity for self-renewal, evidenced by the ability to regenerate new osseous tissue when serially transplanted to secondary recipients, led Friedenstein to postulate that marrow contains a stem cell population, distinct from HSCs, which was responsible for this osteogenic activity. This subpopulation of marrow fibroblastoid cells, which he called stromal cells, isolated via their preferential attachment to plastic, were capable of regenerating the hematopoietic microenvironment of bone marrow and therefore were enriched for the osteogenic stem cells (Friedenstein et al., 1968; Friedenstein and Lalykina, 1970; Friedenstein and Kuralesova, 1971) (Fig. 7).

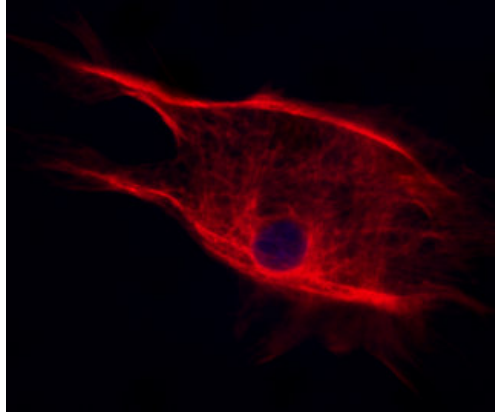


Figure 7: MSCs isolated from the rat bone marrow. (Red: vimentin, Boucherie, unpublished result)

Over the past few decades, analysis of human and rodent long term bone marrow cultures has revealed a large number of adhesion molecules, cytokines and extra cellular matrix proteins expressed by stromal cells that function as binding sites, mitogens and maturation factors for hematopoietic cell lineages (Deryugina and Muller-Sieburg, 1993; Nagao, 1995). Experiments to identify the nature and the potential of Friedenstein's osteogenic stem cells revealed that marrow stromal cells were capable of differentiation into multiple cell types of connective tissue (osteoblasts, adipocytes, chondrocytes) *in vitro* (Beresford et al., 1992; Umezawa et al., 1992; Dorheim et al., 1993; Dominici et al., 2006) suggesting the existence of a population of mesenchymal stem cell in the bone marrow (Caplan, 1994). Beside bone marrow, MSCs have also been detected in skeletal muscle (Williams et al., 1999), adipose tissue (Zuk et al., 2001), umbilical

cord (Erices et al., 2000), synovium (De Bari et al., 2001), the circulatory system (Kuznetsov et al., 2001) and dental pulp (Gronthos et al., 2000). Therefore, it appears that MSCs reside within the connective tissue of most organs.

The stemness identity of MSCs was evidenced through the characterisation of their multipotent nature (for review (Ortiz et al., 2007)), and MSCs are amongst the more extensively studied stem cell populations with respect to tissue regeneration.

In the particular case of the CNS, MSCs are endowed with neuroprotective and transdifferentiation capacities which results in a positive clinical outcome (see introduction, adult stem cells and treatment of the CNS). In addition, MSCs can be easily isolated and expanded for autologous cell therapies without any rejection problems or ethical issues, making them an ideal tool for stem cell therapy to treat neurodegenerative disorders.

4. Amyotrophic lateral sclerosis

4.1. Introduction

Amyotrophic lateral sclerosis (also known as Lou Gehrig's disease) is a progressive, lethal, degenerative disorder of the CNS with death of MNs. ALS has been first described more than 135 years ago by the French neurobiologist and clinician Jean-Martin Charcot. The name of the disease derives from Charcot's observation of a distinct "myelin pallor" within the lateral portions of the spinal cord, representing degeneration and loss of the axons of upper MNs as they descend from the brain to connect to the lower MNs within the spinal cord. Indeed, the hallmark of this disease is the premature and selective death of upper and lower MNs in the brain and spinal cord, provoking fatal paralysis (Fig. 8). Onset of the disease occurs usually between age 45 and 60, with a typical disease course of one to five years. The incidence (frequency of new cases per year) and the prevalence (the proportion of affected individuals in the population) are 1-2 and 4-6 per 100000, respectively. ALS is therefore an orphan disease (a condition that affects less than 1 patient in 2000 people), but statistics understate the impact of the most common MN disease in human (lifetime ALS risk of 1/600 to 1/1000) which are uniformly lethal. Most incidences of ALS are classified as sporadic (referred to sALS, without any identified genetic component) and approximately 10% of all cases are inherited (known as familial ALS, fALS).

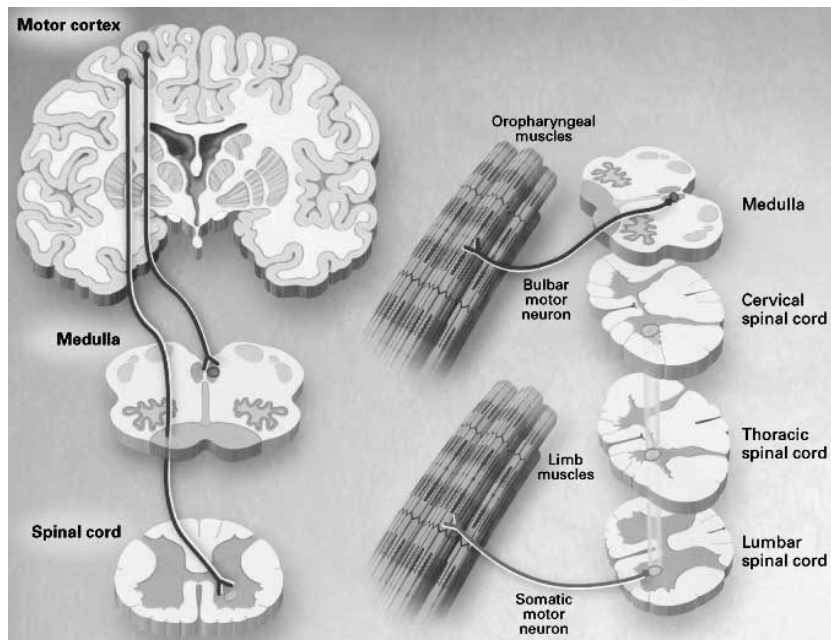


Figure 8: ALS kills specifically MNs in the central nervous system.

Both sALS and fALS are characterized by a progressive dysfunction of lower MNs -muscular atrophy, cramps and fasciculations, which is a muscle contraction visible under the skin that represents the spontaneous firing of a single MN and, as a result, of all the muscle fibers it innervates- and degeneration of cortical MNs – which results in spasticity, a persistent muscle contraction that causes stiffness and interferes with gait, movements and speech. However, muscles that control eye movements and the urinary sphincter are spared. The progression of the disease correlates with muscular weakness, atrophy and paralysis. Denervation of respiratory muscles and diaphragm is generally the fatal event.

ALS progression was initially characterised by the absence of sensory symptoms. Nevertheless, this criteria is now debated (Theys et al., 1999; Amendola et al., 2004; Pugh et al., 2007). The vast majority of ALS patients do not manifest cognitive impairments, resulting in a lock-in syndrome at late stage of the disease. However, ALS progression can sometimes, in rare cases, go along with fronto-temporal dementia (Hosler et al., 2000; Ostojic et al., 2003).

Post mortem analyses of ALS patients have shown that pathological hallmarks of the disease consist in the atrophy of MNs, the swelling of the perikarya and proximal axons, the accumulation of phosphorylated neurofilaments and the presence of Bunina bodies (characteristic proteinaceous inclusions in ALS MNs), Lewy body-like inclusions and ubiquitinated material. In addition, activation and proliferation of astrocytes and microglia are also common in ALS (Ince et al., 1996a). Unfortunately, there is no efficient therapy and the single drug approved by the Food and Drugs administration (FDA) to treat ALS patients, riluzole (initially used in order to limit the glutamatergic transmission), only slightly prolongs survival.

4.2. Genetics of ALS

As mentioned above, 10% of all ALS cases are inherited. Worth mentioning, sALS and fALS produce similar pathological hallmarks, including progressive muscle weakness, atrophy and spasticity in addition to the cellular markers described above. Therefore the study of fALS could provide new insights in the pathological mechanisms implicated in ALS. Specific mutations have been identified. Among

13 loci, 8 genes (superoxide dismutase 1 [SOD1], alsin, senataxin, synaptobrevin/VAMP [vesicle-associated membrane protein]-associated protein B, GLT-1 [glutamate transporter-1], dynactin, mCOX-1 [mitochondrial cytochrome c oxydase subunit-1] and IARS-2 [isoleucine-tRNA synthetase-2]) have been linked to ALS-like human MN disease (Table 2) and several transgenic animals as mice, rats, zebrafishes and flies, have been generated in order to progress in the comprehension of ALS, although models created by mutations in the SOD1 gene are the most widely studied.

4.2.1. SOD1

Mutations in the coding sequence of the SOD1 gene are responsible for up to 25% of fALS. SOD1 protein is a ubiquitously expressed cytosolic metalloenzyme and converts the toxic superoxide anion into hydrogen peroxide. In 1993, 11 missense mutations in the SOD1 gene in 13 different families have been described (Rosen et al., 1993). This number has increased considerably to over 125 mutations distributed throughout the 153 amino acids (Andersen et al., 2003; Andersen, 2006), while the precise functional consequences of these mutations remain unclear. Although the majority of these mutations reduce dismutase activity, others retain their full catalytic function. There is no clear correlation between enzyme activity, clinical progression and disease phenotypes (Andersen et al., 1997; Radunovic et al., 1997; Yamanaka and Cleveland, 2005). It is likely that the enzymatic properties of mutant SOD1 are not implicated in ALS but rather its misfolding and the subsequent aggregation, entrapment of other vital

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proteins and disruption of organelles. Surprisingly, sporadic and SOD1 mutant-mediated fALS are clinically indistinguishable. Worth mentioning, wild-type SOD1 can acquire toxic properties under oxidative stress (Ezzi et al., 2007) which could link SOD1 also to sALS. Interestingly, the oxidation of wild-type SOD1 is a phenomenon that may promote its aggregation (Rakhit et al., 2004; Furukawa and O'Halloran, 2006). Considering evidence of oxidative damage in sporadic ALS patients (Bowling et al., 1993; Ihara et al., 2005) and the abundance of SOD1 protein in cells (Pardo et al., 1995), it seems plausible that SOD1 molecules might constitute targets of oxidative damage in sALS.

Nevertheless, even within the familial forms, the time of onset and duration of disease vary. For instance, the missense mutations that lead to the substitution alanine→valine in position 4 (A4V) is associated with short survival times (about one year after disease onset) (Cudkowicz et al., 1998) whereas patients carrying the mutation D90A develop ALS with survival expectancy over 5 years. Evolution of the disease is also closely related to the genetic background of patients. Indeed, in northern Scandinavia, 2-3% of the population is heterozygous for the D90A mutation, which is a benign polymorphism in that population and homozygous individuals for D90A develop slowly progressive MN disease with prolonged survival of more than 10 years (Andersen et al., 1996).

4.2.2. Alsin

Alsin is ubiquitously expressed and abundant in neurons, but its function is not fully understood. Alsin contains three putative guanine nucleotide exchanger factor domains, involving Ras, Rab and Ran motifs. GTPases of the Ras family regulate vesicle transport and microtubule assembly (Topp et al., 2004). The findings that alsin mutants are instable (Yamanaka et al., 2003) and that most patients are homozygous for the mutation indicate that this form of fALS is caused by a loss of function of alsin. Patients develop an atypical form of ALS characterised by a juvenile onset and a slow progression of paralysis that affects predominantly the corticospinal tract. Recently, alsin has been suggested as being important to maintain the integrity of corticospinal axons, characterising this form of ALS as a distal axonopathy (Deng et al., 2007). Moreover, alsin proteins are implicated in the insertion of the glutamate receptor subunit 2 (GluR2) in the 1-4 α - amino -3 hydroxy -5 methylisoazol- 4 propionate (AMPA) receptor at the membrane of MNs. As GluR2-containing AMPA receptors are calcium impermeable, the loss of function of alsin and the consequent loss of GluR2 in AMPA receptor is therefore toxic for neurons through excitotoxicity (aberrant entry of calcium into the neuron) (Reali et al., 2006).

4.2.3. Senataxin

Patients presenting missense mutations in *senataxin* develop an autosomal dominant, juvenile onset motor neuron disease, characterised by muscle weakness and atrophy, with a normal lifespan. *Senataxin* encodes a DNA/RNA helicase domain with strong homology to proteins involved in RNA processing (Borlongan et al., 2004; Chen et al., 2006). Interestingly, altered RNA processing is implicated in two other motor neuron diseases: spinal muscular atrophy (mutations in the SMN [survival motor neuron] gene) and spinal muscular atrophy with respiratory distress (mutations in the IGHMBP-2 [immunoglobulin mu binding protein-2] gene).

4.2.4. Synaptobrevin-associated protein B

Synaptobrevin-associated protein B belongs to a family of intracellular vesicle-associated proteins that is presumed to regulate vesicle transport between the endoplasmic reticulum and the golgi apparatus (Soussan et al., 1999). In addition, this protein is implicated in the transfer of lipids between the endoplasmic reticulum or other organelles and the membrane (Olkkonen, 2004; Holthuis and Levine, 2005; Levine and Loewen, 2006). Patients develop atypical ALS characterized by an adult onset, slow progression and accompanied by an unusual tremor (Nishimura et al., 2004). *In vitro* studies of the mutated form of synaptobrevin implicated in ALS (P56S mutation) demonstrated that the mutant protein losses its function in lipid transfer and induces slow degeneration of MNs (Teuling et al., 2007). Interestingly, altered levels of sphingomyelin, ceramides, cholesterol

ester and altered expression of genes involved in the control of lipid metabolism have been identified in the spinal cord of ALS patients and mutant hSOD1^{G93A} mice (Malaspina et al., 2001; Cutler et al., 2002). Moreover, drosophila carrying a mutation in the homologous gene of human synaptobrevin (DVAP-33A [drosophila VAMP-associated protein of 33 kD A]) underlined its role in synaptic maintenance (Chai et al., 2008).

4.2.5. GLT-1/EAAT-2

Glutamate mediated excitotoxicity can induce neuronal damage and death as a consequence of repetitive firing, which in turn drives calcium entry through calcium-permeable ionotropic glutamate receptors. EAAT-2 (excitatory amino acid transporter-2), located within the membrane of astrocytes, is essential to remove glutamate rapidly from the synapse. A unique case of ALS has been reported, caused by a germline mutation in the EAAT-2 gene (Aoki et al., 1998; Trotti et al., 2001). This mutation affected *N*-linked glycosylation and glutamate clearance. Interestingly, loss of EAAT-2 activity and expression is a hallmark of the vast majority of both sALS and fALS (Trotti et al., 1999; Howland et al., 2002).

4.2.6. Dynactin

Dominant mutations causing adult-onset, slow progressive, atypical motor neuron disease accompanied by early vocal cord paralysis have been identified in the p150 subunit of dynactin, a component of the dynein complex involved in axonal transport. It is suspected to cause MN death because of an aberrant trafficking (Puls et al., 2003; Laird

et al., 2008). Interestingly, mutant SOD1 protein (responsible for 25% of fALS) and not wild-type SOD1 was found to interact specifically with the dynein complex (Zhang et al., 2007a), entrapping the complex in large aggregates (Strom et al., 2008), demonstrating that defect in axonal transport have a large contribution in ALS.

4.2.7. Mitochondria gene mutations

Defects in two mitochondrial genes are known to cause motor neuron disorders with ALS clinical features. Deletion in the mitochondrial mCOX-1 gene results in early adult onset of corticospinal motor neuron loss (Comi et al., 1998). In contrast, a nucleotide substitution in the sequence of the mitochondrial transfer RNA gene causes a late onset, slowly progressive, predominantly lower motor neuron disease (Borthwick et al., 2006).

4.2.8. Genes implicated in ALS susceptibility

Certain populations present genetic variants that modify sALS susceptibility. Thus, polymorphisms within the promoter of VEGF that reduce its expression are associated with an increased risk of ALS as documented in Sweden and Belgium (Lambrechts et al., 2003) but not in London (UK) (Lambrechts et al., 2003) or Utrecht (The Netherlands) (Van Vught et al., 2005). Variants predicted to reduce the activity of angiogenin (ANG) have been identified in Irish and Scottish populations (Greenway et al., 2004). These two proteins are implicated in angiogenesis. Therefore, blood supply and cellular oxygen pressure are important determinants of MNs viability, in addition to the neuroprotective properties of VEGF (Storkebaum et

al., 2005). Polymorphisms in the genes that encode for the neurofilament heavy subunit (Figlewicz et al., 1994; Al Chalabi et al., 1999) and the survival motor neuron protein (Veldink et al., 2001; Corcia et al., 2002) are also incriminated as risk factors for the development of ALS.

Gene	Chromosome	Inheritance	Clinical features
Medelian genes			
<i>sod1</i>	21q22	Autosomal dominant	Typical ALS
<i>alsin</i>	2q33	Autosomal recessive	Juvenile onset, slowly progressive, predominantly corticospinal
<i>senataxin</i>	9q34	Autosomal dominant	Adult onset, slowly progressive
<i>synaptobrevin</i>	20q13	Autosomal dominant	Typical ALS
<i>eaat-2</i>	11p12-13	-	Single case
<i>dynactin</i>	9p13	Autosomal dominant	Adult onset, slowly progressive, early vocal cord paralysis
Mitochondrial genes			
<i>mcox-1</i>	Mitochondrial DNA	Maternal	Single case, predominantly corticospinal
<i>iars-2</i>	Mitochondrial DNA	Maternal	Single case, predominantly lower motor neuron
Mendelian loci			
-	15q15-21	Autosomal recessive	Juvenile onset, slowly progressive

-	16q12	Autosomal dominant	Typical ALS
-	20p13	Autosomal dominant	Typical ALS
-	9q21-22	Autosomal dominant	Typical ALS, frontotemporal dementia
-	9p	Autosomal dominant	Typical ALS, frontotemporal dementia
-	Centrosome chromosome X	X-linked dominant	Typical ALS

Table 2: Genes that predispose to ALS

4.3. Mechanisms of death in ALS: lessons from mutant SOD1-mice

The selective death of MNs in ALS leads researchers to focus on pathological mechanisms involved in this specific population of neurons. Although numerous dysfunctions have been linked to motor neuron death, ALS-linked toxicity appeared to be decisive in non-neuronal cells with the emerging concept of “non-cell autonomous” death of MNs.

4.3.1. ALS & MNs

4.3.1.1. *Early dysfunction of MNs in ALS*

Embryonic MNs isolated from the spinal cord of mutant hSOD1^{G93A} mice are more susceptible to toxic insults (Van Den et al., 2000; Raoul et al., 2002; Spalloni et al., 2004) and have modified

electrophysiological properties and altered excitability (Pieri et al., 2003; Spalloni et al., 2004). Moreover, these neurons are highly sensitive to the activation of the death pathway triggered by the Fas death receptor (Raoul et al., 2002; Raoul et al., 2006), apparently activated at presymptomatic stages in hSOD1^{G93A} mice (Raoul et al., 2002; Wengenack et al., 2004; Raoul et al., 2006).

4.3.1.2. *Protein aggregates*

Abnormal accumulation of intermediate filaments with other proteins or “aggregates” is a pathological hallmark shared by sALS and fALS, and also occurs in transgenic animal models expressing mutant SOD1 (Gurney et al., 1994; Bruijn et al., 1997; Bruijn et al., 1998; Wong et al., 2000; Watanabe et al., 2001) where aggregates develop before the onset of clinical symptoms.

Nevertheless, their contribution to motor neuron death remains to be recognised. Several hypotheses have been established, including inhibition of the proteasome machinery (Kabashi et al., 2004), decreased chaperon activity (Bruening et al., 1999; Tummala et al., 2005), deregulation of organelles such as the golgi (Urushitani et al., 2008), the endoplasmic reticulum (Nishitoh et al., 2008) and mitochondria (see below), entrapment of vital proteins (Pasinelli et al., 2004) and aberrant axonal transport linked to intermediate filaments accumulation and aggregation of the dynein complex with mutant SOD1 (see below) (Fig. 9).

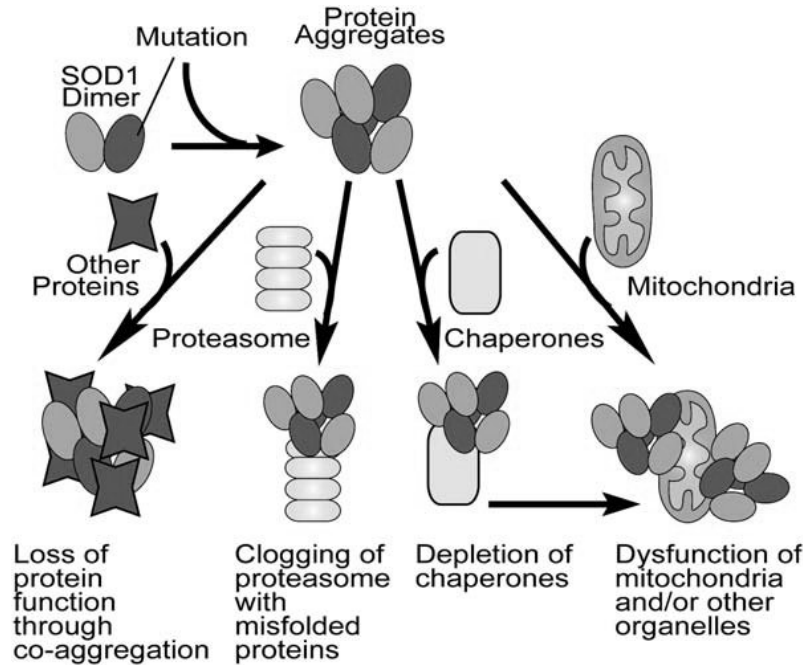


Figure 9: Toxicity of ALS causing by protein aggregates (Boillee et al., 2006a).

In the particular case of mutation in the SOD1 gene, aggregation of SOD1 protein should be in relation with its conformational instability. The propensity of a protein to aggregate is a complex property reflecting a combination of several biophysical and physico-chemical parameters such as net charge, hydrophobicity, native state stability and intrinsic structure. Therefore, an observed increase in the rate of protein aggregation, for any given protein variant, is not always accompanied by a specific defect such as destabilisation of the native state or a decrease in net charge. Interestingly, mutated proteins that

cause a more severe disease have a shorter half-life and are more likely to form aggregates (Sato et al., 2005; Lindberg et al., 2005).

4.3.1.3. Mitochondria & apoptosis

Biochemical markers of apoptosis can be detected in the terminal stage of human and transgenic ALS models (Durham et al., 1997; Pasinelli et al., 1998; Pasinelli et al., 2000; Vukosavic et al., 2000; Li et al., 2000; Guegan and Przedborski, 2003; Bacman et al., 2006) and histopathological observation correlated ALS with vacuolisation and dilatation of mitochondria (Afifi et al., 1966; Hirano et al., 1984). As mitochondria are the main effector of apoptosis, dysfunction of the formers may contribute to MN death via apoptosis.

Studies of SOD1 mice showed that mutant SOD1 protein accumulates in mitochondria, (Liu et al., 2004; Vijayvergiya et al., 2005; Deng et al., 2006; Bergemalm et al., 2006). Mutant SOD1 has been found in both the intermembrane space and the matrix, as well as in both the inner and outer membranes of spinal cord and brain mitochondria (Higgins et al., 2002; Pasinelli et al., 2004; Liu et al., 2004; Vijayvergiya et al., 2005; Deng et al., 2006; Bergemalm et al., 2006), whereas they are largely excluded from similar preparation from liver mitochondria (Liu et al., 2004). This close proximity could be implicated in mitochondria dysfunction. Indeed, cytochrome c translocation and activation of caspase 1, the earliest event in caspase related-apoptosis, is detected in non-symptomatic ALS mice followed by the subsequent activation of caspase 9 and caspase 3 (Pasinelli et al., 1998; Vukosavic et al., 1999; Pasinelli et al., 2000; Vukosavic et al., 2000; Li et al., 2000; Almer et al., 2001; Bacman et al., 2006). In

addition, the entrapment of B-cell lymphoma-2 protein (BCL-2) with mutant SOD1 in detergent resistant aggregates could block its anti-apoptotic function (Pasinelli et al., 2004) and accelerate MNs death. Moreover, mutant SOD1 associated with the mitochondria membrane could impede the protein transport via the membrane-spanning multisubunit translocators (TOM and TIM) which are responsible for the passage of the majority of the 1500 proteins required for its function (Liu et al., 2004). Moreover perturbations in the generation of adenosine tri-phosphate (ATP) via oxidative phosphorylation and especially dysfunction of the complex I have been proposed, but experimental results are somehow contradictory (Bowling et al., 1993; Browne et al., 1998; Higgins et al., 2002; Mattiazzi et al., 2002; Liu et al., 2004; Kirkinezos et al., 2005; Browne et al., 2006; Damiano et al., 2006). Finally, early impairment in mitochondrial calcium-buffering capacity occurring in mutant SOD1 prior to symptom onset and only in disease relevant tissues may be the more meaningful hypothesis to link mitochondria directly to neuron death through excitotoxic mechanism (Damiano et al., 2006) (Fig. 10).

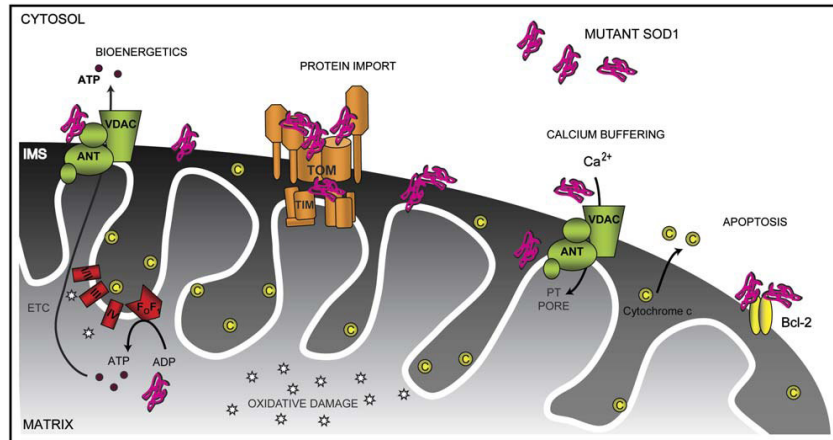


Figure 10: Mutant SOD1-mediated damage in mitochondria (Boillee et al., 2006a).

4.3.1.4. *Compromised axonal transport*

Another characteristic of ALS is the reduced axonal transport, described first in patients (Sasaki and Iwata, 1996) and transgenic mouse models of the disease (Sasaki et al., 2005). The transport of molecules and organelles is a fundamental cellular process that is particularly important for the development, function and survival of neurons, and especially MNs which present a very long axon (up to 1 m in human). This impaired axonal transport in patients and rodents may be related to the alteration of the axonal structure (Kawamura et al., 1981; Hirano et al., 1984; Wong et al., 1995; Bruijn et al., 1998; Damiano et al., 2006), especially the inappropriate misaccumulation of neurofilaments, the most abundant structural component of large myelinated axons. Additional evidence for a link between impaired axonal transport and motor neuron disease arose from the discovery of a mutation in the p150 subunit of dynactin, which is responsible for

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the recruitment of dynein (a motor powering retrograde axonal transport along microtubules (Goldstein and Yang, 2000)). Patients carrying this mutation suffer from a slowly progressive form of lower MNs disease (Puls et al., 2003). Interestingly, mice presenting a mutation in the dynein gene or overexpressing a protein which disrupts the dynein/dynactin complex, have impaired motor neuron function and viability (Witherden et al., 2002; LaMonte et al., 2002; Hafezparast et al., 2003). These observations favour the view that disturbance and attenuation of axonal transport might be a unitary feature of all forms of ALS, contributing to MNs death by blocking the anterograde transport of proteins from the cell body to the synapse and the retrograde transport of protein such as growth factors. In accordance to this hypothesis, several SOD1 protein mutants implicated in ALS development have been shown to interact with the dynein/dynactin complex and this interaction is critical for the formation of large aggregates containing mutant SOD1, resulting in perturbed axonal transport (Strom et al., 2008) (Fig. 11).

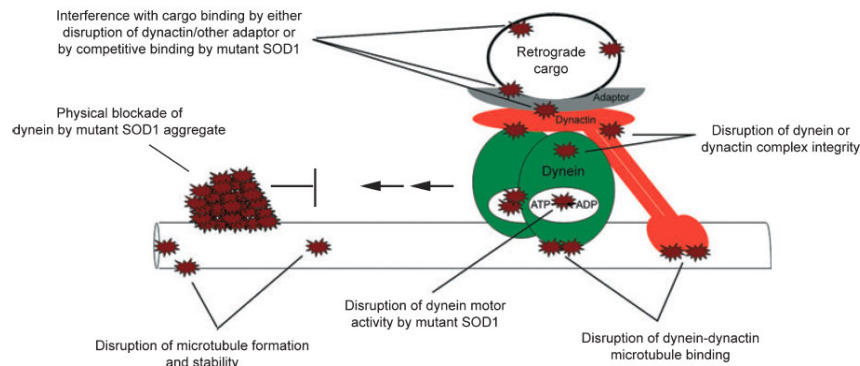


Figure 11: Possible mechanism by which mutant SOD1 could interfere with dynein-dynactin motor activity (Strom et al., 2008).

4.3.2. ALS: cellular environment represents a deadly neighbor

The archetypal vision of neurotoxicity in neurodegenerative diseases was based on the idea that a specific neuronal population is particularly vulnerable to a cumulative toxic agent such as intraneuronal accumulation of toxic mutant protein. Nevertheless, the lack of degeneration (Pramatarova et al., 2001; Lino et al., 2002) or the very late appearance of the disease (Jaarsma et al., 2008) upon synthesis of mutant SOD1 solely within neurons provided the original evidence that disease possibly does not arise strictly from damage caused by the mutant protein within MNs. Disease onset was deferred after partial removal of the ubiquitously expressed mutant *sod1* solely in MNs (Boillee et al., 2006b; Takahashi et al., 2007). The rate of disease progression after disease onset was, however, not affected. Thus, mutant expression within MNs is an essential contributor to

disease instigation, but only a negligible player in disease progression. Indeed, chimeric mice with MNs expressing high levels of mutant SOD1 surrounded by normal neighbour cells survive longer (Clement et al., 2003).

There is now convincing data supporting a non-cell autonomous mechanisms in which neurodegeneration is robustly influenced by the toxicity of mutant protein expression in non-neuronal cells in the vicinity of neurons such as astrocytes (Shin et al., 2005; Custer et al., 2006; Nagai et al., 2007; Di Giorgio et al., 2007; Takahashi et al., 2007), microglia (Liberatore et al., 1999; Beers et al., 2006; Boillee et al., 2006b), oligodendrocytes (Yazawa et al., 2005) and endothelial cells (Zhong et al., 2008), which all have intimate contact with neurons.

4.3.2.1. Cross-talk between astrocytes & MNs

Astrogliosis is a prominent characteristic of sALS and fALS. This phenomenon was considered secondary to the loss of MNs. However, accumulating evidence indicate that dysfunction of astrocytes may be critical in ALS.

4.3.2.1.1. Neurotrophic factors

Astrocytes provide crucial influences to the neurons they support (reviewed in (Maragakis and Rothstein, 2006)). They are well known for their essential role in providing neurons with neurotrophic factors that are known to affect MNs, such as GDNF, BDNF, CNTF and VEGF. Indeed, decreased availability of neurotrophic factors for MNs death has been proposed. Interestingly, transgenic mice with low

VEGF level (after modification of the *veg**f* promoter) showed progressive MNs loss (Oosthuysen et al., 2001). Additionally, in the context of genetic forms of ALS, mutants SOD1 proteins were shown to alter the posttranscriptional processing, reducing the level of VEGF (Lu et al., 2007). Together, these results suggest that decreased neurotrophic factor levels may accelerate the degeneration of MNs.

4.3.2.1.2. *Excitotoxicity*

Astrocytes are also implicated in the catabolism of several amino acids, especially the rapid clearance of glutamate, the primary excitatory neurotransmitter in the CNS, which is taken up from the synaptic cleft by glutamate transporters (GLT-1/EAAT-2 and GLAST [glutamate-aspartate transporter]/EAAT-1 [excitatory amino acid transporter-1]). Astrocytic glutamate transporters are of prime importance in protecting MNs against glutamate excitotoxicity (aberrant entry of calcium after overstimulation of AMPA receptors by glutamate) because of the weak ability of MN to buffer calcium (Palecek et al., 1999). Altered glutamate handling is one of the few mechanisms which link sporadic and familial ALS. Thus, in both transgenic hSOD1^{G93A} animals (Bruijn et al., 1997; Howland et al., 2002; Dunlop et al., 2003) and humans with sALS (Rothstein et al., 1992; Shaw, 1994; Fray et al., 1998; Sasaki et al., 2000) or fALS (Rothstein et al., 1995), GLT-1 transporter expression and activity are focally lost. This is likely to be a consequence of the disease as dysfunctional MNs in ALS produce an aberrant quantity of reactive oxygen species (ROS) (due to mitochondria dysfunction) which can

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selectively inactivate GLT-1 (Rao and Weiss, 2004; Yin et al., 2007), in addition to promote the cleavage of the transporter by caspase 3 (Boston-Howes et al., 2006). Therefore, loss of GLT-1 and the subsequent excitotoxicity fits perfectly in the role of disease accelerator. Worth mentioning, the glial inwardly rectifying potassium channel (Kir) has recently been implicated as a contributor of MN death via excitotoxicity. The Kir channels contribute significantly to the astrocyte resting membrane potential. Therefore these channels influence the transport of various molecules including neurotransmitters such as glutamate. Interestingly, mice with genetically inactivated Kir undergo extensive MN death and it has been shown that presymptomatic mutant hSOD1^{G93A} mice present lower Kir channel levels (Kaiser et al., 2006). Therefore, perturbation of the buffering of extracellular potassium during neuronal activity is another potential contributor to excitotoxicity in ALS.

As indicated above, MNs are very sensitive to excitotoxicity because of their low capacity to buffer calcium and the high expression of calcium permeable ionotropic glutamate receptors on their membrane (Carriedo et al., 1996; Van Den et al., 2000; Tateno et al., 2004; Van Damme et al., 2005). Worth mentioning, AMPA receptors become calcium impermeable by the insertion of a GluR2 subunit. Nevertheless, several mutations implicated in ALS can decrease the trafficking of GluR2 towards AMPA receptors and therefore decrease the number of calcium impermeable AMPA receptors. Indeed, as mutations in *alsin* directly down regulate the presence of GluR2 at the membrane of MNs (see genetics of ALS - *alsin*), mutant SOD1 affects

astrocytes which are in turn not able to secrete factors implicated in the expression of GluR2 in MNs (Van Damme et al., 2007).

4.3.2.1.3. *Secretion of selective toxic factor for MNs*

Studies with *in vitro* models of the interaction between astrocytes carrying *sod1* mutations and MNs derived from embryonic spinal cord (Nagai et al., 2007) or ESCs (Di Giorgio et al., 2007) demonstrated that ALS-astrocytes decrease the viability of MNs. Noteworthy, this toxicity is specific to MNs, as no effect was detected on sensory neurons and interneurons, which corresponds to ALS specificity. Though this toxicity appeared mediated by soluble factor(s), these toxic species remain unidentified so far. However, this study demonstrated that MNs derived from wild-type and transgenic animals are sensitive to this toxic species. Worth mentioning, mutant-SOD1 protein expressed at high levels solely in astrocytes of transgenic mice produces astrogliosis, but is not sufficient to induce MN degeneration (Gong et al., 2000). Considering that partial SOD1 gene excision in astrocytes did not affect disease onset but slowed disease progression (Yamanaka et al., 2008), it is likely that these unidentified toxic factor(s) from astrocytes *in vivo* do not kill MNs but rather aggravate toxicity of intraneuronal SOD1.

4.3.2.1.4. *Conclusion*

Astrocytic activation is now a well accepted feature of sALS and fALS and may be implicated in disease progression after onset since ALS mice carrying the mutated human SOD1 gene in all cells except astrocytes present an increased survival in late stages of the disease

(Yamanaka et al., 2008). It is likely that this result is due to the decreased secretion of toxic factor(s) (Nagai et al., 2007; Di Giorgio et al., 2007) but not due to the decreased accumulation of glutamate in the synaptic cleft because loss of GLT-1 is not prevented (Yamanaka et al., 2008). Worth mentioning, the decreased expression of the mutant SOD1 in astrocytes delayed activation of microglial cells, another key cell type in ALS (Yamanaka et al., 2008). Therefore, this new mechanism by which astrocytes activate microglia (one explanation could be the secretion of mutant SOD1 by astrocytes, which is a potent activator of microglial cells (Urushitani et al., 2006)) may also explain the contribution of astrocytes in disease progression.

4.3.2.2. *Microglia, when ALS turns into an immune disease*

Microglial cells, the macrophages of the CNS, have conventional roles in the elimination of neural cells during development and in maintaining normal neuronal survival by removing toxic cellular debris. As key components of the immune system in the CNS, their main role is linked to neuroinflammation. Microglia, with neurotoxic and neurotrophic effects, are mobilized in essentially all examples of disease and injuries in the CNS (Yin et al., 2007). Historically, the appearance of immune cells in the brain or spinal cord has been perceived as a signal of pathology and imminent tissue destruction (Galea et al., 2003). Indeed, many substances produced by microglia are neurotoxic and have been implicated in the onset and progression of neurodegenerative diseases (Feuerstein et al., 1998; Schroeter and Jander, 2005). However, immune system activation is also a necessary

response to tissue injury or infection throughout the body. It has therefore been suggested that ‘neuroimmune’ responses could be enhanced to augment CNS repair (Hauwel et al., 2005). Nevertheless, aberrant inflammation is now well characterised as a contributor of ALS.

4.3.2.2.1. Evidence for contribution of microglia in ALS

First evidence of inflammation in ALS has been described 18 years ago (Engelhardt and Appel, 1990) and was largely confirmed both in the brain and spinal cord of ALS patients (McGeer et al., 1991; Kawamata et al., 1992; Troost et al., 1993; Turner et al., 2004; Henkel et al., 2004) as well as mutant SOD1 mouse models (Hall et al., 1998; Kriz et al., 2002; Beers et al., 2006). The implication of microglial cells in ALS was demonstrated by evidencing the consequences of treating mutant hSOD1^{G93A} mice with minocycline, an tetracycline derivative with anti-inflammatory properties, previously shown to inhibit microglia activation (Yrjanheikki et al., 1999). Minocycline was potent in increasing survival of ALS mice (Kriz et al., 2002; Van Den et al., 2002; Zhu et al., 2002). Nevertheless, since minocycline exerts an anti-apoptotic effect on neurons, it was unclear in which cells minocycline was active (Zhu et al., 2002). However, forcing the activation of the immune system using chronic administrations of lipopolysaccharide (LPS) or by deletion of the fractalkine receptor, exacerbates SOD1 mutant-mediated disease in mice (Nguyen et al., 2004; Cardona et al., 2006), providing compelling evidence that

neurotoxicity from microglial cells plays a crucial role in ALS. This implication for damage by mutant SOD1 within microglia was further studied using a transgenic mouse model in which the mutant *sod1* transgene was partially but specifically suppressed in the myeloid lineage, including microglial cells. This partial deletion of the transgene produced no delay in disease onset but survival was greatly extended through pronounced slowing of disease progression (Boillee et al., 2006b). In addition, the replacement of the entire microglia population (by crossing hSOD1^{G93A} mice with PU.1 knock-out mice, which are not able to generate the myeloid lineage, followed by wild-type bone marrow transplantation) had no effect on disease onset but extended survival by slowing disease progression (Beers et al., 2006). As a similar transplantation with bone marrow from mutant hSOD1^{G93A} mouse did not generate disease in wild-type mice (Beers et al., 2006), we have now compelling evidence that, as already showed for astrocytes, expression of the mutated transgene solely within microglia is not sufficient to trigger MN disease, but does drive rapid disease progression.

4.3.2.2.2. Mechanisms of toxicity related to microglia activation in ALS

In spite of the multiple indications supporting the contribution of microglia to MN death, it remains to be elucidated through which mechanisms microglial cells exert a toxic function in ALS. During ALS progression, microglial cells become activated and upregulate the production of proinflammatory molecules. One attractive

hypothesis is that microglial cells become activated in response to the mutant SOD1 in their cytoplasm, resulting in the increased activation of intracellular pathways and the secretion of toxic inflammatory mediators. This hypothesis is supported by the demonstration of Boillee and colleagues who showed that the partial suppression of the SOD1 mutant gene in microglial cells increases the survival of hSOD1^{G85R} mice (Boillee et al., 2006b) and *in vitro* evidence indicated that microglia from the CNS of ALS mice produce substantially more inflammatory mediators compared to microglia expressing wild-type SOD1 (Weydt et al., 2004; Xiao et al., 2007).

Nevertheless, the mechanisms of cell death triggered by microglial cells are complex. With respect to its enhanced production by activated ALS-microglia, tumor necrosis factor- α (TNF- α) was thought to be a good candidate, until it was shown that the deletion of the gene encoding TNF- α did not affect disease progression (Gowing et al., 2006). In the same way, inducible nitric oxide synthase (iNOS) expression (and nitric oxide (NO) production) as well as interleukin-1 (IL-1) expression are increased in ALS models but deletion of the corresponding genes did not affect the development of the disease (Son et al., 2001; Nguyen et al., 2001). Cyclooxygenase-2 (COX-2) (the enzyme responsible for the synthesis of prostaglandins) is produced in abundance by microglia in the spinal cord of ALS patients (Yasojima et al., 2001; Yiangou et al., 2006) and hSOD1^{G93A} rodents (Almer et al., 2001). However, the use of COX-2 inhibitors prolong survival of hSOD1^{G93A} mice (Drachman et al., 2002; Pompl et al., 2003). Worth mentioning, prostaglandin-2 (PGE-2), the main

product of COX-2, is increased in the cerebrospinal fluid (CSF) of ALS patients (Drachman et al., 2002). High concentrations of PGE-2 induce apoptosis in neurons via the PGE-2 receptor EP2 and the subsequent activation of caspase-3 (Takadera et al., 2002), or via the release of glutamate from astrocytes leading to the subsequent excitotoxicity (Bezzi et al., 1998; Sanzgiri et al., 1999). However, results about the implication of PGE-2 are not clear as this prostaglandin has been shown to protect MNs against excitotoxicity (Bilak et al., 2004).

Recently, studies have examined the concept that oxygen radicals produced in microglial cells could contribute to microglia-mediated toxicity. Indeed, microglia produce large amount of reactive oxygen species (ROS) during inflammation via the nicotinamide adenine dinucleotide phosphate oxydase (NADPH oxydase), which is essential for the protection against microorganisms. However, NADPH oxydase is also upregulated in sALS, fALS and mutant hSOD1^{G93A} mice. Although this could simply reflect the strong microgliosis as also evidenced by the increased release of TNF- α , iNOS and IL-1, deletion of the gene encoding the catalytic subunit of the NADPH complex (gp91^{phox}) extended the survival of mutant hSOD1^{G93A} mice (Wu et al., 2006). New data provide a direct link between mutant SOD1 and NADPH oxydase. Actually, in addition to catalyzing the conversion of superoxide (O₂⁻) to oxygen peroxide (H₂O₂), SOD1 is directly implicated in the regulation of O₂⁻ production via interaction with the NADPH oxydase activator Rac-1. Under reducing conditions SOD1 binds Rac-1 and activates it as well as the subsequent NADPH,

whereas under oxidising conditions SOD1 “releases” Rac-1. Apparently, mutant SOD1 amplifies the production of ROS by locking Rac1 in its activated state even under oxydising conditions (Harraz et al., 2008). Therefore, it is likely that enhanced production of ROS and the related oxidative damages are components of the non-autonomous cell death of MNs mediated by microglial cells.

4.3.2.2.3. Conclusion

An important question is how microglial cells are activated in ALS. The death of MNs could be a very attractive explanation, in addition to glutamate accumulation. However, microglia activation in ALS is a very early phenomenon (Ince et al., 1996b), occurring prior to MN death and loss of GLT-1. As mentioned above, misfolded mutant SOD1 is a potent activator of microglial cells (Urushitani et al., 2006) and it is proposed that astrocytes and MNs secrete mutant SOD1 through the unusual specific interaction of the mutant SOD1 protein with the chromatogranin-mediated secretion pathway (Urushitani et al., 2006). Therefore, microglial cells in ALS mice, which apparently secrete more inflammatory mediators, may be activated by astrocytes and MNs via secretion of mutant SOD1. Nevertheless, the implication of distinct subpopulation of immune cells appears to be different, as CD4⁺ T-cells have been evidenced to support neuroprotection (Beers et al., 2008).

4.3.2.3. Endothelial cells & the breakdown of the blood-brain-barrier

The role of blood vessels in the pathogenesis of neurodegenerative disorders and especially ALS is poorly understood. Recent reports have documented ultrastructural changes of the blood-brain-barrier (BBB) in the spinal cord of ALS mice (Garbuzova-Davis et al., 2007a; Garbuzova-Davis et al., 2007b). More precise analyses of the BBB in asymptomatic ALS mice detected a strong reduction of expression of proteins specifically responsible for the maintenance of tight junctions which mediate paracellular permeability between adjacent endothelial cells and accumulations of extracellular fluid (oedema) between the capillaries and MNs. In addition, this study highlighted a reduction of the length of capillaries in the spinal cord and a reduction of the blood flow (Zhong et al., 2008). Together, these results demonstrate that changes to the vasculature probably contribute to the degeneration of MNs. Disruption of the BBB mediated by reduced levels of tight junction results in microhemorrhages that promote the release of neurotoxic molecules for neurons (Regan and Guo, 1998), and reductions in microcirculation (hypoperfusion) of the spinal cord, indicating endothelial cells as possible contributors to the non-cell autonomous death of MNs in ALS.

4.3.2.4. The muscle enigma

Muscle atrophy is one of the earliest events detectable in hSOD1^{G93A} rodents and the dying back pattern of degeneration suggests that muscle abnormalities precede MN death. hSOD1^{G93A} mice display significant muscle energy defects long before neurodegeneration, such

as increased nutrient uptake, modified expression of genes involved in carbohydrate and lipid metabolism, increased mitochondrial uncoupling protein-3 content, decreased ATP levels and altered mitochondrial respiration (Dupuis et al., 2003; Dupuis et al., 2004). Moreover, muscles from ALS patients and hSOD1^{G93A} mice express Nogo-A (Dupuis et al., 2002), which is a repellent for axonal regeneration in the CNS (Schwab, 2004). Worth noting, the absence of Nogo-A increases hSOD1^{G93A} mice lifespan (Jokic et al., 2005) and its overexpression cause nerve terminal retraction (Jokic et al., 2006). Surprisingly, decreased expression of the human SOD1 protein by siRNA or gene excision via the CRE-Lox system specifically in muscles did not modify the progression of the disease (Smith et al., 2006). Thus questions persist on the real contribution of muscles to non-cell autonomous MN death in ALS.

4.4. Pre-clinical treatment

The generation of transgenic animals developing ALS produced ideal tools for preclinical studies of potential ALS therapies. Currently, over 150 different therapeutic agents or strategies have been tested in mutant hSOD1^{G93A} rodents, the most efficient are summarised below.

4.4.1. Anti-oxidants

Considering the role of SOD1 as an anti-oxidant and the implication of oxidative stress in ALS, several therapies aiming at targeting oxidative stress were evaluated. Treatment with catalase, putrescine-modified catalase, ginseng and vitamin E significantly slowed disease

onset with negligible influence on animal survival (Gurney et al., 1996; Reinholz et al., 1999; Jiang et al., 2000). Application of NOS inhibitors, creatine, red wine extract and SOD1/catalase mimetics extended survival, but did not change the age of onset (Klivenyi et al., 1999; Jung et al., 2001; Amodio et al., 2006). Finally, celastrol, green tea and Neu2000 delayed both onset and endpoint (Koh et al., 2006; Kiaei et al., 2006; Shin et al., 2007). One promising anti-oxidant appears to be a manganese-porphyrin which, when given at symptomatic stage, robustly increased life expectancy (Crow et al., 2005).

4.4.2. Anti-glutamatergics

Treatments with anti-excitotoxic agents were found to predominantly shift survival, rather than disease onset. Therefore, it is consistent with the fact that excitotoxic death is a late phenomenon. For example, glutamate carboxypeptidase II inhibition, glutamate receptor antagonism and pharmacologic upregulation of EAAT-2 by centriaxone or GPI-1046 significantly prolonged life (Van Damme et al., 2003; Ghadge et al., 2003; Rothstein et al., 2005; Ganel et al., 2006).

4.4.3. Anti-aggregation

Aggregate formation is a hallmark of sALS and fALS. Pharmacological induction of heat shock proteins (HSPs) by arimoclomal therapy enhanced survival in ALS mice, even when administrated at disease onset (Kieran et al., 2004). However, a direct effect on mutant SOD1 misfolding, aggregation and HSPs was not

demonstrated. In contrast to ubiquitous genetic overexpression, extracellular delivery of HSP70 affords protection in ALS mice due to a possible peripheral mode of action (Gifondorwa et al., 2007). The microtubule stabilising compound noscapine was also beneficial by normalising slow axonal transport of tubulin (Fanara et al., 2007).

4.4.4. Anti-apoptotics

End stage MNs display characteristics of apoptosis. Agents that block apoptosis in ALS mice affect the disease endpoint as expected. Administration of lithium or of a broad-spectrum caspase inhibitor zVAD-fmk slowed the appearance of symptoms, while the latter decreased also mortality (Li et al., 2000; Shin et al., 2007). Treatment with rasagiline, phenylbutyrate and a janus kinase-3 inhibitor (WHI-P131) increased mouse survival (Trieu et al., 2000; Waibel et al., 2004; Yazawa et al., 2005). Worth mentioning, valproate was neuroprotective in two studies, but only disease-modifying in one (Sugai et al., 2004; Rouaux et al., 2007). In fact, valproate was completely ineffective against distal neuropathology in ALS mice, dissociating proximal cell death from mouse survival (Rouaux et al., 2007).

4.4.5. Immunomodulatory drugs

Inflammation plays a critical role in ALS. Administration of COX-2 inhibitor, NADPH oxydase inhibitor, cyclosporine, lenalidomide, thalidomide and sulindac suppressed inflammation and prolong life (Drachman et al., 2002; Klivenyi et al., 2004; Karlsson et al., 2004; Kiaei et al., 2006; Harraz et al., 2008). Inhibition of glycogen synthase

kinase 3 and matrix metallo peptidase-9 prior to symptom onset was also beneficial in mice (Lorenzl et al., 2006; Koh et al., 2007). The antibiotic minocycline, which is recognised to suppress microglia activation, is protective in ALS mice (Kriz et al., 2002; Van Den et al., 2002; Zhu et al., 2002).

In a totally distinct approach, ALS mice were immunised against mutant SOD1 (Ezzi et al., 2007), because secretion of misfolded SOD1 by MNs was reported *in vitro* and *in vivo* (Turner et al., 2005; Urushitani et al., 2006) and could cause inflammation in the spinal cord and potentiate neurodegeneration (Urushitani et al., 2006). Active and passive immunization against mutant SOD1 considerably extended life (Ezzi et al., 2007).

4.4.6. Neurotrophic factors

Reflecting the practical limitations of CNS delivery, therapy using systemic neurotrophic factors has notably failed in ALS patients (Penn et al., 1997; Borasio et al., 1998; Miller et al., 2006). However, intrathecal delivery of IGF-1 provided mild benefit in ALS mice (Sato et al., 2005), whereas intraventricular delivery of VEGF in ALS rats delayed symptom onset and extended survival (Storkebaum et al., 2005). Importantly, this mode of delivery was most effective in suppressing forelimb paralysis onset, consistent with a rostro-caudal gradient of VEGF, highlighting the difficulty to introduce neurotrophic factors into the entire CNS. Direct intraspinal injection of HGF into spinal cord of symptomatic ALS rats was efficient to prolong survival (Ishigaki et al., 2007).

Another solution is to use retrogradely transported viral vectors coding for neurotrophic factors. In ALS mice, intramuscular injection of adeno-associated viruses or lentiviruses encoding cardiotrophin-1, GDNF, IGF-1 and VEGF significantly delayed disease onset. Furthermore, viral expression of GDNF, IGF-1 and VEGF in mice greatly enhanced survival (Bordet et al., 2001; Acsadi et al., 2002; Wang et al., 2002b; Kaspar et al., 2003; Azzouz et al., 2004).

4.4.7. Antisense therapies

Gene silencing has yielded exciting results in ALS rodents. Lentiviral-mediated delivery of shRNA targeting the mutant transgene via muscle or spinal injection dramatically slowed down disease progression (Ralph et al., 2005; Raoul et al., 2005). Furthermore, SOD1 knockdown in ALS rats by continuous brain infusion of antisense oligonucleotides moderately improved survival (Smith et al., 2006).

4.4.8. Cell therapies

Cell therapies are a very exciting challenge in neurodegenerative disorders such as ALS because, depending of the type of cell, they can display a range of therapeutic effects on MNs but also on surrounding non-neuronal cells, in addition to the possibility of giving rise to healthy, functional neural cells.

4.4.8.1. Stem cell therapies

4.4.8.1.1. Neural stem cells

Transplantation of spinal cord-derived human NSCs into the lumbar spinal cord of hSOD1^{G93A} rats at presymptomatic stage delay disease onset by 7 days and increased survival by 11 days (Xu et al., 2006). Nevertheless, there was no evidence for MN replacement by grafted cells however, higher levels of GDNF and BDNF were found in the spinal cord and the cerebrospinal fluid, likely derived from donor cells (Fig. 12).

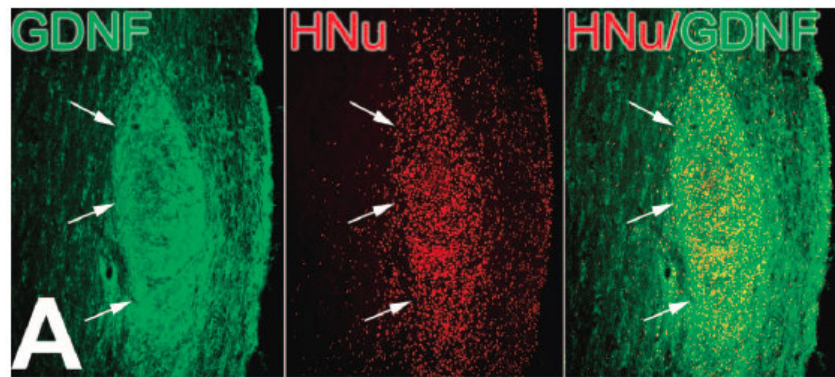


Figure 12: Human neural stem cells produce growth factors in the spinal cord of rodent ALS (green: GDNF, red: human nuclei) (Xu et al., 2006).

Then, another study biased neuronal differentiation of NSCs purified from adult mouse brain (on the basis of the expression of stage-specific embryonic antigen-4 [SSEA-4] and CXCR-4) by priming them *in vitro* into a MN phenotype. hSOD1^{G93A} mice showed a 3-weeks delay in onset and survival. Nevertheless, NSC-derived MNs

did not display integration in the neuronal network or projection to muscular targets but rather secreted growth factors such as VEGF and IGF-1 which correlate with a partial preservation of host MNs (Corti et al., 2007). Thus, it appears that the beneficial effect of intraspinal injection of NSCs was mediated mainly by delivery of these growth factors to MNs (Fig. 13).

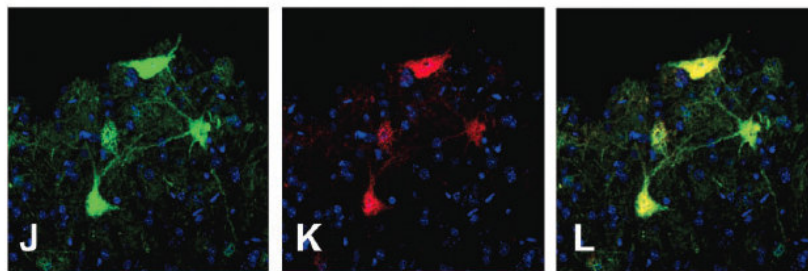


Figure 13: Graft of neural stem cell-derived MNs (green: HB9-GFP, red: acetylcholine transferase) in the spinal cord of rodent ALS (Corti et al., 2007).

In attempt to preserve host MNs, two studies used NSCs derived from human cortex and genetically modified in order to over-express GDNF. Klein and colleagues (Klein et al., 2005) predifferentiated NSCs with CNTF to induce higher contents of astrocytes able to over-express GDNF. Nevertheless, transplanted cells showed limited migration and GDNF secretion only within the grafted region, neither resulting in protection of MNs nor effecting disease onset or duration. The lack of benefit of GDNF release in that study could be attributed to an ineffective anatomical targeting as most of the grafts were placed in the white matter. The same group repeated the same

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experiment using undifferentiated NSCs. GDNF was more widely expressed as a consequence of higher migration, which causes a robust MN survival. NSCs kept an undifferentiated phenotype in proximity to degenerating MNs and NSCs not over-expressing GDNF were without effect on MN survival, demonstrating that this neuroprotection was subsequent to GDNF production around MNs. Nevertheless, protection of MNs from death was without influence on disease progression as connections between MN and muscles at the neuromuscular junction were lost (Suzuki et al., 2007) (Fig. 14).

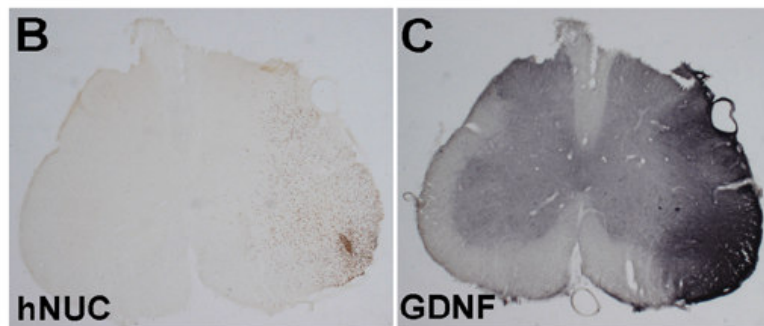


Figure 14: Over-expression of GDNF by human neural stem cells in the spinal cord of rodent ALS (Left: human nuclei, right: GDNF) (Suzuki et al., 2007).

4.4.8.1.2. Bone marrow stem cells

Bone marrow transplants into irradiated presymptomatic hSOD1^{G93A} mice delay disease onset and prolong survival by 14 days (Corti et al., 2004). In that study, after grafting bone marrow, stem cells were identified within the CNS and skeletal muscles but a recent study

demonstrated that expression of mutant SOD1 in muscles did not affect disease progression (Smith et al., 2006), suggesting that the beneficial effect was derived from implantation into the CNS. Interestingly, transplanted cells contributed to almost one-third of the microglial population (Fig. 15). Yet, microglial cells carrying the mutated SOD1 gene have been identified as a potent actor of ALS pathology (see ALS: cellular environment move in as deadly neighbors). Therefore, the substitution of mutant microglia by healthy microglia may be a therapeutic approach to reduce inflammation. A proof-of-concept was realised by Beers and colleagues (Beers et al., 2006) who bred mice lacking the myeloid lineage (which includes microglial cells) ($PU.1^{-/-}$) with $hSOD1^{G93A}$ mice. Subsequent wild-type bone marrow transplantation replaced the entire microglial population in $hSOD1^{G93A}$ mice, slowing down progression of the disease after onset as also observed when mutated *sod1* is deleted specifically in microglia (Boillee et al., 2006b).

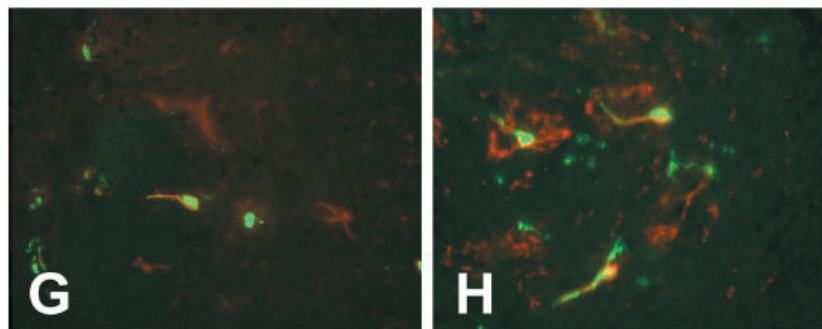


Figure 15: Generation of microglia cells in the spinal cord of rodent ALS by bone marrow transplantation (green: GFP, red: F4/80) (Corti et al., 2004).

4.4.8.1.3. *Mesenchymal stem cells*

Very recently, intraspinal implantation of human MSCs was realised in asymptomatic hSOD1^{G93A} mice. Human cells presented an undifferentiated phenotype but provided beneficial effects to hSOD1^{G93A} rodents. No extensive data was presented about the lifespan of male hSOD1^{G93A} mice but motor performances were improved. The study was stopped before emergence of motor symptoms in females, but the number of MN appeared to decrease more slowly in treated female hSOD1^{G93A} mice at this stage. However, in both males and females decreased astrogliosis and microgliosis were observed (Vercelli et al., 2008), which is in accordance to the anti-inflammatory properties of MSCs. Another study using intrathecal delivery of human MSCs into the cisterna magna of ALS mice observed migration into the brain (Habisch et al., 2007). However, no therapeutic effect was noticed. Human MSCs migrated only into the brain which is not affected in rodent models of ALS and not into the spinal cord where neuroprotective effects are needed. In addition to a small number of injected cells, this negative result could arise from a wrong localisation of MSCs in the CNS. MSCs have been also used as “biopump” in muscles to secrete virus-encoding GDNF at the neuromuscular junctions (Suzuki et al., 2008). This procedure was efficient to preserve neuromuscular junctions and MNs but the influence of GDNF is not obvious compared to GFP-expressing MSCs, which suggest that non-modified MSCs present potent capacities to protect neuromuscular junctions.

4.4.8.2. Other cell therapies

4.4.8.2.1. Sertoli cells

Sertoli cells are normally found within the testes where they act as “nurse” cells and provide an immunoprivileged environment for the developing spermatogonia. Several groups have employed cell therapy using isolated Sertoli cells for treatment of diseases in the CNS such as PD, where transplanted Sertoli cells improved the viability and function of both co-grafted and endogenous dopaminergic cells in the brain (Sanberg et al., 1997; Liu et al., 1999). In hSOD1^{G93A} mice, Sertoli cells (Fig. 16) exerted a strong protection of MN cell bodies but only for those in close proximity, leading the authors to speculate about production of growth factors and immunomodulation but no data were provided to underline these mechanisms in the spinal cord (Hemendinger et al., 2005).

4.4.8.2.2. Umbilical cord blood cells

Umbilical cord blood cells (UCBCs) contain a heterogeneous population of HSCs, T-cells and B-cells. Moreover, lymphocytes from UCBCs produce great amounts of anti-inflammatory cytokines (Rainsford and Reen, 2002), suggesting that UCBCs could ameliorate ALS via immunomodulation. Although preliminary studies demonstrated the beneficial outcome after infusion of UCBCs in hSOD1^{G93A} mice (Pinto et al., 2000; Garbuzova-Davis et al., 2003), it was only recently demonstrated that UCBCs could modulate the host immune inflammatory system. Th2-cytokines, which are anti-inflammatory (IL-4 and IL-13), are provided by donor T-cells.

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Moreover, decreased proinflammatory cytokines production in the spinal cord and microglia proliferation were also observed but treatment of mice with cyclosporine A may interfere with the latter beneficial effect (Garbuzova-Davis et al., 2008).

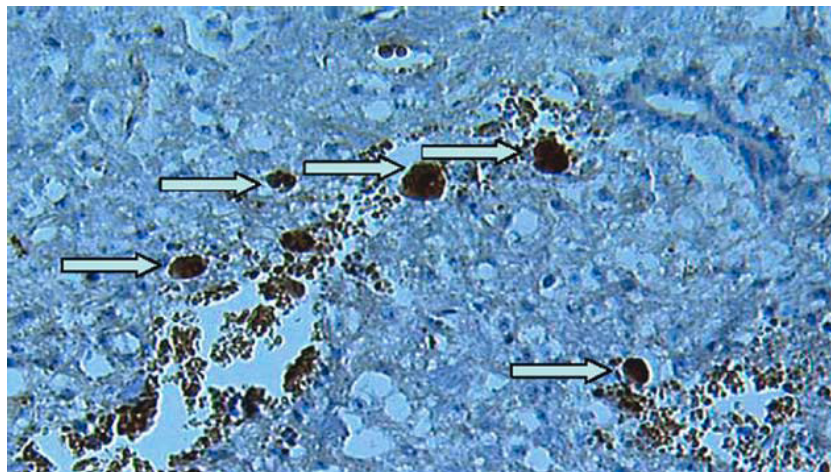


Figure 16: Implantation of Sertoli cells in the spinal cord of rodent ALS (brown: aggregation of GATA-4 positive Sertoli cells) (Hemendinger et al., 2005).

4.5. Clinical trials

A great deal of research has been done with the use of cellular and animal models, in particular with rodents transgenic for mutated human SOD1 genes. Disappointing is the lack of therapeutic advances based on findings from research in animals. Many therapeutic agents have been tested on rodent models of ALS, but they failed to be translated into clinical practice. The only disease modifying

medication for patients with ALS is riluzole. It was developed as an inhibitor of glutamate release with anti-excitotoxic effects, and in clinical trials its use has resulted in the extension of survival by approximately 3 months. A recent disappointment has been the trial of minocycline (a second generation tetracycline with anti-inflammatory properties), a preliminary report suggests that it may accelerate disease progression in humans (Gordon et al., 2007), despite having promising effects on hSOD1^{G93A} mice (Kriz et al., 2002; Van Den et al., 2002; Zhu et al., 2002). However, treatment was started before symptom onset in mice and after manifestation of the disease in humans, which suggests that timing of the treatment is highly relevant in rodent preclinical trials.

Nevertheless, a recent, very exciting report by Fornai and colleagues pointed out lithium as a treatment of ALS (Fornai et al., 2008). Lithium is a compound used as a mood stabiliser but is also endowed with neuroprotective properties (Raoul et al., 2005; Sarkar et al., 2005; Bian et al., 2007; Feng et al., 2008; Leng et al., 2008). In ALS mice, lithium prolongs survival, even when given at symptomatic stage, by protecting MNs and decreasing gliosis. Worth mentioning, lithium facilitates clearance of SOD1. Indeed, degradation of SOD1 is linked to the autophagy pathway (Kabuta et al., 2006). Thus, these beneficial effects could be related to the promotion of autophagy by lithium (Berridge et al., 1989; Sarkar et al., 2005) and a pilot trial enrolling 44 ALS patients presented encouraging result in combination to riluzole (Fornai et al., 2008).

Great expectations are raised by cell therapy in ALS. Stem cells demonstrate many characteristics of the different treatments used in hSOD1^{G93A} mice such as anti-oxydant, anti-inflammatory and anti-excitotoxic properties, in addition to release of growth factors and the possibility to replace lost or dysfunctional cells of the CNS. Proof-of-concept for stem cell therapy comes from Mazzini and colleagues who implanted autologous MSCs directly into the thoracic spinal cord of 9 patients with definitive ALS (no information was provided about the sporadic or familial nature of the disease) (Mazzini et al., 2006; Mazzini et al., 2008). Extensive expansion of MSCs did not involve modifications such as chromosomal alterations or cellular senescence. The long-term follow-up (more than 4 years) confirm that intraspinal injection of MSCs in ALS patients is safe as no signs of toxicity or abnormal growth of the cells were detected, nor did patients reveal any psychological consequences. Four of them showed a trend toward a transient slowing down of the decline (according to the ALS-functional rating scale, Norris score, bulbar score and medical research council strength scale) but four died due to respiratory complications. Given the short number of patients and the great variability of the disease however, no definite conclusion is possible, a new phase 1 study is being carried out to verify these data in a larger number of patients. Recently, reimplantation of bone-marrow cells, collected from the systemic circulation of ALS patients after mobilization by G-CSF (granulocyte colony stimulating factor) treatment and reimplantation showed no adverse effect, providing a supplementary source of stem cell (Cashman et al., 2008).

5. Aim of the study

Considering the accumulation of data implicating astrocytes in the pathogenesis of ALS, cell therapies aiming at replacing astrocytes at site of lesions constitute promising therapeutic strategies. Focusing on approaches which could be easily translated to the treatment of ALS patients, we decided to consider the potential of adult MSCs. Adult stem cells can be easily harvested from the patient bone marrow, avoiding issues concerning embryonic material, and can be used in autologous grafts without risk of destruction of grafted cells by the immune system.

Previous works in our laboratory has established a protocol for the *in vitro* differentiation of adult MSCs from Wistar rats into cells characterized by the expression of astrocytic markers (glutamine synthetase, S100- β) and functional high affinity glutamate transporter. The first objective of this thesis was to characterize differentiated adult MSCs from Sprague-Dawley rats for their capacity to express and secrete neuroprotective growth factor. In particular, we decided to study the production of GDNF which is crucial for MN survival and also investigated the pharmacological regulation of this production. The characterization of adult MSCs from Sprague-Dawley rats allowed us to investigate whether autologous graft in patients suffering from familial ALS could be efficient to replace dysfunctional astrocytes, as a rat model of familial ALS has been generated in this strain.

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Secondly, as we were interested in the possibility to use these cells *in vivo*, we explored whether the progression of a disease in the CNS triggers endogenous repair mechanisms involving stem cells recruitment. This study was conducted in the spinal cord of a rat model of familial ALS where lesions are well characterized. Finally, our motivating results in adult MSCs plasticity toward the glial lineage and the mechanism of cell attraction developed in the CNS of rat developing a familial form of ALS prompted us to examine the beneficial effect possibly brought by adult MSCs transplantation.

Results

1. In vitro generation of cells displaying astrocyte characteristics from adult mesenchymal stem cells.

Adapted from “*In vitro* evidence for impaired neuroprotective capacities of adult mesenchymal stem cells derived from a rat model of familial amyotrophic lateral sclerosis (hSOD1^{G93A})”. *Published in Experimental Neurology (2008)*.

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Results

As an alternative to neuronal substitution, protection of neurons by stem cells is an attractive challenge in the development of efficient therapies of neurodegenerative diseases. When giving preference to autologous grafts, the bone marrow constitutes a valuable source of adult stem cells. In this perspective, we studied the acquisition of typical glial-neuroprotective functions by cultured mesenchymal stem cells (MSCs) exposed to growth factors known to promote the differentiation of neural stem cells into astrocytes.

In these conditions, MSCs showed increased transcription and expression of the high affinity glutamate transporter GLT-1 and functional studies revealed increased aspartate uptake activity. In addition, differentiation was shown to endow the cells with the capacity to respond to riluzole which triggers a robust up-regulation of the GDNF production. In parallel, MSCs derived from the bone marrow of a transgenic rat model of familial ALS (hSOD1^{G93A}) were also characterized. Unexpectedly, cells from this rat strain submitted to the differentiation protocol showed modest capacity to take up aspartate and did not respond to the riluzole treatments.

Together, these data highlight the neuroprotective potential attributable to MSCs, supporting their use as valuable tools for the treatment of neurodegenerative disorders. However, the cells from the transgenic animal model of ALS appeared deficient in their capacity to gain the

neuroprotective properties, raising questions regarding the suitability of autologous stem cells grafts in future therapies against familial forms of this disease.

1.1. Introduction

ALS is a fatal neurodegenerative disease characterized by the progressive and selective death of MNs in the cerebral cortex, brainstem and spinal cord (Cleveland, 1999). While most cases are sporadic, a minority of patients (10%) shows a familial history and several mutations have been associated with these inherited cases. Best known are those affecting the gene encoding the superoxide dismutase 1 (SOD1) (Rosen et al., 1993) and transgenic animals carrying these mutations are widely used as experimental models since they recapitulate the principal features of the disease. Recently, the rapid progression of the disease was correlated with the recruitment of neural progenitors in the degenerating lumbar spinal cord of rodents carrying the predominant human SOD1^{G93A} mutation (Chi et al., 2006; de Hemptinne et al., 2006). This finding may hold clues for the development of new treatments using stem cells in order to slow the progression of the disease. As a putative alternative to neural stem cells, MSCs are frequently considered in experimental approaches aimed at promoting nervous repair in models of brain injuries (Chen et al., 2001) and spinal cord lesions (Cizkova et al., 2006). However, the restorative outcome obtained with these cells certainly surpasses the reasonable expectations of their transdifferentiation into functional neurons. In ALS, the substantial and effective replacement of damaged neurons by these stem cells is very improbable, considering the complexity to specifically drive the differentiation into functional MNs and their integration in the local

nervous circuitry. After grafting into the brain, MSCs could give rise to astrocytes (Azizi et al., 1998; Kopen et al., 1999), the properties of which could promote neuronal survival or protection (Clement et al., 2003). The acquisition of typical astrocyte features by grafted stem cells could be of potential benefit in the treatment of ALS as recent evidences suggest a dysfunction of glial cells in this disease (Boillee et al., 2006b; Nagai et al., 2007). However, recent studies raised doubts regarding the genuine properties of bone marrow and neural stem cells derived from familial mouse models of ALS (Corti et al., 2004; Liu and Martin, 2006). The purpose of the present work was to study the potential of rat MSCs to acquire “*chaperone*” characteristics related to astrocytes when maintained in culture conditions that would promote glial maturation (Johe et al., 1996; Maric et al., 2003; de Hemptinne et al., 2004). Focusing on putative neuroprotective properties of these cells, we evaluated their ability to produce the glial derived neurotrophic factor (GDNF) and to take up extracellular glutamate. GDNF is a potent survival factor produced by astrocytes which protects MNs after injuries (Li et al., 1995) and constitutes a promising therapeutic agent for the treatment of ALS (Wang et al., 2002b). Besides, dysfunction of glial glutamate transport is considered as a key pathogenic mechanism of ALS (Rothstein et al., 1995) and any approach contributing to enhance the uptake of this excitotoxic amino-acid is considered as potentially valuable for the treatment of the disease (Rothstein et al., 2005). In contrast to MSCs from wild-type animals that undoubtedly show glial-related protective capacities after differentiation, cells derived from a transgenic rat model of

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familial ALS (carrying the hSOD1^{G93A} mutation) appear to some extent defective in these properties. Together these results should certainly encourage further studies regarding the neuroprotective potential of MSCs but raise questions regarding the appropriateness of the use of autologous stem cells based therapies against familial forms of neurodegenerative disorders such as ALS.

1.2. Material and methods

Cell preparation.

The strain of transgenic Sprague-Dawley rats carrying the human SOD1 gene with the G93A mutation (NTac:SD-Tg(SOD1G93A)L26H, (Howland et al., 2002)) was initially obtained from Taconic Farms Inc, NY. All experiments were conducted on cells obtained from heterozygous animals or wild-type littermates which were all genotyped immediately after birth (de Hemptinne et al., 2006). MSCs were isolated from femurs and tibias of 8 weeks old animals by adhesion to non-coated tissue culture plasticware and propagated *in vitro* for up to 15 passages as previously described (de Hemptinne et al., 2004). All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC). For differentiation, cultures were maintained in a medium consisting of DMEM/F12 (Invitrogen), enriched with the defined growth factors supplement G5 (Invitrogen), diluted 1/100, as suggested by the manufacturer (composition: insulin 500 µg/ml, human transferrin 5 mg/ml, selenite 0.52 µg/ml, biotin 1 µg/ml, hydrocortisone 0.36 µg/ml, bFGF 0.52 µg/ml and EGF 1 µg/ml). Medium was complemented with bFGF 10 ng/ml (Peprotech, UK) during 5 days. Thereafter, the medium was renewed and bFGF was substituted with CNTF 10 ng/ml (Peprotech) for another 5 days. The entire differentiation protocol lasted for 10 days.

Immunocytochemistry.

Cells grown on 15 mm round poly-L-lysine (Sigma) coated glass coverslips, were fixed with ethanol 95% for 20 min at room temperature and permeabilized in PBS (phosphate buffer solution)/1% triton X-100 for 10 min. Non specific labelling was blocked by incubating the cells in PBS/non fat dry milk (15 mg/mL) for 1 h at 37°C. The cells were immunostained (overnight at 4°C) with antibodies against nestin (1:500; Chemicon), glutamine synthetase (GS) (1:1000; Sigma) and Glutamate Transporter-1 (GLT-1) (1:1,000; Chemicon). As secondary antibody, Cy3-conjugated anti-mouse IgG, Cy3-conjugated anti-rabbit IgG and Cy3 conjugated anti-guinea pig IgG (1:500; all from Jackson Immunoresearch Laboratory, Belgium) were used (1 h incubation at room temperature). Nuclei were stained for 30 min with the nuclear dye, 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1:5,000; Sigma). After staining, cells were overlaid with Fluoprep (Biomerieux, Belgium) and examined with an Olympus IX70 inverted fluorescent microscope coupled to a CCD camera (T.I.L.L. photonics, Germany). Excitation light (540, 400 nm wavelength for Cy3 and DAPI, respectively) was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. photonics, Germany). Digital images of fluorescent signals were acquired using appropriate filters and combined using the TILLvisION software (T.I.L.L. photonics, Germany).

GDNF promoter activity.

The pGDNF^{-986/+587} plasmid construct harbouring the firefly luciferase cDNA under the control of a rat genomic sequence that putatively contains the GDNF promoter was previously described (Caumont et al., 2006). This promoter region corresponds to a 1,572 bp fragment situated within the 5' untranslated region (5'UTR) of the rat GDNF gene. MSCs were transfected with pGDNF^{-986/+587} plasmid along with the phRG-TK (allowing constant expression of renilla luciferase) (Promega) using LipofectAMINETM (Invitrogen). After transfection, cells were maintained in OptiMEMTM (Invitrogen) supplemented with 10% FBS (fetal bovine serum). When indicated, riluzole was added 48 h after transfection during 24 h in OptiMEMTM. At the end of incubations, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) as previously described (Caumont et al., 2006). The results are presented as relative luciferase activity, which corresponds to the firefly related signal normalised to the Renilla signal, expressed in percent of the control.

Measures of GDNF release.

MSCs were cultivated in 12-well plates in OptiMEMTM (Invitrogen) with 0.5% BSA (bovine serum albumine). Culture media (300 µl) was collected after a 24 h-drug treatment and kept at -80°C. Secreted GDNF was quantified using an enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human GDNF CytoSets, Biosource, Belgium).

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Tritiated D-aspartate uptake assay.

Glutamate uptake velocity was measured as previously described (de Hemptinne et al., 2004), using [^3H]-D-aspartate (20 nM, Perkin Elmer, USA) as substrate. The specific uptake velocity (measured after 6 min) was estimated after subtracting the data obtained using Na^+ -free Krebs buffer. When indicated, the inhibitor of glutamate transporters *L-trans*-pyrrolidine-2, 4-di-carboxylic acid (t-PDC) (Tocris, UK) 1 mM and the GLT-1 inhibitor dihydrokainate (DHK) (Ocean Product International, Canada) 100 μM , were added 6 min before the addition of the substrate.

Western blot.

MSCs were homogenised in a buffer containing 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 20 mM 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), supplemented with a protease inhibitor cocktail (Sigma). Fifty μg of total protein extract were electrophoresed on 12% (hSOD1) and 10% (GLT-1) sodium dodecyl sulfate-polyacrylamide gel and proteins were transferred to nitrocellulose. Immunoblots were probed with anti-hSOD1 (1:500; BD Pharmingen), anti-GLT-1 (1:2,000; Chemicon) Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit (1:3,000), anti guinea pig (1:2,000) IgG (Sigma). Immunoreactive proteins were revealed using the ECL Western blotting detection system (Amersham Biosciences, Belgium).

Signals were quantified using a Kodak imaging station and analysis software (Kodak). To control for equivalent loading, blots were stripped and reprobed with an anti-actin antibody (1:750).

Real-time PCR.

Total RNA was isolated from MSCs grown in Petri dishes using Tripure (Roche, Switzerland) as detailed by the supplier. Real-time PCR (polymerase chain reaction) was performed for quantification of the genetic expression of GLT-1, nestin and GS using a MyQ™ unicolor real-time PCR detection system (Bio-Rad, Belgium). Reactions were carried out in a total volume of 25 µL containing 2 ng cDNA template of each sample, 0.375 µM primers (GLT-1 forward : GCGAATACCAAGGAGT, GLT-1 reverse : TTCATCCCGTCCTTGAAGTC, nestin forward: AGAAGATGAGGGTGACACG, nestin reverse: ATCTGTCAGGATTTGGGATGG, GS forward: TGGACAAAGTGATGGAGACG, GS reverse: CAACTCATGACAGGGTGTGG) and SYBR® green super mix (100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl₂, SYBR green I, 20 mM fluorescein) (Bio-Rad). The PCR protocol was conducted as follows: 30 s denaturation at 95°C, 45 s annealing at 60°C (for GLT-1 and GS) or 58°C (for nestin), 15 s elongation at 79°C for 40 cycles. The fluorescence signal was monitored at the end of each elongation step. Upon completion of PCR, amplification products were melted by gradually increasing the temperature in order to validate the

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specificity of the amplification. For quantitative analysis, amplifications were performed in the same conditions with serial dilutions of a mix of the cDNA templates. Each sample was normalized to the relative amplification of GAPDH. Calculation of cycle threshold values, standard curve preparation and relative quantification of mRNA in the sample were performed using the post-run data analysis software provided with the iCycler system.

1.3. Results

Differentiated MSCs express typical markers and morphology of glial cells

MSCs from wild-type and transgenic (hSOD1^{G93A}) rats were propagated in culture monolayers and maintained beyond passage 15 and characterized for their phenotype and putative differentiation (de Hemptinne et al., 2004). Immunocytochemical and FACS (Fluorescent Activated Cell Sorting) analyses indicated that MSCs from both strains were CD45-negative and CD90-positive, selected markers for haematopoietic and stromal bone marrow cells, respectively (Wexler et al., 2003). The mesenchymal identity of both populations was confirmed by evidencing their differentiation into osteogenic and adipogenic phenotypes (Fig. 17).

During their proliferation, MSCs from both wild-type and transgenic animals were undistinguishable, exhibiting a similar typical fibroblast-like morphology (Fig. 18). In contrast, during *in vitro* differentiation, these cells progressively evolve into totally distinct morphologies. Thus cells from wild-type animals adopted a glial-like profile extending ramified processes while cells from hSOD1^{G93A} animals developed only few, but very long processes, usually leading to bipolar cells. Immunocytochemical analyses revealed that before differentiation, cells from both animal strains showed modest nestin expression, a marker of neuroprogenitor cells. Despite the lack of expression of typical astrocytic (GFAP) and neuronal (MAP-2

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[microtubule associated protein-2]) markers (data not shown), differentiation of the cells coincided with the induction of nestin and GS in the entire population of MSCs cells from both wild-type and transgenic animals (Fig. 18). Since hSOD1^{G93A} protein accumulates in neurons and glial cells during the progression of the disease, we examined the expression of this protein in MSCs from transgenic rats by Western blotting using an antiserum that selectively targets the human form of the enzyme. Densitometric analysis of the signals revealed that differentiation was associated with a significant increase in hSOD in these cells (84.3 ± 11.9 % increase in hSOD1, $p < 0.001$, student's t-test) (Fig. 18).

Figure 17: ICC analysis revealed that after 15 passages, cultured MSCs from wild-type rats are CD45 negative (a) and CD90 positive (b). Both signals were revealed with FITC coupled secondary antibodies (green) and nuclei were stained with DAPI (blue). These results were confirmed by FACS (c, d). MSCs from hSOD1^{G93A} rats show a similar staining pattern in ICC (e, f) and FACS analysis (g, h). FACS plots show isotype control IgG staining profile (red) vs. specific antibody staining profile (green).

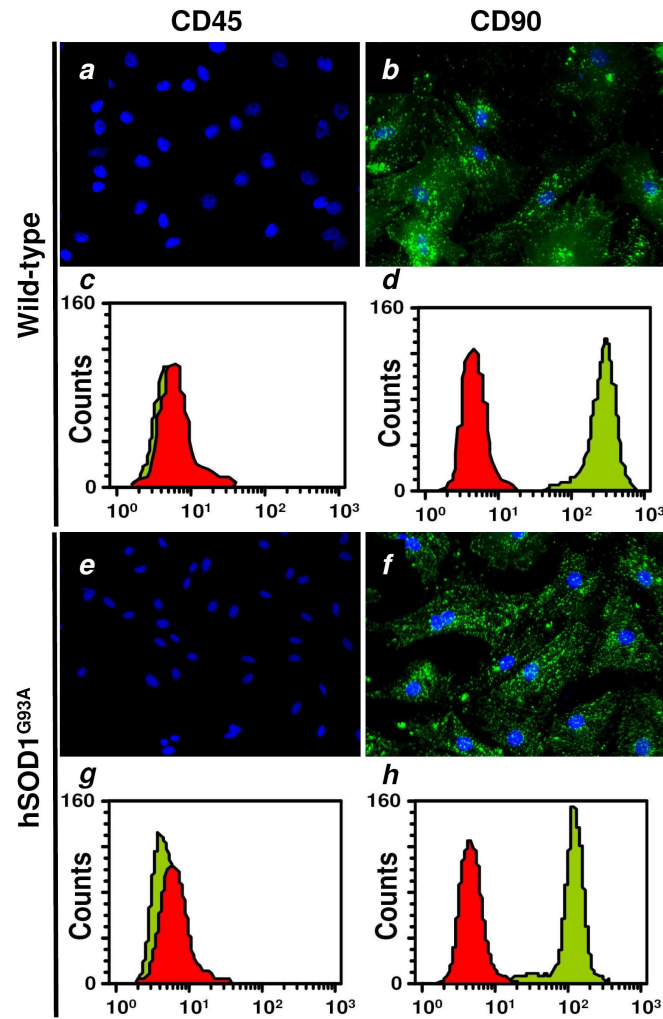


Figure 17: Characterization of cultured MSCs derived from the bone marrow.

MSCs display astrocytic regulation of GDNF production after differentiation

Expression of GDNF was evaluated at the transcriptional level using a luciferase reporter system and simultaneously, GDNF release was measured by ELISA (Fig. 19). MSCs maintained in proliferation conditions showed a basal transcription of the GDNF gene which was significantly increased after 24 h incubation with riluzole (up to 152% at 10 μ M, $p < 0.01$). Nevertheless, while differentiation only modestly increased the constitutive activity of the GDNF promoter ($p > 0.05$), the effect of riluzole was drastically enhanced, as induction reached up to 505% ($p < 0.001$) at 10 μ M. The modulation of GDNF concentration in the medium from the MSCs appeared consistent with data from the luciferase assay. Thus, in cells from wild-type animals, differentiation did not significantly change the basal secretion of GDNF, but markedly increased the response to riluzole.

In MSCs derived from hSOD1^{G93A} animals, the constitutive activity of the GDNF promoter appeared slightly higher as compared to cells from wild-type animals. Nevertheless, neither differentiation, nor incubation with riluzole were found to significantly increase luciferase activity in cells from the transgenic rats, suggesting that the regulation of GDNF gene was impaired in cells carrying the hSOD1^{G93A}.

MSCs increase glutamate transporter GLT-1 expression, maturation and functionality after differentiation

When maintained in proliferation conditions, a substantial aspartate uptake activity was evidenced (2.7 ± 1.0 pmol/mg prot/min). In the presence of the non-selective glutamate transporters inhibitor t-PDC, the uptake was abolished while only partial inhibition (27%) was obtained using DHK, a selective blocker of GLT-1. This suggests that most of the uptake activity is achieved through the GLAST or EAAC1. In differentiated MSCs, the uptake velocity was robustly increased (reaching 4.9 ± 1.4 pmol/mg prot/min) and the use of DHK revealed that this 85% increase mostly reflected an upregulation of substrate uptake through GLT-1.

Accordingly, there was no difference in the uptake velocity between differentiated and non-differentiated cells when tested in the presence of DHK (Fig. 20a). Indeed, increase expression of this transporter was evidenced by real-time PCR (Fig. 20b). Finally, the influence of cell differentiation on GLT-1 expression was also examined by Western blot, revealing a modification of both the intensity and gel mobility of immunoreactive proteins, ranging from 56 to 72 kDa. This could indicate that increased substrate uptake activity was correlated with the post-translational modification of GLT-1 after cell differentiation (Fig. 20c). In MSCs derived from hSOD1^{G93A} rats, the substrate uptake velocity (1.0 ± 0.4 pmol/mg prot/min) appeared significantly lower with comparison to cells from wild-type littermates in proliferation conditions and was not inhibited by DHK.

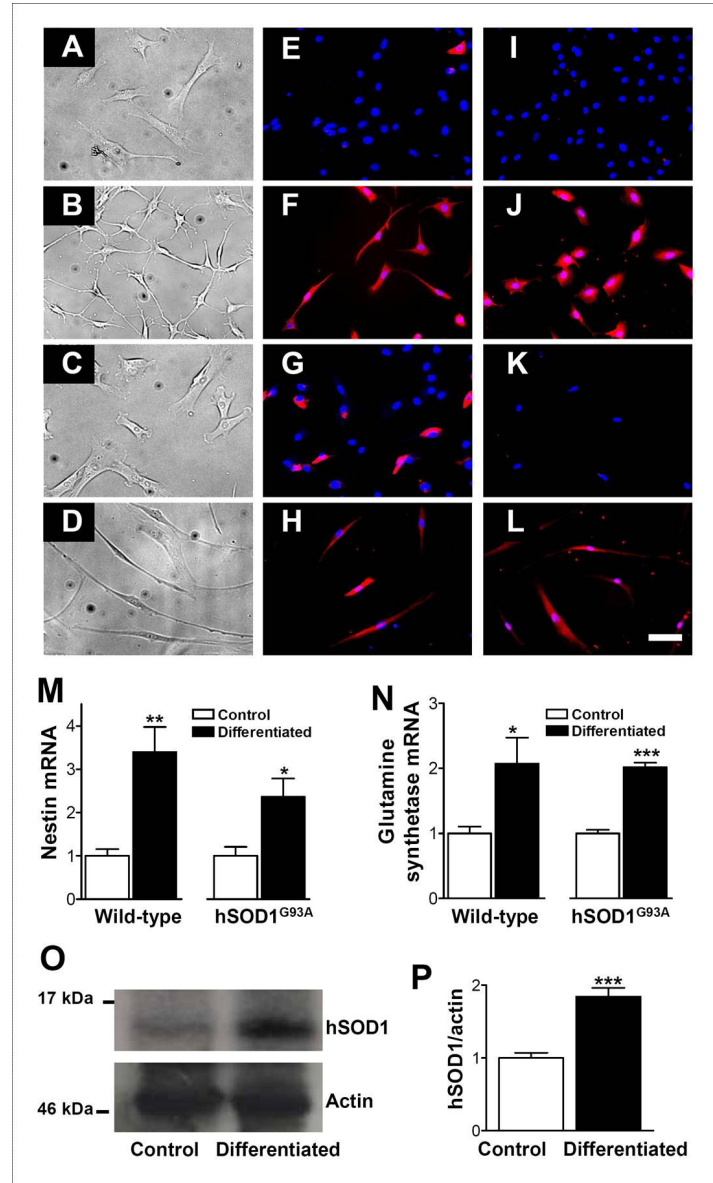


Figure 18: Acquisition of astroglial morphology and markers by MSCs.

Astrocytic differentiation of mesenchymal stem cells *in vitro*

Figure 18: Cultured MSCs from wild-type (A) and transgenic rats (C) showed a typical fibroblast-like morphology. After differentiation, MSCs from wild-type animals developed long and ramified processes (B) whereas cells from hSOD1^{G93A} rats adopted a bipolar morphology with few very long processes (D). Cells from both animal strains showed modest nestin expression in proliferative conditions (wild-type: E, hSOD1^{G93A}: G) and GS was not detected (wild-type: I, hSOD1^{G93A}: K). Differentiation was accompanied by an increased expression of nestin (F, H, M) and GS (J, L, N) mRNA and the corresponding proteins were expressed in the entire population of MSCs from both wild-type and hSOD1^{G93A} rats. Scale bar = 100 μ m. Western blot analysis showed that differentiation of cells from hSOD1^{G93A} rats leads to a robust accumulation of hSOD1 (upper panel). The lower panel shows detection of actin validating gel loading and electrophoresis (O) and quantification (P). (Data shown are representative of at least 6 independent experiments, student's t test, *p<0.05, **p<0.01, ***p<0.001).

Besides, though differentiation significantly increased the uptake velocity to 3.3 ± 0.9 pmol/mg prot/min, this activity appeared clearly lower as compared to MSCs from wild-type animals and only partial inhibition was obtained with DHK (Fig. 20a). This impaired GLT-1 function in cells from the transgenic animal contrasted with the increase in the corresponding mRNA upon exposure to differentiation medium, similar to that found with MSCs from wild type animals (Fig. 20b). Western blot analysis of GLT-1 in differentiated cells revealed differences between samples from wild-type and transgenic animals. Thus, the lower band (56 kDa) was systematically detected and changes in its expression after exposure to differentiation

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conditions were consistent with real-time PCR data. In contrast, noticeable differences in the intensity and position of other bands were clearly evidenced, and in particular, the above mentioned 72 kDa form of the GLT-1 was not induced in cells from hSOD1^{G93A} rats (Fig. 20c).

Figure 19: Luciferase activity measured in transiently transfected MSCs and its modulation observed after exposure to riluzole for 24 h. Data are expressed as relative firefly luciferase activity normalised to the renilla luciferase activity and finally expressed in percentage of the control (MSCs from wild-type rats). B: Quantification of GDNF in MSCs media conditioned during 24 h in the absence or in the presence of riluzole. Data shown are means with S.E.M. from at least 3 independent experiments. (One-way ANOVA, Tukey's post hoc test; *p<0.05, **p<0.01, ***p<0.001 for a given cell type; ###p<0.001 for differentiated cells). WT and G93A refer to cells from wild-type animals and transgenic animal respectively; diff., differentiated.

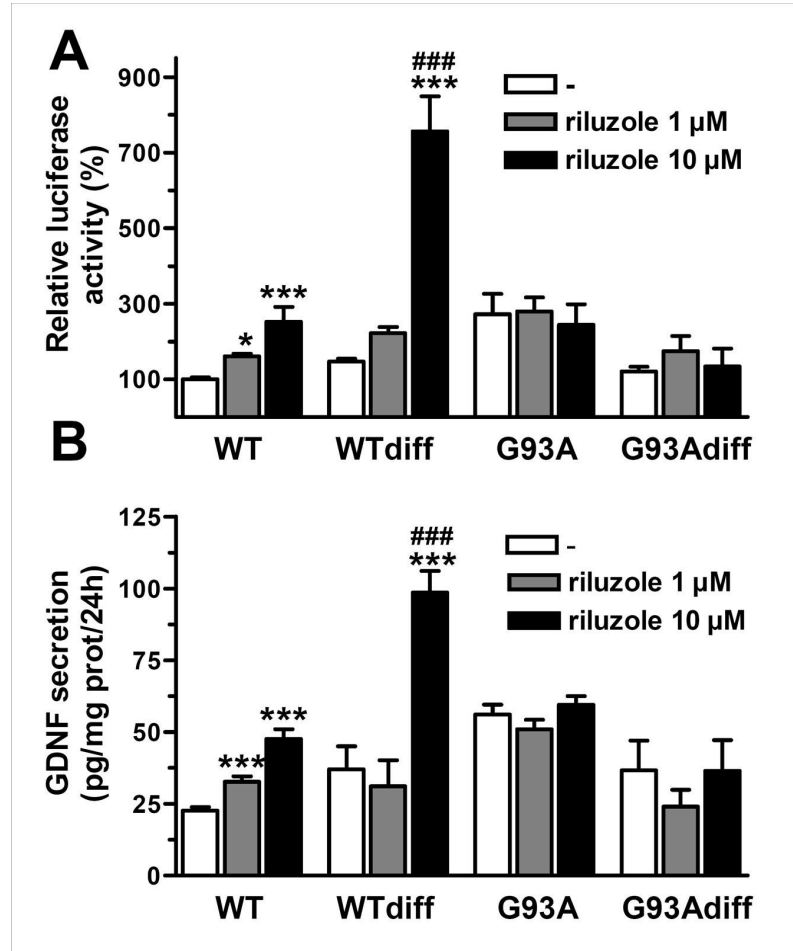


Figure 19: Modulation of GDNF expression and production by MSCs.

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Figure 20: Aspartate uptake velocity, GLT-1 mRNA levels and the corresponding protein were examined in MSCs. A: The influence of cell differentiation on the [³H]-D-aspartate (20 nM) uptake in MSCs from both strains of rats. The implication of high-affinity glutamate transporters was validated by showing inhibition with the non-selective blocker t-PDC while the GLT-1 dependent uptake was evaluated using the selective uptake competitor DHK. Data shown are means with S.E.M. from at least 3 independent experiments. Statistical analysis (one-way ANOVA followed by tukey's post hoc test) was performed in order to investigate the influence of the inhibitors (*p<0.05, **p<0.01, ***p<0.001) or to compare uptake velocities in differentiated and non differentiated cells (###p<0.001). B: Real-time PCR quantification of GLT-1 mRNA. Data shown are means with S.E.M. from at least 3 independent experiments. Statistical analysis (student's t-tests) shows the influence of cell differentiation (**p<0.01, ***p<0.001). C: Immunoblotting analysis showing the influence of differentiation on the level of GLT-1 (upper panel) in protein extracts from MSCs. The lower panel shows detection of actin performed in order to validate gel loading and electrophoresis. Shown are blot from a typical experiment performed at least 4 times with similar results.

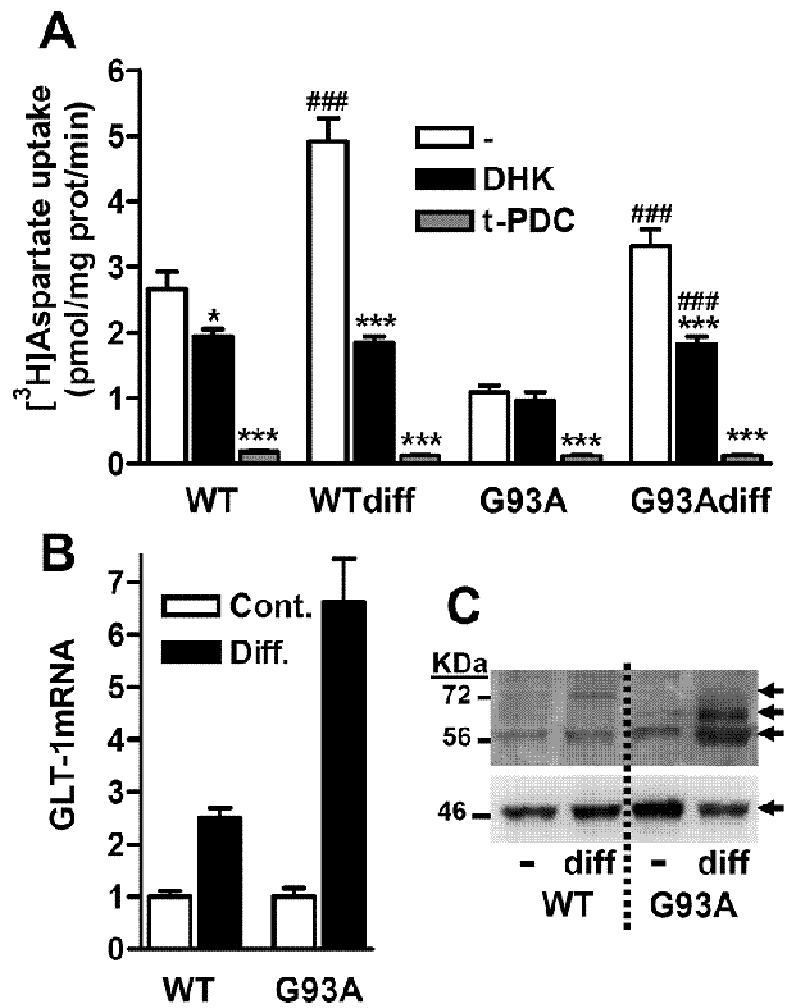


Figure 20: Expression and activity of GLT-1 in MSCs.

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1.4. Conclusion

Recent studies have suggested that the death of MNs in ALS is not cell autonomous since altered functions of other cell types such as astrocytes (Nagai et al., 2007) and microglia (Boillee et al., 2006b) appear to actively participate in the progression of the disease. Indeed, healthy glial cells provide essential metabolic and physical supports for adjacent MNs (Clement et al., 2003; Beers et al., 2006). Therefore, as an alternative or an adjunct to neurons replacement, the restoration of spinal and brain glial cell activities should constitute a valuable form of cell-based therapy for ALS. Limitation in cell availability for nervous system repair has motivated the search for surrogate sources of stem cells that could be differentiated along nervous lineages. Indeed, convincing findings suggest that adult stem cells can overcome their intrinsic restrictions upon exposure to novel chemical or cellular environments. Thus, the exposure of MSCs obtained from the bone marrow to appropriate conditions was shown to promote their differentiation into neurons and astrocytes, as suggested in both *in vitro* and *in vivo* studies (Azizi et al., 1998; Bossolasco et al., 2005; Deng et al., 2006). Nevertheless, trans- differentiation of MSCs is still a matter a debate (for review (Krabbe et al., 2005; Phinney and Isakova, 2005)). Cultures of MSCs were exposed to soluble factors commonly used to enhance the proliferation and differentiation of neural cells. Combination of bFGF and EGF was shown to favour the commitment of neural stem cells into neuronal and glial phenotypes (Maric et al., 2003) while CNTF efficiently promotes the

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differentiation of rodent neural stem cells into astrocytes (Johe et al., 1996).

In these conditions, MSCs i) were found to strongly express nestin, an intermediate filament found in stem/progenitor cells in the CNS, ii) adopted a typical glial morphology, extending ramified processes and iii) showed increased GS expression, which is typically associated with astrocyte metabolism. Intriguingly, the lack of GFAP expression in the differentiated cultures inevitably raises questions regarding the acquisition of a mature astrocyte phenotype by MSCs. Nevertheless, we carried on the characterization of the potential neuroprotective properties offered by these cells when adopting the differentiated phenotype. Obviously, several studies reported on the potential benefit of MSC grafts in animal model of neurodegenerative disorders with no substantial proof of cell differentiation (i.e. GFAP expression). Indeed, these cells were shown to stimulate endogenous neurogenesis (Munoz et al., 2005) and angiogenesis (Ball et al., 2007), to restore blood flow and blood brain barrier (Borlongan et al., 2004) and to reduce inflammatory processes (Zappia et al., 2005). Classically produced by glial cells in the CNS, GDNF is among the most potent trophic factors known to protect and rescue neuronal cells (Li et al., 1995). Indeed, therapeutic approaches based on the administration of GDNF have been frequently proposed in ALS (Wang et al., 2002b), Parkinson (Dowd et al., 2005) and Huntington diseases (McBride et al., 2006).

Herein, we observed that differentiation of MSCs only modestly increased the basal activity of the GDNF promoter and the release of the corresponding protein. However, differentiated cells showed a robust increase in the sensitivity to the neuroprotective drug riluzole, which substantially promoted the transcription of the GDNF gene and the release of GDNF. As previous studies evidenced a glial specificity of the influence of riluzole on GDNF production (Mizuta et al., 2001; Caumont et al., 2006), this finding further suggests a glial-related identity of MSCs after differentiation.

An attractive feature of MSCs resides in the possibility to develop autologous stem cell therapies for neurological disorders. Therefore, the differentiation of MSCs into cells bearing defined astroglial properties was investigated with cultures of cells derived the bone marrow of transgenic rats carrying the hSOD1^{G93A} transgene. Mimicking cells from wild-type animals, differentiation of cells from the transgenic rats coincided with increased expression of the typical markers such as nestin and GS. However, contrasting with the stellate morphology adopted by the former, cells carrying the hSOD1^{G93A} mutation showed a bipolar morphology, suggestive of a distinct differentiation potential. Noteworthy, immunoblotting studies evidenced that the differentiation of cells from the transgenic rats was accompanied with a robust accumulation of hSOD1 protein. Aggregation of misfolded hSOD1 in neurons but also in glial cells could be implicated in various cellular dysfunctions and is thought to participate in the pathogenesis of the disease (Bruijn et al., 1997).

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Herein, impaired acquisition of neuroprotective properties by MSCs from the transgenic rats was observed as both luciferase based promoter assays and ELISA failed to evidence riluzole mediated induction of GDNF expression and release by these cells. Nevertheless, basal secretion of GDNF appeared higher in MSCs derived from hSOD1^{G93A} rats, suggesting that the expression of the mutated hSOD1^{G93A} protein could be harmful for MSCs which in turn activate mechanisms of protection. Although the molecular mechanism involved in the positive modulation of GDNF gene transcription in glial cells remains obscure, recent study have implicated a MAPK [mitogen activated protein kinase]/ERK [extracellular signal-regulated kinase] kinase dependent signalling pathway (Caumont et al., 2006). Since increased activity of p38 MAPK is documented in ALS (Bendotti et al., 2005; Veglianesi et al., 2006), one could suggest that increased activation of p38 MAPK is responsible for the increased GDNF promoter activity. However, its accumulation in protein aggregates was detected in rodent models of familial ALS (Tortarolo et al., 2003; Bendotti et al., 2004) This could reduce the availability of MAPK in MSCs from the transgenic rats, explaining the lack of modulation by riluzole.

Glutamate-mediated excitotoxicity resulting from repetitive firing and elevation of intraneuronal calcium through ionotropic glutamate receptors is frequently incriminated in the pathogenesis of ALS (Van Damme et al., 2005). In both patients and animal models, the pathology is associated with a reduction in the expression and activity

of the glial transporter GLT-1 (Rothstein et al., 1996; Howland et al., 2002). Therefore, the restoration of glutamate homeostasis within lesioned areas constitutes one of the promises of therapies involving stem cells (Rothstein et al., 2005). The present study demonstrates the specific upregulation of functional GLT-1 during the *in vitro* differentiation of MSCs derived from the bone marrow. Increased GLT-1 function was apparently associated with modification of its post-translational processing giving rise to high molecular weight GLT-1 immunoreactive proteins (72 kDa) which likely correspond to glycosylated, functional transporters. Intriguingly, in cells carrying the hSOD1^{G93A} transgene, both GLT-1 mRNA and the corresponding protein appeared significantly more abundant as compared to cells from wild-type animals. However, this induction mainly gives rise to less-functional transporters, as revealed by low substrate uptake values. Indeed, immunoblot studies revealed the absence of the above mentioned 72 kDa immunoreactive band, suggesting that increased transcription and translation failed to engender mature proteins. Worth mentioning, a link between reduced glycolysation and decreased functionality of GLT-1 was already reported in a case of ALS involving a mutation of GLT-1 (Trotti et al., 2001).

In summary, the key observation of our data is the impairment of MSCs from hSOD1^{G93A} rats to acquire neuroprotective properties. More work is needed to test whether MSCs from hSOD1^{G93A} rats exhibit a default of differentiation (supported by a lack of glial specific regulation of GDNF and GLT-1 in addition to the

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morphology) or if they are as differentiated as MSCs from wild-type rats but present dysfunction in neuroprotective capacities (supported by the increased nestin and glutamine synthetase expression). Similarly, previous studies have shown that neural stem cells from hSOD1^{G93A} mice display subtle alterations in proliferation capacity and phenotypic characteristics, contributing to an impaired adult neurogenesis (Liu and Martin, 2006). Similarly, haematopoietic stem cells from ALS2 mice show dysfunctions in restoring lymphopoiesis and haematopoiesis (Erie et al., 2007). Finally, grafts of freshly isolated bone marrow cells from hSOD1^{G93A} mice into the same animal strain failed to provide any benefit with respect to the disease progression whereas cells from wild-type animals significantly prolonged their lifespan (Corti et al., 2004). Though questions persist regarding the option of autologous grafts of stem cells in the future therapies against familial form of neurodegenerative disorders such as ALS, our study should encourage to consider the neuroprotective potential of MSCs when developing stem cells based approaches of neurological disorders.

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2. Neurodegeneration in amyotrophic lateral sclerosis is permissive for migration stem cell migration.

Adapted from “Unilateral induction of progenitors in the spinal cord of hSOD1^{G93A} transgenic rats correlates with an asymmetrical hind limb paralysis”. *Published in Neuroscience letters (2006)*.

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Transgenic rats expressing a mutated form of the human Cu/Zn superoxide dismutase (hSOD1^{G93A}) develop an amyotrophic lateral sclerosis (ALS)-like phenotype, including motor neurone degeneration and reactive gliosis in the spinal cord. Considering that MSCs are able to display astrocytic characteristics and could rescue MNs, we examined the presence of stem cell recruitment mechanisms in the lumbar spinal cord of these rats at the end-stage of the disease.

Immunohistochemical data clearly demonstrated the induced expression of the stem cell factor (SCF) reported as a chemoattractant and survival factor for neural stem cells as well as nestin (neuro-epithelial stem cell intermediate filament) in the spinal cord sections. While the stem cell factor immunolabelling appeared diffuse throughout the gray matter, nestin labelling was restricted to clusters within the ventral horn. Finally, immunohistochemical detection of the stem cell factor receptor (c-Kit) revealed its specific induction which coincided with nestin immunolabelling.

Together, these results are indicative of endogenous recruitment of neural progenitors within lesioned tissues and could support the development of treatments involving endogenous or exogenous stem cells.

2.1. Introduction

ALS is a neuromuscular disorder characterised by progressive motor dysfunction leading to paralysis, muscular atrophy and death. The pathology results from the death of large MNs in motor cortex, brainstem and the spinal cord (Kikuchi et al., 1999). ALS occurs in both sporadic and familial forms (Siddique and Deng, 1996), the latter representing 5-10 % of reported cases. Approximately 15-20 % of familial ALS cases has been linked to autosomal dominant inheritance of mutated forms of SOD1 (Deng et al., 1993; Rosen et al., 1993). While oxidative stress and disturbed control of the glutamatergic neurotransmitter system have been implicated, the precise cellular mechanism underlying the anatomically selective neurodegeneration in this disease has still to be identified (Cluskey and Ramsden, 2001; Castro et al., 2002).

Recently, transgenic rats carrying the human SOD1^{G93A} (hSOD1^{G93A}) gene have been generated (Nagai et al., 2001) and constitute a dedicated experimental animal model of ALS. At the end-stage of the disease, they show motor neuron degeneration in the spinal cord with decreased expression of MAP2 and tissue vacuolisation. Simultaneously, a pronounced gliosis is observed in the spinal cord and the brainstem (Howland et al., 2002; Dunlop et al., 2003). Gliosis is a prominent feature of several diseases of the CNS in which reactive astrocytes invade lesioned areas and participate in both neurone replacement as well as neuroprotection thanks to their ability to take up excitotoxic glutamate and to release neurotrophic factors

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(Bresjanac and Antauer, 2000; Chen and Swanson, 2003; Pekny and Nilsson, 2005).

In this context, the present study aimed at examining presence of endogenous neural progenitors in the lesioned tissues of hSOD1^{G93A} which could indicate the emergence of repair processes during the progression of the pathology. We investigated the expression of SCF. Initially characterised as a key factor in the regulation of haematopoiesis (Andrews et al., 1994), SCF acts as a chemoattractant and survival factor for neural stem cells (NSC) (Erlandsson et al., 2004). Hence, its tyrosine kinase receptor (c-Kit) is present on NSCs and its activation triggers cell migration both *in vivo* and *in vitro* (Sun et al., 2004; Erlandsson et al., 2004). Finally, the expression of nestin (neuro-epithelial stem cell intermediate filaments) was also examined. This intermediate filament protein expressed by proliferating neuro-epithelial cells during the development of the CNS is also expressed by NSCs in the adult mammals and classically used to identify adult neural progenitors in culture (Mignone et al., 2004). The results of this immunohistochemical study reveal modulated expression of these markers in the spinal cord of end-stage transgenic animals. We suggest that these changes figure an adaptation of the organism in response to the degenerative process and we propose that it could provide a biological support for treatments involving endogenous or exogenous stem cells.

2.2. Material and methods

Animals.

Transgenic Sprague-Dawley rats harbouring a 12-kb *EcoRI/BamHI* restriction fragment of the human SOD1 gene with the G93A mutation (hSOD1^{G93A}) were previously described (Mignone et al., 2004) and obtained from Taconic Farms Inc, NY. Animals housed in plastic cages were kept at constant temperature ($23 \pm 1^\circ\text{C}$) and relative humidity (40-60 %) on a 12 h light, 12 h dark cycle with access *ad libitum* to both food and water. All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC). Newborn rats were analyzed for the presence of the transgene by PCR performed on genomic DNA extracted from tails biopsy obtained a few hours after birth. Tissue from each animal was incubated with proteinase K (100 mM TrisHCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) at 56°C overnight and after centrifugation, precipitation and washes, purified DNA was quantified. 500 ng of genomic DNA were subjected to PCR using specific primers SOD-i3f (5'-GTGGCATCAGCCCTAATCCA-3') and SOD-E4r (5'- CACCAGTGTGCGGCCAATGA-3') and the program consisted in 30 cycles of denaturation ($95^\circ\text{C}/ 45\text{s}$), hybridisation ($60^\circ\text{C}/ 45\text{s}$) and extension ($72^\circ\text{C}/ 45\text{s}$). 20 μl of each sample were electrophoresed on a 1% agarose gel and visualised using

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ethidium bromide. Transgenic animals were diagnosed by the presence of a 160 bp amplification product.

Tissue analysis.

The behaviour of each animal was routinely monitored by testing extension reflex of the hindlimb when rats are suspended by the tail. As the animal's condition dramatically deteriorated, rats were anaesthetised, their spinal cord was dissected and fixed in formalin 10% for 48-72 h. Tissues from aged-matched wild-type littermates were processed simultaneously for control. After rinsing in 0.1 M phosphate buffered saline (PBS, NaCl 0.137 M, KCl 2.68 mM, KH_2PO_4 1.5 mM, Na_2HPO_4 8 mM, pH 7.4) and progressive dehydration in ethanol 70°, 90° and 100° (24 h each), tissues were immersed in toluol (16 h) and embedded in paraffin. Five- μm sections of tissues from at least 5 different animals were cut in a microtome and apposed on coated glass microscope slides. Sections were deparaffinized with toluol, rehydrated in alcohol and rinsed in distilled water. Non-specific labelling was blocked by incubating the sections in PBS containing 1% bovine serum albumin for 1 h at room temperature. Sections were then incubated during 1 h with primary antibodies i.e. a guinea pig anti GLT-1 antibody (Chemicon 1:200), a mouse anti-nestin antibody (Chemicon, Hampshire, UK - 1:200), a rabbit anti-GFAP antibody (DAKO, Heule, Belgium - 1:400), a mouse anti-MAP2 antibody (Sigma, Bornem, Belgium - 1:200), a rabbit anti-SCF antibody (Chemicon - 1:250) and a rabbit anti-C-kit antibody (Sigma, Bornem, Belgium - 1:100). Secondary antibodies (all from

Jackson Immunoresearch Laboratory, De Pinte, Belgium), applied for 1 h at room temperature were fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (1:200), FITC-conjugated anti-rabbit IgG antibody (1:200) and cyanin3 (Cy3)-conjugated antiguinea pig IgG antibody (1:200). The Tyramide Signal Amplification Kit (Invitrogen-Molecular Probes, Merelbeke, Belgium) was used for SCF and c-Kit detection following manufacturer's advice, in order to enhance specific signals. Cell nuclei were stained during 15 min with the nuclear dye 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1:5000). After 3 rinses with PBS, the preparations were mounted in Fluoprep and examined using an Olympus IX70 inverted fluorescent microscope coupled to a CCD camera (T.I.L.L. photonics, Martinsried, Germany). Excitation light (485, 540 and 400 nm wavelength for FITC, Cy3 and DAPI, respectively) was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. photonics, Martinsried, Germany). Digital images were acquired using appropriate filters and combined using the TILLvisION software (T.I.L.L. photonics, Martinsried, Germany).

2.3. Results

Each litter contained both wild-type and transgenic pups and therefore, each animal has been genotyped for the presence of the hSOD1^{G93A} gene by PCR on genomic DNA. The behaviour of each animal was routinely monitored and the apparition and progression of the symptoms was recorded (Table 3 and fig. 21a). In most cases, motor disability appeared after 4½-5 months and began asymmetrically at the hind limb. Animal's condition rapidly declined and complete paralysis was observed within 9-17 days after detection of the first symptoms. At that stage, animals were sacrificed and tissues were immediately processed for immunohistological analysis.

Lumbar spinal cord sections from end-stage hSOD1^{G93A} rats were investigated for the expression of MAP2 and GFAP in order to evaluate the extent of neuronal loss and the progression of the gliosis. In accordance with data from the literature, MAP2 immunolabelling was strongly decreased in the spinal cord of transgenic rats as compared to wild-type littermates (Fig. 21b-b'). In contrast, while GFAP labelling was almost absent in tissue sections from wild-type animals, an intense immunoreactivity was detected throughout the grey matter of the spinal cord of hSOD1^{G93A} rats (Fig. 21c-c'). At this stage of the disease, no difference in MAP2 or GFAP labelling could be observed between the left and right sides of the spinal cord sections. Consistent with previous studies, GLT-1 immunolabelling appeared reduced throughout the lumbar spinal cord of transgenic rats

Neurodegeneration in ALS is permissive for stem cell migration

Table 1

Behavioural data: the progression of the disease was examined in 22 animals

Side of initial symptoms	Number of animals	Symptoms onset (days after birth)	Symptoms duration (days)	Animal survival (days after birth)
Right	9	140.4 $\pm$ 4.3	11.3 $\pm$ 1.7	151.8 $\pm$ 4.0
Right and left	6	140.2 $\pm$ 4.0	14.17 $\pm$ 3.3	154.3 $\pm$ 2.0
Left	7	143.0 $\pm$ 4.6	10.57 $\pm$ 1.5	153.6 $\pm$ 5.0

Symptoms onset, symptoms duration and animal survival are given in days $\pm$ S.E.M.

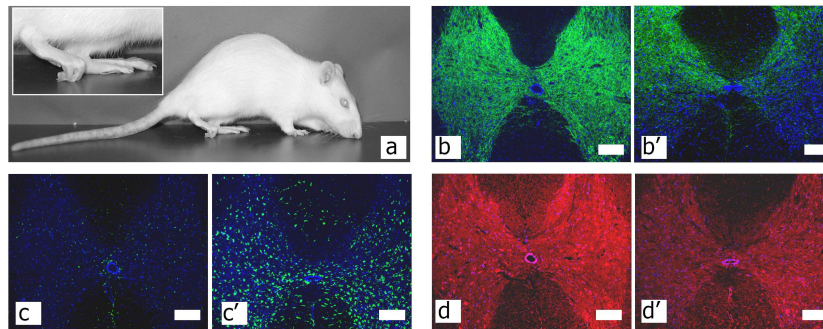


Figure 21: Immunohistochemical detection of MAP2, GFAP and GLT-1 in the lumbar spinal cord of end-stage hSOD1^{G93A} rats.

Figure 21: Paralysis of ALS rats started asymmetrically (a). The expression of MAP2 (b and b'), GFAP (c and c') and GLT-1 (d and d') was detected in tissue sections using specific antibodies visualised with a FITC-coupled secondary antibody (green). Cell nuclei are stained with DAPI (blue). Images shown are taken within the grey matter in sections from wild-type (b, c, d) or transgenic rats (b', c', d') and are representative of several sections obtained from at least five different animals. scale bar = 200 μ m.

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and a focal loss of this transporter was evidenced at high magnification (Fig. 21d-d'). In view of the degeneration affecting the lumbar spinal cord, sections from this region were probed for the expression of the SCF. In sections from wild-type animals, only few disseminated pericaryons within the grey matter showed SCF immunoreactivity whereas the density of positive cells was higher in the white matter (Fig. 22a).

In contrast, intense and widely spread labelling was detected in both the grey and white matters of the spinal cord of hSOD1^{G93A} rats (Fig. 22a'). Considering the intense and widespread labelling of SCF, the nestin immunoreactivity was studied in sections from the spinal cord in order to detect the presence of putative neuroglial progenitors. Careful examination of several sections from the lumbar spinal cord of wild-type animals did not permit to detect nestin-positive cells (Fig. 22b). In contrast, nestin appeared abundantly expressed in tissue sections from hSOD1^{G93A} rats (Fig. 22b'). Important variations in the extent of nestin immunoreactivity were observed between sections from different hSOD1^{G93A} rats. Thus, for some animals, nestin-positive cells were present as disseminated clusters while for others, labelling concerned large areas in the grey matter. This labelling was highest in the upper part of the ventral horn and interestingly, was mainly restricted to a single side of the spinal cord sections. Further confrontation of these immunohistochemistry data with behavioural data from the same animals revealed an anatomical relationship between the induction of nestin and the progression of the pathology.

Thus, as paralysis initially develops asymmetrically, induction of nestin was mainly restricted to the ipsilateral side of the predominant symptoms observed at the early stages of the disease. The relationship between SCF expression and the presence of nestin-positive clusters in the spinal cord of transgenic animals was further investigated by examining the expression of c-Kit (Fig. 22c). As shown in Fig 3b, nearly all nestin-positive cells showed positive c-Kit labelling (Fig. 22d). Noteworthy, significant proportion c-Kit positive/nestin negative cells were present at the centre of the clusters of nestin immunoreactivity.

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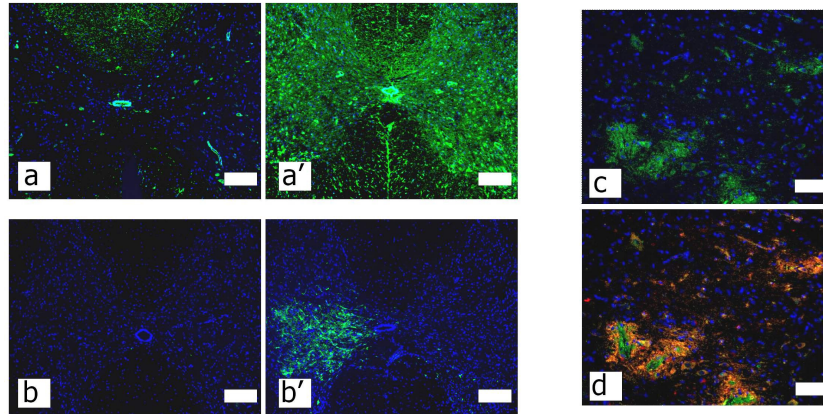


Figure 22: Immunohistochemical detection of SCF, nestin and c-Kit in the lumbar spinal cord of end-stage hSOD1^{G93A} rats.

Figure 22: The expression of SCF (a and a') and nestin (b and b') was detected in tissue sections using specific antibodies visualised with a FITC-coupled secondary antibody (green). The Tyramide Signal Amplification Kit was used to enhance the detection of SCF immunoreactivity. Cell nuclei are stained with DAPI (blue). Images shown are taken within the grey matter in sections from wild-type (a and b) or transgenic rats (a' and b') and are representative of several sections obtained from at least five different animals. The expression of c-Kit (green) (c) was detected in tissue sections using a specific antibody and the tyramide Signal Amplification Kit. Cell nuclei are stained with DAPI (blue). (d) merged images of the detection of c-Kit (green) and Nestin (red). Images shown are taken within the grey matter in sections from transgenic rats and are representative of several sections obtained from at least three different animals. Scale bar= 200 μ m.

2.4. Discussion

The present study aimed at examining the existence of endogenous repair processes that develop in the lumbar spinal cord of severely disabled hSOD1^{G93A} rats. These transgenic animals developed progressive and asymmetrical paralysis at the age of 4½-5 months and died within 9-13 days after emergence of the first symptoms. Immunolabelling studies on lumbar spinal cord sections confirmed extensive neurodegeneration that coincides with a robust induction in GFAP expression, characteristic of reactive astrocytes (Eddleston and Mucke, 1993). Changes in astrocytes morphology and metabolism are thought to be indicative of the cellular adaptation in response to extensive neuronal death and prolonged exposure to oxidative stress (Ridet et al., 1997). Beside their role in the control of glutamate homeostasis, astrocytes appear to coordinate tissue remodelling, both in physiological and pathological conditions. Indeed, reactive astrocytes overexpress a variety of growth factors, including VEGF and FGF₂ and thereby, may directly influence the recruitment, proliferation and differentiation of endogenous stem cells or progenitors (Horner and Palmer, 2003). In this perspective, an original observation of the present study is the robust induction of the SCF expression in lumbar spinal cord of hSOD1^{G93A} rats where extensive gliosis was evidenced. Immunohistochemical labelling showed that SCF is mainly restricted to the perikaryons of MNs in control animals, while intense labelling was detected throughout the lumbar spinal cord of transgenic rats. This coincided with a previous study indicating that

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the SCF expression is induced within zones of traumatic brain injury (Zhang et al., 1999) where it may participate in the recruitment of NSCs, as shown both *in vitro* and *in vivo* (Sun et al., 2004). While accelerated neurogenesis favouring functional recovery has been documented after cerebral ischemia (Jin et al., 2001; Jiang et al., 2001), the factors regulating this process in the adulthood remain poorly understood. Precisely, a putative role for the SCF in the CNS is to induce neurogenesis (Jin et al., 2002) and to promote migration and survival of NSCs (Erlandsson et al., 2004). Indeed, the SCF was shown to increase the expression of several growth factors in cultured neurones and glial cells, suggesting that SCF could indirectly support neuronal survival and/or functions.

Based on these reports, the increased expression of the SCF in regions of the spinal cord of transgenic animal models of ALS suggests that the progression of the disease could be associated with the local recruitment of neuronal progenitors. This hypothesis was reinforced by the detection of several clusters of nestin-positive cells in the lumbar spinal cord of hSOD1^{G93A} rats, while absolutely no expression was observed in wild-type animals. Similarly, induction of nestin mRNA was recently reported in tissue samples obtained from the ventral horn of the lumbar spinal cord of ALS patients (Strong et al., 2004). Nestin is an intermediate filament used as a common marker of progenitor cells from several lineages. The presence of nestin within adult tissues is considered as indicative of regenerative potential (Wiese et al., 2004) and its detection in tissue sections from

hSOD1^{G93A} rats at the end-stage of the disease provides indications for the emergence of endogenous repair processes. Interestingly, in the present study, nestin was only expressed on one side of the lumbar spinal cord sections and further analysis of data revealed that the asymmetrical induction of this progenitor marker systematically correlated with the ipsilateral side of the hind limb showing the first signs of paralysis at the early stages of the disease. The SCF produces its biological effects of chemotaxis, proliferation and survival by activating its tyrosine kinase receptor c-Kit, which is found on several cell types in the brain (Zhang et al., 1999), including stem cells or progenitors. The majority of the nestin-positive cells in our study, immunostained positive for c-Kit. This is in accordance with an other study, where Erlandsson and colleagues reported that among the population of nestin-positive cells in the brain, more than 90% express c-Kit (Erlandsson et al., 2004). Furthermore, increased expression of both SCF and c-Kit was previously demonstrated in a model of brain injury (Zhang and Fedoroff, 1999), suggesting a role for this signalling system during repair/scar processes.

In summary, the present study provides evidence for the emergence of adaptive changes in the spinal cord of transgenic animal models of ALS. Induction of SCF- and nestin-immunoreactive cells is indicative of molecular and cellular responses to the progression of the neurodegenerative process. These changes have been examined at the end-stage of the disease and further studies are needed to examine their progression before and during the onset of the symptoms. Indeed,

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previous studies have highlighted the existence of presymptomatic biochemical indices of the pathology, in particular the progression of the gliosis and vacuolisation within nervous tissue (Howland et al., 2002). While gliosis is frequently associated with tissue replacement, limiting the possible regeneration, recent studies have highlighted the role of reactive astrocytes as key actors in the active repair processes taking place within lesioned tissues (Liberto et al., 2004). Thus, beside the documented production of classical growth factors (Yamamoto et al., 1996) these cells could also release the SCF and contribute to progenitor recruitment. Contrasting with most other neurodegenerative diseases, ALS is characterised by its rapid and dramatic progression (Kanazawa, 2001). Therefore, one would suggest that these endogenous repair mechanisms that develop in the lesioned areas are certainly insufficient to limit the expansion of local neuronal damages. However, the demonstration of their existence may hold clues for the development of novel therapies in ALS, based on either the use of pharmacological approaches that would enhance the efficiency of endogenous mechanisms or on exogenously applied stem cells (Silani et al., 2004).

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recipients of T       Fellowships and EH is Senior Research Associate of the F.N.R.S

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3. Chimerisation of astroglial population in the lumbar spinal cord by adult mesenchymal stem cells transplantation prolongs survival of a rat model of amyotrophic lateral sclerosis

Adapted from “Chimerisation of astroglial population in the lumbar spinal cord by adult mesenchymal stem cells transplantation prolongs survival of a rat model of amyotrophic lateral sclerosis”. Submitted.

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Glial cells dysfunctions are crucial in the death of MN. Hence, glial cells replacement by stem cell therapy is a promising therapeutic strategy. We previously demonstrated that MSCs can differentiate into cells displaying astrocytic functions and that signals responsible for the migration of stem cells are present in the lumbar spinal cord of a rat model of ALS.

Herein, we injected MSCs into the cerebrospinal fluid of symptomatic hSOD1^{G93A} rats, a transgenic animal model of familial ALS, expressing a mutated form of the human superoxide dismutase. MSCs were found to infiltrate the nervous parenchyma and migrate substantially into the ventral grey matter where motor neurons degenerate. Even though overall astrogliosis was not modified, MSCs differentiated massively into astrocytes at the site of degeneration. The intrathecal delivery of MSCs and the subsequent generation of healthy astrocytes at symptomatic stage decreased motor neuron loss in the lumbar spinal cord, preserving motor functions and extending the survival of hSOD1^{G93A} rats. This neuroprotection was correlated with decreased inflammation, as evidenced by a lower proliferation of microglial cells and a reduction of COX-2 and NOX-2 expression.

Together, these data highlight the protective capacity of adult MSC-derived astrocytes when grafted into the central nervous system and illustrate an attractive strategy to target excessive inflammation in ALS.

3.1. Introduction

Lou Gehrig's disease or amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by the progressive and selective death of MNs in the cerebral cortex, brainstem and spinal cord, resulting in extensive paralysis and death of the patient within 3-5 years after onset (Cleveland, 1999). While most cases are sporadic, some patients show a familial history and several mutations have been associated with these inherited cases (for review (Boillee et al., 2006a)). Best known are those affecting the gene encoding superoxide dismutase 1 (SOD1) (Deng et al., 1993) and transgenic rodents carrying the human gene with these mutations are widely used as experimental models. Indeed, these animals recapitulate the principal biochemical and behavioural features of the disease (for review (Turner and Talbot, 2008)). Studies conducted with rodent models of ALS have provided several hypotheses for MNs dysfunction and death, including aggregation of the mutated protein (with secondary entrapment of other vital proteins), mitochondrial dysfunction, excitotoxicity (due to the excessive glutamate transmission), defects in axonal transport, oxidative stress and decreased availability of growth factors (for review (Boillee et al., 2006a)).

The potential benefit of stem cell therapies in the treatment of ALS has been highlighted in several recent publications. Highly convincing are those studies showing that intraspinal injections of neural stem cells (NSCs) delayed the onset and prolonged animal survival through

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a neuroprotective potential attributed to the local production of trophic factors (Xu et al., 2006; Corti et al., 2007). Enhanced survival was also reported after systemic injections of bone marrow stem cells from wild-type animals in irradiated mice expressing mutated hSOD1 or in double transgenic mice lacking myeloid cells (hSOD1/PU.1^{-/-} mice). In this model, stem cells differentiated massively into microglial cells, highlighting the role played by microglia in the progression of the disease (Corti et al., 2004; Beers et al., 2006). Indeed, while the origin of motor neuron degeneration in ALS remains largely elusive, exciting data suggest a non-autonomous mechanism for motor neuron death (Pramatarova et al., 2001; Lino et al., 2002), implicating glial cells in the progression of the disease (Boillee et al., 2006b; Yamanaka et al., 2008). Hence, targeting glial cells activation appears as a promising strategy to slow down ALS progression. Moreover, studies conducted with chimeric mice have shown that increasing the proportion of healthy wild-type non neuronal cells in the vicinity of MNs expressing mutated hSOD1 decreased the severity of the disease (Clement et al., 2003). Altogether, these studies suggest that a positive outcome could be obtained by replacing or enriching abnormal glia with healthy glial cells. With respect to accumulating data showing that MSCs can transdifferentiate into astrocytes when grafted into the central nervous system (Azizi et al., 1998; Kopen et al., 1999), these cells represent an attractive tool for cell therapies aiming at ameliorating the neuronal environment.

We herein report on the benefit obtained after a single intrathecal transplantation of late passage cultured MSCs into the lumbar spinal cord of transgenic rats carrying the hSOD1^{G93A} gene. MSCs were delivered into the cerebrospinal fluid at clinical onset and the integration of grafted cells in the spinal cord parenchyma was characterised. In addition to evaluating animal survival, the progression of the disease was also examined in behavioural studies. In addition, biochemical analyses were conducted to evaluate the influence of grafted cells on MNs and glial cells of the spinal cord.

3.2. Material and methods

Animals.

The strain of transgenic Sprague-Dawley rats carrying the human SOD1 gene with the G93A mutation (NTac:SD-Tg(SOD1G93A)L26H (Howland et al., 2002)) was initially obtained from Taconic Farms Inc, NY. All experiments were conducted on heterozygous animals which were all genotyped immediately after birth (de Hemptinne et al., 2006). Rats were maintained at 21°C with relative humidity 55% and a 12h light/dark cycle. Food and water were supplied *ad libitum*. All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

MSCs cultures and transplantation.

MSCs were isolated from femurs and tibias of 8-week-old male wild-type Sprague-Dawley rats by adhesion to non-coated tissue culture plasticware, propagated *in vitro* for 15 passages and characterised as previously described (Boucherie et al., 2008). Bromodeoxyuridine (BrdU, Sigma, Bornem, Belgium) (10 μ M) was added to the medium 72 h before transplantation. MSCs were harvested by trypsinisation, washed 3 times and resuspended in OptiMEM (Invitrogen, Merlbeeke, Belgium) at a density of 130.000 cells. μ L⁻¹ for injection. Cell suspension or vehicle (OptiMEM) were injected into the cerebrospinal

fluid with an intrathecal catheter as previously described (Yaksh and Rudy, 1976). Briefly, under volatile anesthesia (sevoflurane; Sevorane®, Abbott, IL), a PE-10 tubing (Becton Dickinson, Erembodegem, Belgium) was inserted through a small hole in the *cisterna magna* and directed caudally for 6 - 7 cm within the intrathecal space so that the tip reached the lumbar enlargement. MSCs or vehicle were then slowly injected in a total volume of 15 µL followed by a 10 µL isotonic saline flush. Immediately after the injection, the catheter was gently removed and the wound was sutured in two separated layers (muscle and skin) with 3-0 silk. Volatile anesthesia was then stopped and the animals were allowed to recover. Rats showing neurologic deficits were immediately euthanised. As MSCs were obtained from wild-type animals of the same strain (Sprague-Dawley), no immunosuppressant was given.

Disease monitoring.

Disease onset was predicted by the observation of decreased spontaneous activity (passive behavioural testing). Every 2 days, the distance walked by the rats was monitored from postnatal day 80 on a 60cm x 60cm open field during 2 min, using a video tracking system (EthoVision XT, Noldus, Wageningen, The Netherlands). To evaluate the progression of the disease, hSOD1^{G93A} rats were daily monitored between postnatal day 80 and end-stage by measuring body weight and motor score. Motor score is a global behavioural scoring evaluating the motor function in rats modelling ALS initially described by Matsumoto *et al.* (Matsumoto et al., 2006).

Tissue and cell analysis.

Rats were killed by transcardiac perfusion with cold (4°C) saline solution (0.9% NaCl) followed by cold 4% paraformaldehyde (dissolved in PBS) under deep anesthesia with xylazine (0.5 mL.kg⁻¹) and ketamine (1.6 mL.kg⁻¹). Immediately after perfusion, the lumbar spinal cord (L1-L5) was removed and post-fixed overnight in 4% paraformaldehyde at 4°C. Tissues were embedded in paraffin and sectioned (10 µm thick). For immunostaining, non specific staining was blocked by incubating sections in PBS/ 5% non fat dry milk/ 0.3% Triton X-100 for 1 h at 37°C. Sections were stained (overnight at 4°C) with antibodies against CD11b (1:100, Serotec, Oxford, UK), stem cell factor (SCF) (1:250, Chemicon, Hampshire, UK), phospho c-Kit (1:100, Sigma), microtubule associated protein 2 (MAP2) (a gift from Pr. Brion, Université Libre de Bruxelles, Belgium) and glial fibrillary acidic protein (GFAP) (1:300, Dako, Biognost, Heule, Belgium). As secondary antibodies, FITC & Cy3-conjugated anti-mouse or anti-rabbit (1:200; all from Jackson ImmunoResearch Laboratory, DePinte, Belgium) were used (1 h incubation at 20°C). The tyramide signal amplification kit (Invitrogen) was used to enhance the specific signal of SCF and c-kit. Nuclei were stained for 30 min with the nuclear dye 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1:5,000, Sigma). For BrdU staining, DNA was denatured by immersing sections in HCl 2N at 40°C. After 20 min, sections were washed twice in borate buffer (pH 8.5) 30 min and stained with anti-BrdU (1:50, Serotec).

For immunocytochemistry, cells were grown on 15 mm round poly-L-lysine (Sigma) coated glass coverslips, fixed with ethanol 95% for 20 min at 20°C and permeabilised in PBS/1% triton X-100 for 10 min. Non specific labelling was blocked by incubating the cells in PBS/non fat dry milk (15 mg.mL⁻¹) for 1 h at 37°C. The cells were immunostained (overnight at 4°C) with antibodies against c-Kit (1:50, Santa Cruz Biotechnology, Heidelberg, Germany). As secondary antibody, FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratory) was used (1 h incubation at 20°C). Nuclei were stained for 30 min with DAPI (1:5000, Sigma). After staining, sections or cells were overlaid with Fluoprep (Biomerieux, Brussels, Belgium) and examined with an Olympus IX70 inverted fluorescent microscope connected to a charged couple device (CCD) camera (T.I.L.L. Photonics, Martinsried, Germany). Excitation light (480, 540, 400 nm wavelength for FITC, Cy3 and DAPI, respectively) was obtained from a xenon lamp connected to a monochromator (T.I.L.L. Photonics). Digital images of fluorescence signals were acquired using appropriate filters and merged using the TILLvisION software (T.I.L.L. Photonics).

For Nissl staining, sections (10 µm) were incubated with cresyl violet for 3-5 min, rinsed in 95% ethanol and dehydrated in 100% ethanol. Finally, sections were mounted with Eukitt (Kindler GmbH & Co, Freiburg, Germany) and examined for quantification of motor neurons.

Quantitative analysis.

For quantitative analysis of healthy motor neurons, serial cross-sections of the lumbar spinal cord were made at 10 μm thickness and process for Nissl staining. Every 10th section was examined in the ventral grey matter (both left and right). Identified neurons were then analysed for cell area using the following criteria: i) the presence of a large single nucleolus located within the nucleus, surrounded by light blue staining cytoplasm, ii) a cell soma area over 100 μm^2 and iii) localization within lamina IX of the ventral horn. Cell count and morphometry were analysed using NIH ImageJ software. Quantification of GFAP and CD11b positive cells were realized in ventral grey matter (both left and right) by both cell count and staining area calculation using NIH imageJ software. High magnification pictures were taken in the Lamina IX for the grey matter and the spinothalamic tract for the white matter.

RNA extraction, reverse transcription and real-time PCR.

Total RNA was isolated from spinal cord using TriPure (Roche Diagnostic, Vilvoorde, Belgium), as detailed by the supplier. The reverse transcription was carried out with the iScript cDNA synthesis Kit, using 1 μg of total RNA in a total reaction volume of 20 μL . Real-time PCR was performed for quantification of the genetic expression of cyclooxygenase-2 (COX-2), gp91^{phox} (NAPDH oxydase catalytic subunit), CD11b, GFAP and SCF using a MyQTM unicolor real-time PCR detection system (Bio-Rad, Nazareth, Belgium). The

reaction was carried out in a total volume of 25 μ L containing 2 ng cDNA template of each sample, 0.375 μ M primers (COX-2 forward: 5'-GTGTGAGTGGTAGCCAGCAA-3', COX-2 reverse: 5'-CCCACAGGAGGATCTGAAAA-3', gp91^{phox} forward: 5'-CCTTTCCTGCATCTGGGTCTCC-3', gp91^{phox} reverse: 5'-CGCCCTTTGCCTCCATTCTC -3', CD11b forward : 5'-CTGCCTCAGGGATCCGTAAAG-3', CD11b reverse : 5'-CCTCTGCCTCAGGAATGACATC-3', GFAP forward : 5'-TGGCCACCAGTAACATGCAA-3', GFAP reverse: 5'-CAGTTGGCGGCGATAGTCAT-3', SCF forward: 5'-CTCGTGGCATGTATGGAAGA-3', SCF reverse: 5'-TGTCAGATGCCACCATGAAG-3', GAPDH forward: 5'-CCCCCAATGTATCCGTTGTG-3', GAPDH reverse : 5'-TAGCCCAGGATGCCCTTTAGT-3') and SYBR[®] green super mix (100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM MgCl₂, SYBR green I, 20 mM fluorescein) (Bio-Rad). The PCR protocol was conducted as follows: 30 s denaturation at 95°C, 45 s annealing at 60°C, 15 s elongation at 79°C for 40 cycles. The fluorescence signal was monitored at the end of each elongation step. For quantitative analysis, amplifications were performed in the same conditions for each target gene with serial dilutions of a mix of the cDNA templates. Each sample was normalised to the relative amplification of GAPDH. Calculation of cycle threshold values, standard curve preparation and relative quantification of mRNA in the sample were performed using the “post

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run data analysis” software provided with the iCycler system (Bio-Rad).

Immunoblotting studies.

Lumbar spinal cord was homogenised and protein isolated with TriPure (Roche Diagnostic). Fifty µg of total protein extract were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and proteins were transferred to nitrocellulose. Immunoblots were probed with an anti-GLT-1 (1:2,000, Chemicon). Primary antibodies were detected with horseradish peroxidase-conjugated anti-guinea pig (1:2,000) IgG (Sigma). Immunoreactive proteins were revealed using the ECL Western blotting detection system (Amersham Biosciences, Diegem Belgium). Signals were quantified using a Kodak imaging station and analysis software (Kodak, Rochester, NY). To control for equivalent loading, blots were stripped and reprobed with an anti-actin antibody (1:750, Sigma).

Statistical analyses.

All data are presented as mean with SEM. Differences between groups were examined for statistical significance using Student's t test or analysis of variance (ANOVA) followed by a Tukey post-hoc analysis for multiple comparison

3.3. Results

Intrathecal injection of adult MSCs

With the aim to study the integration of MSCs in the spinal cord parenchyma of animals with established ALS symptoms, a cell suspension was injected into the cerebrospinal fluid of hSOD1^{G93A} rats at postnatal day 90. Indeed, according to previous studies (Matsumoto et al., 2006), passive behavioural testing evidenced a decreased spontaneous motor activity of these transgenic rats at 87.0 ± 4.8 d of age (data not shown). Rats received a single intrathecal injection of $2 \cdot 10^6$ BrdU-labelled MSCs via a catheter at the lumbar level and were sacrificed 35 days later (postnatal day 125). Immunodetection of BrdU revealed the presence of transplanted cells in the white matter (Fig. 23A) and a substantial infiltration of MSCs into the grey matter within the ventral horn, where extensive motor neuron degeneration is evidenced (Fig. 23B).

Through the local production of specific chemicals, lesioned tissues constitute attractive niches for stem cells. The SCF is recognised as a chemoattractant for NSCs and MSCs in the injured nervous parenchyma (Bantubungi et al., 2007). Confirming our earlier observation (de Hemptinne et al., 2006), increased expression of the SCF mRNA was detected in the lumbar spinal cord of transgenic rats as compared to wild-type animals (112% increase, $p < 0.001$) (Fig. 23E). Furthermore, while SCF immunostaining was predominantly associated with MNs in wild-type animals (Fig. 23C),

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the staining appeared more diffuse in transgenic rats (Fig. 23D) where it was likely associated with surrounding glial cells. Noteworthy, *in vitro* characterisation of cultured MSCs revealed the constitutive expression of c-Kit, the receptor for SCF (Fig. 23F). Similarly, in spinal cord sections from the grafted transgenic rats, BrdU-positive cells detected in the grey matter stained positive for activated (phosphorylated) c-Kit (Fig. 23G-I), suggesting that the homing of MSCs in the spinal cord could involve a SCF-dependent migratory signalling pathway.

Figure 23: MSCs were injected intrathecally into the lumbar CSF at postnatal day 90, when spontaneous motor activity of transgenic rats starts to decrease. (A): detection of MSCs (BrdU positive nuclei in red) migrating through the white matter (n=5). (B): BrdU positive cells were detected in the grey matter of the ventral horn (n=5). (C): SCF immunostaining is detected in MNs of the lumbar spinal cord of wild-type rats (n=5). (D): In hSOD1^{G93A} rats, the expression of SCF is absent in MNs (star), but appears associated with glial cells surrounding MNs (n=5). (E): The SCF mRNA is increased by 112% in the spinal cord of transgenic rats (125 days) (Student's t test, ***p<0.001, mean value with SEM, n=5). (F): MSCs were characterised *in vitro* for the expression of C-Kit, (G-I): which is activated in MSCs (BrdU positive nuclei in red) in the spinal cord of hSOD1^{G93A} rats (green), suggesting the implication of the C-Kit/SCF pathway in their migration (n=3). Scale bar: 200µm in A-B, 40 µm in C-D, 20 µm in F-I.

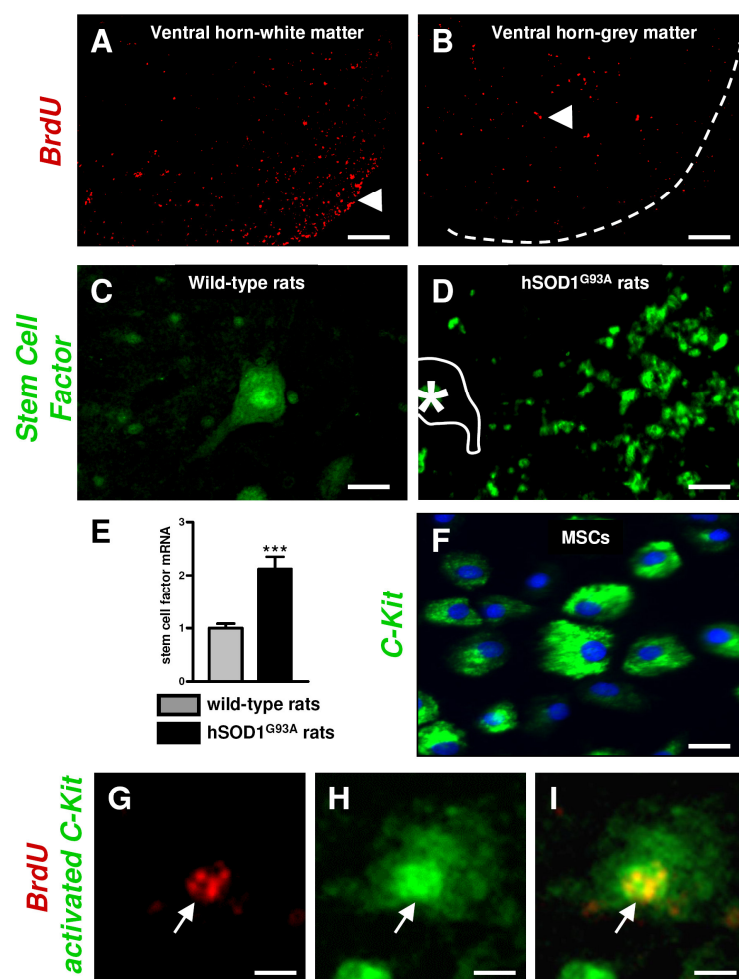


Figure 23: Migration of MSCs into the spinal cord of hSOD1^{G93A} rats.

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MSCs prolong survival in hSOD1^{G93A} rats

To evaluate the influence of spinal MSCs grafts on the disease, hSOD1^{G93A} rats were injected with either vehicle (sham) or MSCs and their behaviour was monitored until end-stage in order to compare the onset of the paralysis, the progression of the disease and the lifespan of the animals. After transplantation at postnatal day 90, sham and cell-grafted rats were housed in mixed cages. Their weight and their motor score (Matsumoto et al., 2006) were measured every day. Even though dysfunction and death of MNs correlate with progressive reduction of body weight, likely resulting from denervation induced-muscular atrophy, it is documented that paralysis arises later, when approximately 50% of the total population of MNs disappeared. In sham and cell grafted rats, the onset of amyotrophy was detected at the same age (107.0 ± 2.3 d and 110.2 ± 1.2 d respectively, $p>0.05$) (Fig. 24A-B).

Figure 24: (A-B) Body weight loss in hSOD1^{G93A} rats revealing that the MSC injection was without influence on the onset of the disease. (C-D): Animal motor score, reflecting the progression of the disease, illustrates that paralysis occurred later in MSC-grafted rats. (E): Animal death was delayed and the lifespan of hSOD1^{G93A} rats was increased by 16 days after MSC injection. (F): The overall survival of MSC-grafted ALS rats from the onset of amyotrophy was increased by 13 days. (G): However, the progression of the disease was not modified after paralysis onset. (H): duration between onset of body weight loss and paralysis onset. Histograms show mean values with SEM (n =12). Statistical analysis were performed using the Student's t test (***) refers to $p<0.001$.

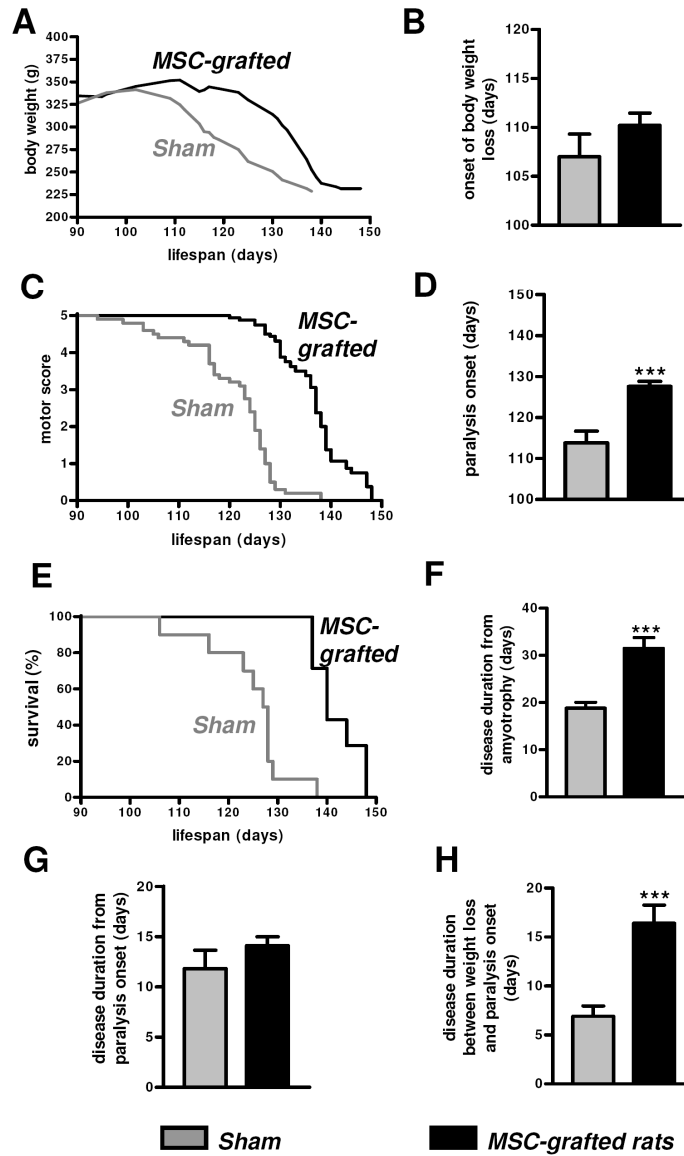


Figure 24: Influence of MSC grafts on severity of ALS.

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However, the progression of the disease appeared slower in cell grafted animals, as first signs of paralysis (motor score 4) were detected 14d later as compared to sham animals (127.6 ± 1.2 d and 113.8 ± 2.9 d respectively, $p < 0.001$) (Fig. 24C-D). Consistently, the life expectancy of hSOD1^{G93A} rats injected with MSCs was increased by 16 days (141.7 ± 1.2 d vs 125 ± 2.5 d for sham animals, $p < 0.001$) (Fig. 24E). Indeed, the duration of the disease (from amyotrophy onset to end-stage) was increased by 72% in cell grafted animals as compared to sham animals (respectively 31.5 ± 2.3 d vs 18.3 ± 1.2 d, $p < 0.001$) (Fig. 24F).

Nevertheless, when focusing on the paralysis, the progression of the disease appeared not particularly influenced by MSCs as revealed by a non significant increase in the duration of the symptomatic stage (11.8 ± 1.8 d for sham rats and 14.1 ± 0.9 d for MSCs-grafted rats, $p > 0.05$) (Fig. 24C, 24G). Altogether, these data indicate that the beneficial influence of MSCs is principally observed at the early stage of motor neuron death, between the onset of amyotrophy and the first signs of paralysis (6.9 ± 1 d for sham rats and 16.4 ± 1.8 d for MSCs-grafted rats, $p < 0.001$) (Fig. 24C, 24H).

MSCs protect MNs in hSOD1^{G93A} rats

As MSCs were shown to slow down the progression of the disease, their influence on the survival of MNs in the lumbar spinal cord was investigated. Transgenic rats were sacrificed after 125d (35d post-grafting), when the motor score testing was showing a highly

significant difference between sham and cell injected rats (2.8 ± 0.2 and 4.4 ± 0.2 respectively, $p < 0.001$). MNs were counted on *Nissl*-stained sections ($10 \mu\text{m}$) taken every $50 \mu\text{m}$ in the L4 part of the spinal cord (Fig. 25A, 25C, 25E). MNs and axon densities were also identified by MAP2 staining (25B, 25D, 25F). At this stage, ALS progression was associated with a 70% reduction in the number of MNs in hSOD1^{G93A} rats as compared to wild-type rats (3.5 ± 0.4 MNs per section vs 11.6 ± 0.4 MNs in sections from wild-type animals) and a severe reduction of axonal projections through the white matter.

MSCs-grafted rats had twice as many MNs as compared to sham animals (6.0 ± 1.3 MNs per section) and a higher density of axons. Obviously, this was significantly lower as compared to healthy wild-type animals, but these data evidence that grafted MSCs significantly delay the loss of MNs in the ventral horn of the spinal cord of the transgenic rats (Fig. 25G). Additional immunohistochemical studies revealed that none of the BrdU-positive cells detected in the spinal cord of grafted animals stained positive for the neuronal markers MAP2 (not shown), excluding any transdifferentiation of MSCs into mature neurons.

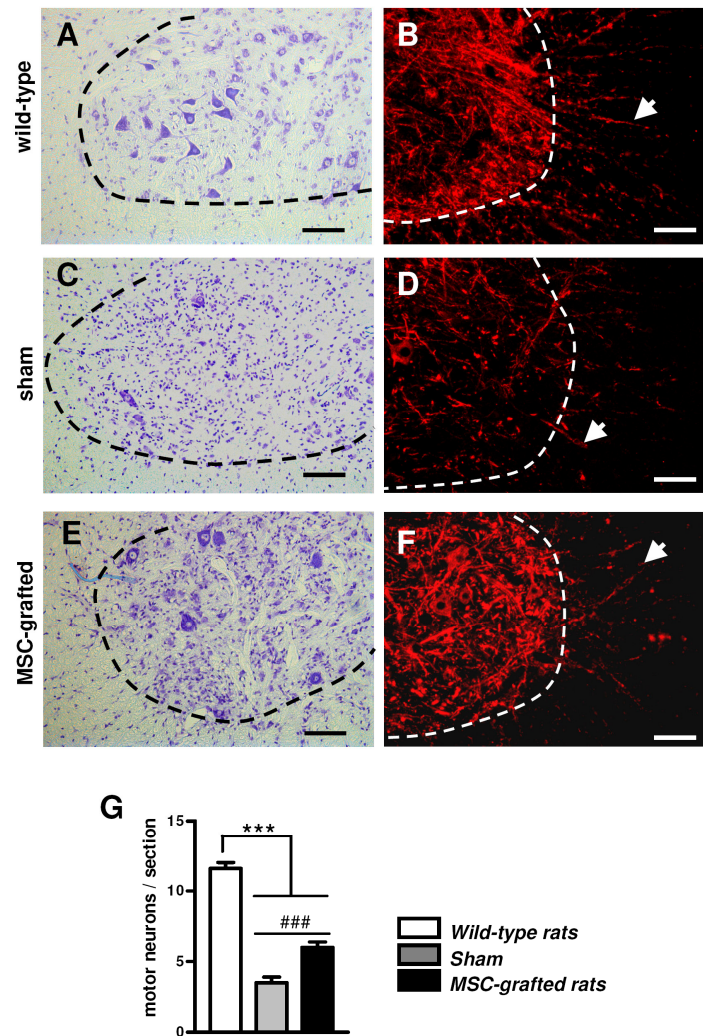


Figure 25: Protection of MNs by MSCs.

Figure 25: (A,C) Nissl staining and (B,D) MAP2 staining evidencing the severe loss of MNs observed at postnatal day 125 in the lumbar spinal cord of sham hSOD1^{G93A} rats as compared to wild-type animals. Arrows indicate the staining of axonal projections in the white matter (E,F): Injection of MSCs at onset resulted in the substantial preservation of MNs detected at day 125. (G): Quantification of MNs evidences a 70 % decrease in sham animals whereas MSCs-grafted rats conserved 52.5 % of the MNs population (n = 12, mean values with SEM, one-way ANOVA followed by Tukey's post hoc test, ***p<0.001). Scale bar: 200 μ m in A, C, E, 130 μ m in B, D, F.

MSCs participate in astrogliosis

Consistent with previous reports, a strong astrogliosis was detected in hSOD1^{G93A} rats as evidenced by an intense GFAP immunostaining throughout the lumbar spinal cord sections and by a robust increase in the GFAP mRNA level, quantified by real-time PCR (12.5 fold at postnatal day 125). The graft of MSCs was without significant influence on this glial activation process. Thus, the density of GFAP positive cells in lumbar ventral grey matter was similar in sham and cell-injected rats (122.5 ± 3.2 vs 124.2 ± 12.7 cells per sections, respectively, $p>0.05$) (Fig. 26A-C). Accordingly, the GFAP staining area (Fig. 26D) and the induction of GFAP mRNA quantified in samples of lumbar spinal cord (12.54 ± 2.2 and 9.36 ± 2 fold increase for sham and MSC-grafted rats as compared to wild-type animals, $p>0.05$) (Fig. 26E) were not modified in cell injected animals .

As overall astrogliosis was not modified, we focused on lamina IX where motor neurons are localised. In this area, MSCs massively

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contributed to the astrocytes population as 43.3% of BrdU-positive cells express GFAP in the grey matter (lamina IX) (Fig. 26F-I). Quantitative analysis also indicated that up to 31.4% of GFAP positive cells detected in this region were BrdU-positive, revealing that the graft of MSCs resulted in a substantial and local chimerisation of the astrocyte population at the site of the lesions. Similar quantifications revealed a modest (7.7%) differentiation of MSCs into astrocytes in the white matter (Fig. 26I), suggesting that transdifferentiation is promoted by the microenvironment found at the proximity of degenerating MNs. Excitotoxicity resulting from insufficient glutamate uptake by astrocytes is thought to participate in the progression of ALS. Accordingly, the expression of the principal glutamate transporter GLT-1 appeared dramatically decreased in the spinal cord of transgenic rats. Despite the substantial infiltration of MSC-derived astrocytes in the lumbar spinal cord of grafted rats, immunoblotting studies failed to evidence the induction of GLT-1 expression, suggesting that the benefit brought by MSCs did not result from an increased capacity of extracellular glutamate clearance (Fig. 26J).

Grafted MSCs decrease inflammation

Neuroinflammation, consisting in the activation and proliferation of resident microglia is a well described pathogenic feature of ALS (Ajami et al., 2007). Accordingly, round, phagocytic microglial cells were evidenced in both sham and MSC-grafted hSOD1^{G93A} rats (Fig. 27A-B). The number of microglial cells was quantified after

immunostaining of CD11b, a specific marker of the myeloid lineage which includes microglial cells. Injection of MSCs significantly decreased (by 62%) the proliferation of CD11b-positive cells in the ventral grey matter (Fig. 27C), and this was correlated with a 44% decreased CD11b staining area (Fig. 27D) and a 51% decreased expression of CD11b mRNA (Fig. 27E). Beside a typical phenotypic switch, activation of microglia is associated with an increased production and release of reactive oxygen species (ROS) and inflammatory mediators. This response is easily evidenced by measuring changes in the expression of NADPH oxidase (NOX-2) and COX-2. For sham rats, strong elevations of *cox-2* and *gp91^{phox}* (the catalytic subunit of NOX-2) expression (3.2 ± 0.4 and 12.8 ± 1.9 fold increases as compared to wild-type, respectively) were detected in the lumbar spinal cord. In sample from MSC-grafted rats, the induction of these enzymes appeared severely reduced (2.0 ± 0.3 and 8.0 ± 0.9 fold increase for *cox-2* and *gp91^{phox}* respectively, as compared to wild-type) (Fig 27F-G), confirming that MSCs substantially decreased ALS associated inflammation processes.

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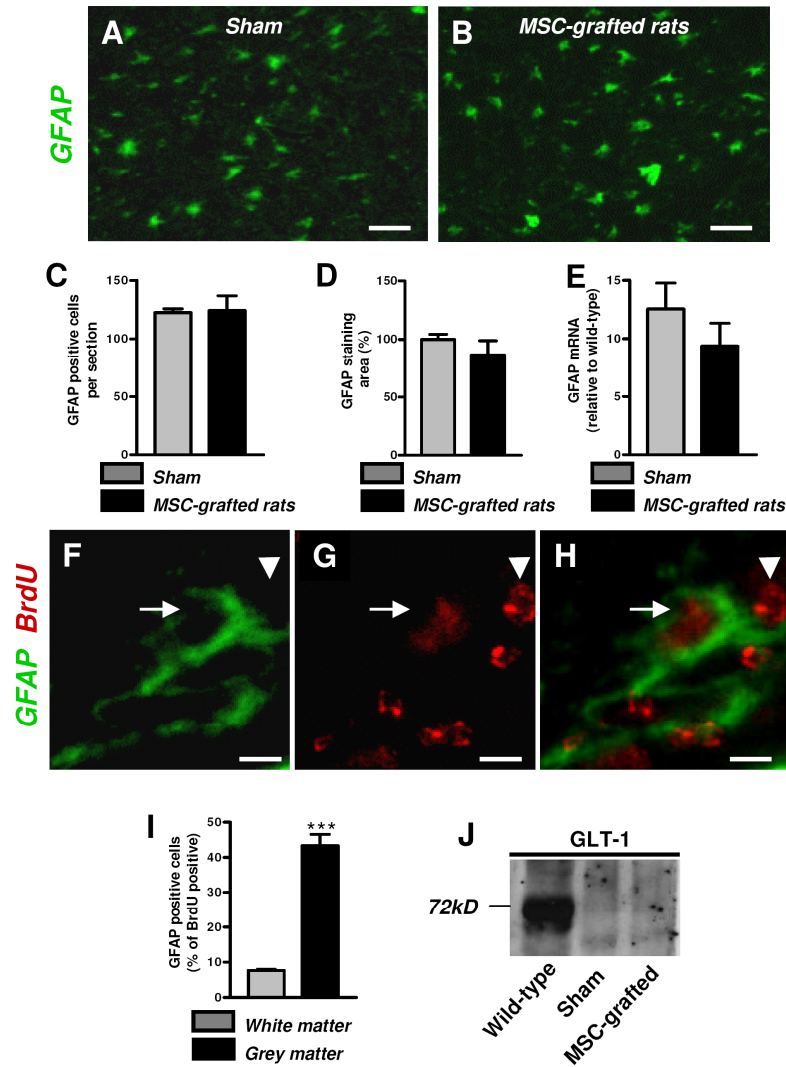


Figure 26: MSCs participate in astrogliosis in hSOD1^{G93A} rats

Generation of astroglial cells from MSCs

Figure 26: (A-B): GFAP staining (green) revealing astrogliosis in the lumbar spinal cord of 125 days-old rats during ALS progression in sham and MSC-grafted animals. **(C):** Quantification of astrocytes (GFAP-positive cells) in the grey matter of the ventral horns and **(D):** GFAP staining area and **(E):** quantitative PCR analysis of *gfap* expression indicate that astrogliosis was not changed after MSC injection. **(F-H):** Co-localisation of GFAP and BrdU staining (arrows) illustrating grafted MSCs expressing GFAP in the grey matter. (Arrow: GFAP⁺/BrdU⁺ cells, arrow head: GFAP⁻/BrdU⁺ cells) **(I):** Quantitative analysis of GFAP and BrdU co-staining in Lamina IX and in the ventral white matter (spinothalamic tract) of MSC grafted rats. **(J):** Immunoblotting analysis of GLT-1 expression in the lumbar spinal cord of wild-type and transgenic rats, evidencing the loss of transporter expression in hSOD1^{G93A} rats which is not modified after graft of MSCs. Histograms show mean values with SEM (n=5 animals per condition) and statistical analysis was performed using the Student's t test (***) refers to p<0.001). Scale bar: 80 µm in **A** and **B**, 20 µm in **F-H**.

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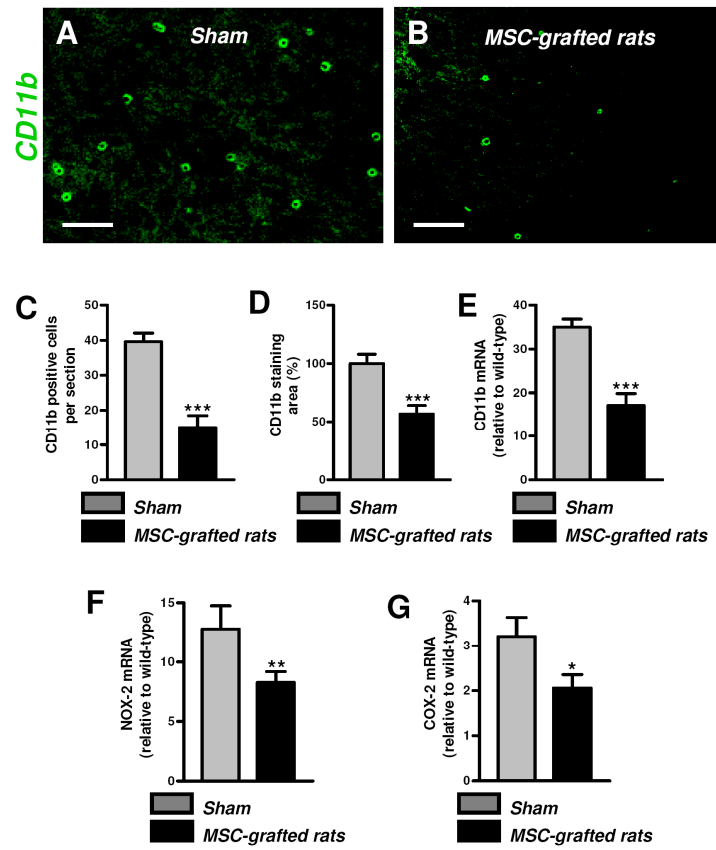


Figure 27: Decreased microgliosis in MSC-grafted hSOD1^{G93A} rats.

Figure 27: (A-B) CD11b staining (green) was used to detect microglia in the lumbar spinal cord of 125 day-old sham MSCs-grafted animals. (C): Quantifications of CD11b positive cells revealed that injection of MSCs reduced the proliferation of round shaped phacocytic microglia in the ventral grey matter. (D): Quantitative analysis of CD11b staining area and (E) mRNA confirmed the decreased proliferation of microglia in the lumbar spinal cord. (F-G): Quantitative analysis of gp91^{phox} and COX-2 mRNA in these lumbar spinal cord samples revealed a reduced inflammatory profile in MSC-grafted animals. Histograms show mean values with SEM (n= 5 animals per condition). Statistical analysis was performed using the Student's t test (*, ** and *** refer to p<0.05, p<0.01 and p<0.001, respectively). Scale bar: 80 μ m in A and B.

3.4. Discussion

The principal finding of this study is that cultured MSCs grafted in a rat model of familial ALS at disease onset preserve motor performances and significantly prolong animal survival. These benefits were correlated with the infiltration of grafted cells into the grey parenchyma of the lumbar spinal cord where they substantially adopted an astrocytic phenotype. In addition, when examined 35 days after graft, these effects were accompanied by a reduced local microgliosis and decreased inflammatory response. These observations are consistent with recent studies focusing on the interaction between glial cells in the progression and treatment of ALS.

Initiating the treatment in this animal model at clinical onset appears therapeutically pertinent, as for the majority of ALS cases, medical care can only be provided after diagnosis. Intrathecal injection constitutes a rather minimally invasive procedure and previous studies have demonstrated the efficiency of such delivery to implant stem cells in the CNS (Wu et al., 2002; Satake et al., 2004; Lepore et al., 2005; Arnhold et al., 2006), allowing beneficial outcomes in neurological disorders (Pluchino et al., 2003; Kerr et al., 2003; Corti et al., 2006). When grafted at the earliest symptomatic stage, herein characterised by a decreased spontaneous activity, MSCs did not delay the animal body weight loss, a reliable sign of clinical deterioration which reflects the progressive axonal retraction from neuromuscular

junctions. Nevertheless, first signs of paralysis appeared significantly later in grafted animals, an effect that was correlated with the partial preservation of MNs in the spinal cord and presumably their connections with skeletal muscles. There is accumulating evidence demonstrating that in rodent models of ALS, glial cell dysfunction is intimately involved in the severe progression of the paralysis but not in the initiation of body weight loss (Boillee et al., 2006b; Yamanaka et al., 2008). Accordingly, convincing data have highlighted the benefit obtained in animal models of ALS in which the expression of mutated hSOD1 was specifically absent in glial cells (Clement et al., 2003). This suggests that therapeutic approaches aiming at preserving or introducing healthy glial cells in ALS animals could substantially delay the degenerative process. Since the graft of MSCs appeared to reduce the progression of the disease beyond the initiation of amyotrophy, and considering previous studies showing the acquisition of astroglial features by MSCs after transplantation (Azizi et al., 1998; Kopen et al., 1999), we herein focused on the recruitment and putative transdifferentiation of MSCs in the spinal cord of transgenic rats.

When delivered into the CSF of rats expressing hSOD1^{G93A}, MSCs were found to migrate substantially through the white matter tissue, towards the site of neurodegeneration in the ventral grey matter. This infiltration of cells into the lesioned areas could depend on the local secretion of SCF, a chemoattractant molecule able to attract MSCs or NSCs expressing the receptor c-Kit (Bantubungi et al., 2007). Indeed, an increased expression of SCF was measured in the lumbar spinal

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cord of these transgenic rats. Consistently, our previous study revealed that the increased SCF immunostaining in the spinal cord of this rat strain was associated with the recruitment of endogenous neural stem/progenitor cells (de Hemptinne et al., 2006). Worth noting, cultured MSCs derived from adult rat bone marrow were found to express c-Kit, and *in vivo*, cells that integrated into the spinal cord parenchyma stained positive for the activated form of this receptor, emphasising on the recognition of endogenous signals by grafted MSCs. While the majority of MSCs penetrating into the white matter exhibited an undifferentiated phenotype, cells migrating into the grey matter differentiated massively into astrocytes, resulting in the chimerisation of up to one third of this glial subpopulation at the proximity of motor neuron cell bodies. Our previous *in vitro* characterisation demonstrated that late passages cultured MSCs adopt specific astrocytic features when maintained in defined conditions (Boucherie et al., 2008), corroborating those *in vivo* studies that evidenced the acquisition of an astrocyte phenotype by MSCs grafted into the CNS (Azizi et al., 1998; Kopen et al., 1999). These are compelling data supporting the here observed transdifferentiation of grafted MSCs into functional astrocytes in the spinal cord of the transgenic rodents showing ALS. Of note, overall astrocyte activation, commonly observed in degenerative diseases and repeatedly documented in ALS was not modified in grafted rats, implying the participation of MSCs-derived astrocytes in the gliosis process. In chronic disorder such as ALS, glial scars likely result from the proliferation and differentiation of glial progenitors and/or stem cells

rather than from proliferation of resident astrocytes (Lepore et al., 2008; Magnus et al., 2008). This suggests that the grafted stem cells could be recruited to the “gliogenic” niche where they contribute to the local gliotic response and exert a favourable influence on the local environment. Indeed, it was shown that astrocytes expressing the mutated enzyme secrete uncharacterised chemicals that accelerate motor neuron death (Nagai et al., 2007; Di Giorgio et al., 2007). The data reported herein, demonstrating the benefit obtained with wild-type MSCs that are recruited to the site of the lesion where they participate in the astrogliosis surrounding MNs are consistent with these previous studies. A tentative hypothesis would consider that the substitution of endogenous astrocytes by differentiated MSCs could reduce the toxic influence brought by the host astrocytes undergoing activation.

Prevention of excitotoxic damages through efficient glutamate clearance constitutes a well described neuroprotective function of healthy astrocytes. Hence, in ALS, the focal loss of EAAT-2/GLT-1 glutamate transporter expression is observed in affected regions (Howland et al., 2002; Dunlop et al., 2003) likely contributing to motor neuron death (Rothstein et al., 2005). Nevertheless, the recruitment and differentiation of grafted MSCs in the spinal cord was not accompanied by a significant increase in the expression of GLT-1, suggesting a lack of or only a modest influence of MSCs on the control of extracellular glutamate levels. Worth mentioning, reactive oxygen species (ROS) produced in suffering MNs were shown to

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diffuse to nearby astrocytes and disrupt glutamate uptake through transporter destabilisation (Rao et al., 2003; Rao and Weiss, 2004). Accordingly, selective blockade of ionotropic glutamate receptors in hSOD1^{G93A} rats efficiently prevents ROS release from MNs and thereby preserves GLT-1 expression in astrocytes (Suzuki et al., 2007). Consistent with the emerging concept that grafted cells could suffer from host-to-graft disease propagation (Li et al., 2008; Kordower et al., 2008) it is likely that astrocytes derived from MSCs would be exposed to a deleterious influence of the toxic microenvironment.

Concomitant with the chimerisation of the glial population by MSCs-derived astrocytes, a significant reduction in microglia activation and proliferation was evidenced in grafted animals. Even though microglial activation is an established feature of ALS progression in the spinal cord of patients and animal models of the disease (Kawamata et al., 1992; Hall et al., 1998; Kriz et al., 2002; Turner et al., 2004; Henkel et al., 2004), the underlying mechanisms remain largely unresolved. Several pathologic facets associated with ALS could support microglia activation such as accumulation of misfolded hSOD1 protein released by MNs and astrocytes (Urushitani et al., 2006). Expression of mutated hSOD1 in microglia was also shown to contribute to the progression of the disease by increasing the production of proinflammatory mediators (Weydt et al., 2004; Xiao et al., 2007; Harraz et al., 2008). In addition, damages in astrocytes expressing mutant hSOD1 appear to amplify the inflammatory

response arising from microglia and the related release of toxic molecules (Yamanaka et al., 2008). Consistent with a putative neuroprotective role of grafted MSCs and with the immunomodulatory properties of astrocytes (Kostianovsky et al., 2008), one would suggest that astrocytes derived from healthy stem cell could modulate the local inflammatory response that develops in microglia. Accordingly, the present study indicates that chimerisation of astroglial population in the spinal cord attenuated the induction of the inflammatory markers COX-2 and gp91^{phox} (NOX-2 catalytic subunit) in the transgenic rats. Indeed, as previously shown in mice carrying the mutated hSOD1 gene, rats were also shown to present a robust increase in the expression of these genes. These data suggest that reduction of COX-2 and NOX-2 expression in the lumbar spinal cord of grafted rats contributes to the protection of MNs. Up to now, the functional cross-talk between astrocytes and microglia was mainly focused on the study of iNOS, which appears not to be implicated in ALS progression (Son et al., 2001). Nevertheless, COX-2 and NOX-2 are significantly increased in post-mortem spinal cord of ALS patients and transgenic animal model of the disease (Almer et al., 2001; Yasojima et al., 2001; Wu et al., 2006), particularly in microglial cells (Wu et al., 2006; Yiangou et al., 2006). Hence, enhanced productions of ROS and prostaglandins were shown to be strongly associated with ALS progression and the neuroprotective effects obtained through pharmacological inhibition of COX-2 and NOX-2 were correlated with increased lifespan in a mice model of ALS (Drachman et al., 2002; Pompl et al., 2003; Harraz et al., 2008). Worth mentioning, the

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genetic inactivation of NOX-2 in mice expressing mutated hSOD1 did not influence disease onset but extended animal survival (Wu et al., 2006), mimicking the benefit obtained through selective genetic deletion of *sod1* in microglia (Boillee et al., 2006b). These observations highlighting the importance of ROS production by microglia as a key mechanism of toxicity are corroborated by recent data showing the interaction between mutated hSOD1 and Rac1, indirectly leading to the activation of NOX-2 (Harraz et al., 2008).

In conclusion, the present study indicates that the intrathecal injection of MSCs could constitute a valuable therapeutic approach for ALS. Of importance, the benefit is not due to the substitution of dying neurons by stem cells, but to a neuroprotective mechanism involving the chimerisation of the glial cell population. Indeed, our observations provide evidence for the 1) transdifferentiation of transplanted cells into astrocytes in the vicinity of suffering MNs and 2) a decreased microgliosis and neuroinflammation in grafted animals. In accordance with recent reports from the literature, these data suggest that the observed benefits rely on crosstalks between healthy astrocytes and local microglia, which lead to a modification of the deleterious environment of MNs. Additional studies should help to characterise these cellular interactions further and to consider the advantages to manipulate the inflammatory responses using stem cells in degenerative disorders such as ALS.

Conclusion-discussion

ALS is the most widespread motor neuron disease in human. Riluzole, the only FDA approved drug for ALS patients, exhibits a very limited effect on disease progression. Unfortunately, many therapeutic agents tested with success on rodent models of ALS failed to translate into clinical practice. Therefore, it appears essential to develop new treatments for this not curable disease.

1. What is the purpose of stem cell therapy in ALS?

During this thesis, we decided to examine the potential benefit of MSCs in ALS treatment. Considering the particularity of ALS which specifically kills MNs, the ultimate goal of stem cell therapies in neurodegenerative disorders could be the generation and the integration of functional neurons. However, functional MNs substitution appears as a complex and ambitious outcome of cell therapy. Indeed, MNs have been successfully generated from ESCs (Wichterle et al., 2002; Miles et al., 2004), iPS cells (Dimos et al., 2008) and NSCs (Gao et al., 2005) but their integration into the cortex or the spinal cord and formation of neuromuscular junctions with appropriate targets represent important limitations. Nevertheless, Deshpande and colleagues reported on the *in vitro* generation of MNs from ESCs which were able to connect muscles after transplantation in a viral model of MN death (Deshpande et al., 2006). This benefit

was obtained by using the stem cells in combination with pharmacological treatments. Axon outgrowth of transplanted cells was stimulated by implantation of NSCs overexpressing GDNF in the sciatic nerve, creating an attractive cue for ESCs within the peripheral nervous system. In addition, the repulsion created by the myelin for neuron regeneration was decreased via intrathecal administration of dBcAMP along with systemic phosphodiesterase type 4 inhibitor rolipram. Although these findings are therapeutically promising, applications for ALS treatment present serious limitations. Assuming that a transplanted MN axon grows at the fastest rate of a regenerating motor axon ($\pm 4\text{mm/day}$ (Fugleholm et al., 1994)) it would take up to 1 year to reach muscles in an adult organism, which is far too long for ALS patients. Furthermore, the CNS of ALS patients constitutes a hostile environment through excessive inflammation, oxidative stress, excitotoxicity and glial toxicity, which will inevitably affect the viability of new implanted MNs.

A more reasonable strategy to slow disease progression is to provide protection for suffering MNs. Indeed, stem cells can orchestrate a variety of beneficial influences when introduced into the degenerating CNS (Hedlund et al., 2007; Lepore and Maragakis, 2007). For instance, NSCs have been shown to be beneficial in hSOD1^{G93A} rodents, mainly through the secretion of growth factors able to support MN survival (Xu et al., 2006; Corti et al., 2007). It could be also the case for HSCs transplantation, which were characterized for the secretion of trophic factors and the rescue of MNs in another model of neurodegeneration (Cabanès et al., 2007). Nevertheless, another

mechanism was illustrated in an ALS model by Corti and colleagues who evidenced that HSCs were able to substitute for endogenous microglia (Corti et al., 2004). Hence, chimerisation of the microglial population by HSC is valuable for hSOD1^{G93A} rodents (Beers et al., 2006). Indeed, although MNs which are specifically killed throughout the motor cortex and the spinal cord appear to be the primary target of ALS toxicity, it is now well accepted that surrounding glial cells are as critical players as MNs in this pathology. Therefore, the restoration of spinal and brain glial cell activities should constitute a valuable form of cell-based therapy for ALS.

2. Generation of astroglial cells from adult stem cells: between hope and reality.

Genetic manipulations of SOD1 mice and in vitro studies have clearly underlined dysfunctions of the glial population. In this study, we have focused our attention on the substitution of astroglial residents. Astrocytes exert a wide variety of biological activities contributing to the viability of neurons in physiological conditions and their protection under potentially harmful circumstances. Nevertheless, astrocytes are recognized as a major component of MN death during ALS progression (see introduction, cross-talk between MNs and astrocytes).

As a tool for cell substitution, we studied the potential of adult stem cells to generate healthy, functional astrocytes. The use of adult stem cells avoids common issues related to embryonic materials but their

availability is limited in the adult organism. NSCs are natural precursors of astrocytes and adopt this phenotype extensively in a dish or after transplantation into the CNS. Nevertheless, even if NSCs can be isolated from several parts of the brain of the young organism (striatum, cortex, septum, optic nerve (Palmer et al., 1999; Brunet et al., 2004)), NSCs are mostly restricted to specific areas in the adult CNS such as the subventricular zone and the hippocampus (Eriksson et al., 1998), localizations that complicate their isolation for *ex vivo* manipulation. Limitation in stem cells availability from the CNS has motivated the search for surrogate sources of stem cells that could be differentiated along nervous lineages. For many years the general belief was that the tissue residing, multipotent stem cells were developmentally restricted to differentiating into cell lineages of the specified tissue from which they originate. Nevertheless, recent studies have demonstrated that adult stem cells have the capacity to transdifferentiate into cells of completely different tissue lineages or germ layers. For instance, NSCs were shown to give rise to blood cells (Bjornson et al., 1999) and bone marrow stem cells can give rise to neural cells as well (Mezey et al., 2000).

MSCs could be easily and non invasively isolated from patient's bone marrow for purification, *ex vivo* manipulation and large-scale expansion. This methodology offers great hope to generate therapeutic tools and presents also the opportunity to create patient-specific stem cell lines.

In this study, we isolated MSCs from the bone marrow of rats and established cultures of MSC lines. Their capacity to adhere to

uncoated plastic surfaces was sufficient to eliminate hematopoietic cells and obtain a stem cell population authenticated as MSCs by considering their typical phenotype (CD90⁺CD45⁻) and their ability to differentiate into mesenchymal derivatives such as adipocytes or chondrocytes. Then, we exposed these long-term cultivated MSCs (15 passages) to media supplemented with growth factors previously documented to favour the glial commitment of NSCs. Indeed, previous results from our laboratory as well as from other groups demonstrated that the plasticity of MSCs increases with the number of passages, at least for the acquisition of astroglial markers (de Hemptinne et al., 2004; Wislet-Gendebien et al., 2005). As the cells were passaged for longer times, they likely accumulate changes such as genetic or epigenetic events which may contribute to transdifferentiation. For example, transdifferentiation of NSCs into blood cells (Bjornson et al., 1999) was achieved with long-term cultivated stem cells and was not replicated by others with cells from shorter passages (Morshead et al., 2002). Hence, stem cells which had undergone such changes may constitute interesting tools as shown with multipotent adult progenitor cells (MAPCs) isolated by very particular *in vitro* culture conditions and which present an impressive potential of transdifferentiation (Jiang et al., 2002).

Under our optimized differentiation conditions, MSCs strongly express nestin, a marker of stem/progenitor cells and astrocytes in the CNS, adopt a typical glial morphology extending ramified processes and express the GS which is critical for astrocytic conversion of toxic glutamate into glutamine. However, we were not able to detect the

expression of GFAP, the most typical astrocyte marker, inevitably raising questions regarding the acquisition of a mature astrocyte phenotype by MSCs *in vitro*, even if a distinct population of GFAP⁻/GS⁺ astrocytes has been characterized (Walz et al., 1998). The transdifferentiation of MSCs into cells from the ectodermal lineage is still matter of debate, and often based on the expression of specific markers. Nevertheless, protocols leading to a rapid -within hours- transdifferentiation of MSCs into neural cells (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) identified by morphological criteria and induction of neural protein is actively criticized. Paradoxically, increased expression of neural markers which was found to occur without changes in the corresponding mRNA was also observed when protein synthesis was inhibited (Lu et al., 2004). Indeed, several studies evidenced that undifferentiated stem cells maintained in culture express specific neural proteins (Morrison and Weissman, 1995; Goolsby et al., 2003; Wislet-Gendebien et al., 2003; Tondreau et al., 2004; Deng et al., 2006). Moreover, rapid changes in the morphology of stem cells frequently result from disruption of the actin cytoskeleton and retraction of the cell edge (Neuhuber et al., 2004). In our hands, morphological changes required at least 5 days of exposure to the selected inducers, ruling out any bias related to cellular stress. In addition, the detection of neural proteins was always related to an increased transcription of the corresponding genes, suggesting that our observations likely reflect progressive changes in the transcriptome of differentiating MSCs. Based on the lack of GFAP expression, we could suggest that MSCs did not reach full astrocytic maturation

during differentiation. Indeed, a neuronal environment appears to be crucial for the transdifferentiation of MSCs into astrocytes and the subsequent GFAP expression (Azizi et al., 1998; Kopen et al., 1999; Wislet-Gendebien et al., 2003). Moreover, other laboratories were able to detect GFAP expression *in vitro* without any differentiation (Tondreau et al., 2004), highlighting the difficulty to compare several MSC lines isolated and manipulated in different experimental conditions.

Together, these data indicate that the sole detection of a set of selected markers should not be considered as sufficient to assert the transdifferentiation of MSCs and the assessment of specific physiological functions appears crucial. Therefore, we focused on the characterization of the potential neuroprotective properties, valuable for ALS, offered by these cells when adopting the differentiated phenotype. To our knowledge, there are limited studies aiming at characterizing typical astrocyte functions in differentiated stem cells (de Hemptinne et al., 2004; Vanhoutte et al., 2004). In particular, we studied the capacity of MSCs to secrete growth factors and to actively take up glutamate.

Secretion of neurotrophic factors is crucial for the development and the maintenance of neuronal populations (Pascual et al., 2008). In addition, production of neurotrophic factors by glial cells surrounding dying neurons is an essential mechanism of protection after acute or chronic injuries. Classically produced by glial cells in the CNS, GDNF is among the most potent trophic factors known to protect and

rescue MNs (Henderson et al., 1994). Indeed, therapeutic approaches based on the administration of GDNF have been frequently proposed in ALS (Acsadi et al., 2002; Wang et al., 2002b; Suzuki et al., 2007; Suzuki et al., 2008). Worth mentioning, neurotrophic factor secretion from astrocytes is highly regulated in the adult CNS and their production is increased after specific stimuli such as injuries or pharmacological treatments. Interestingly, differentiated cells showed a robust increase in the sensitivity to the neuroprotective drug riluzole, which substantially promoted the transcription of the GDNF gene and the release of GDNF. As previous studies evidenced a glial specificity of the influence of riluzole on GDNF production (Mizuta et al., 2001; Caumont et al., 2006), this finding further suggests a glial-related identity of MSCs after differentiation. Thus, differentiated MSCs appeared to respond to signals specifically implicated in astrocyte function, suggesting that differentiated MSCs could interact with extracellular factors in the CNS. Integration of grafted cells is a requirement for cell-based therapy and could be seen as an indication for the transdifferentiation of MSCs.

Another crucial function of astrocytes is the rapid removal of neurotransmitters from the synaptic cleft, and especially glutamate. Communication between MNs is mediated by the synaptic release of glutamate, where interaction with specific receptors on responding cells allows the entry of sodium, potassium and calcium. Unfortunately, MNs are highly vulnerable to excessive stimulation of ionotropic glutamate receptors and the subsequent calcium entry (see

introduction: cross-talk between astrocytes and MN, excitotoxicity). Thus, excitotoxicity has been recognized as a cause of MNs death and the manipulation of glutamate capture is considered as a valuable therapeutic approach. Indeed, pharmacological stimulation of GLT-1 expression (the principal high-affinity glutamate transporter in the adult CNS) is beneficial for hSOD1^{G93A} mice (Rothstein et al., 2005). Therefore, we evaluated whether differentiated MSCs can have the capacity to control glutamate transmission. We demonstrated the specific upregulation of functional GLT-1 during the *in vitro* differentiation of MSCs derived from the bone marrow. To date, there is no study demonstrating the influence of stem cells on glutamate uptake *in vivo*, thus the implantation of MSCs in a model of excitotoxicity, for instance ALS, should be useful to measure the impact of such restoration.

3. Limitations of patient-specific stem cell

As mentioned above, an attractive feature of MSCs resides in the possibility to develop autologous stem cell therapies. Therefore, the differentiation of MSCs into cells bearing defined astroglial properties was investigated with cultures of cells derived from the bone marrow of transgenic rats carrying the hSOD1^{G93A} transgene. Mimicking cells from wild-type animals, differentiation of cells from the transgenic rats coincided with increased expression of typical neuroglial markers. Noteworthy, the differentiation of stem cells derived from the transgenic rats was accompanied by a robust accumulation of hSOD1

protein. Aggregation of misfolded hSOD1 in neurons but also in glial cells could be implicated in various cellular dysfunctions and is thought to participate in the pathogenesis of the disease (Bruijn et al., 1997). Herein, MSCs from the transgenic rats failed to acquire neuroprotective properties such as GDNF modulation and exhibited a modest glutamate uptake capacity.

Similarly, previous studies have shown that neural stem cells from hSOD1^{G93A} mice display subtle alterations in their proliferation capacity (like MSCs from hSOD1^{G93A} rat, Boucherie *et al.*, unpublished data) and phenotypic characteristics, contributing to an impaired adult neurogenesis (Liu and Martin, 2006). In addition, grafts of freshly isolated bone marrow cells from hSOD1^{G93A} mice into the same animal strain failed to provide any benefit with respect to the disease progression whereas cells from wild-type animals significantly prolonged their lifespan (Corti et al., 2004). Hence, the use of autologous cell transplantation in ALS, although avoiding any risk of immune rejection, does not eliminate the problem that these cells could be affected by the same genetic disorder that contributed to the disease process. Indeed, up to 10% of ALS patients are referred to familial ALS. For instance, astrocytes carrying the mutated SOD1 gene secrete toxic factors for MNs and astrocytes-derived from hSOD1^{G93A} stem cells may likely behave similarly. So, questions persist regarding the option of autologous grafts of stem cells in future therapies against neurodegenerative disorders, especially regarding the detection of mutations related to these diseases. Indeed, this issue is

not restricted to ALS. Thus, α -synuclein, a protein related to a familial form of PD, can influence the fate and differentiation of NSCs (Schneider et al., 2007). Fortunately, stem cell lines can be generated from healthy donors or patients suffering from sporadic case of neurodegenerative disorder. In addition, for the few familial forms of disorders for which mutations are well characterised, genetic correction of these cells should be considered as an option for autologous grafts (Hanna et al., 2007).

4. Implantation of MSCs in a rodent model of ALS

Despite the intense focus on genetic mouse models of ALS, transgenic rats overexpressing the mutated human SOD1 have been generated with the obvious advantage of larger animal size for experimental manipulation. These rats develop progressive neuromuscular symptoms reminiscent of the disease observed in human and mouse, including MN loss, spinal astrogliosis, EAAT2/GLT-1 down regulation (Howland et al., 2002) and inflammation (Boucherie, unpublished data, (Xie et al., 2004)).

Most of the studies involving cell therapy in ALS were based on their direct intraspinal injection (Klein et al., 2005; Hemendinger et al., 2005; Xu et al., 2006; Suzuki et al., 2007; Corti et al., 2007; Vercelli et al., 2008), limiting their penetration into the CNS. ALS in human is a disease with extensive MN loss within both the cortex and the spinal cord. Thus, it is necessary to have a widespread distribution of

transplanted cells. More efficient implantation has been demonstrated after intravenous application (Corti et al., 2004; Beers et al., 2006), where hematopoietic stem/progenitor cells invaded efficiently the CNS to repopulate the microglial population in hSOD1^{G93A} mice.

Intrathecal application constitutes another way to obtain a widespread distribution, while limiting the diffusion to other organs. Intrathecal injection constitutes a rather minimally invasive procedure and previous studies have demonstrated the efficiency of such delivery to implant stem cells in the CNS (Wu et al., 2002; Satake et al., 2004; Lepore et al., 2005; Arnhold et al., 2006), allowing beneficial outcomes in neurological disorders (Pluchino et al., 2003; Kerr et al., 2003; Corti et al., 2006). Delivery of MSCs into the fourth ventricle was already considered for the treatment of hSOD1^{G93A} mice, but without success. This negative result was mainly attributed to the lack of implantation of stem cells in affected regions such as the spinal cord. In order to counteract cell leakage (Ohta et al., 2004) and to target more directly the lumbar spinal cord, MSCs were herein delivered in the CSF through a catheter at the lumbar level. In the case of hSOD1^{G93A} rodents and other neurodegenerative models, stem cells are often implanted at pre-symptomatic stages (Klein et al., 2005; Xu et al., 2006; Corti et al., 2006; Suzuki et al., 2007; Corti et al., 2007; Vercelli et al., 2008). We decided to graft MSCs at disease onset, which is therapeutically relevant, as for the majority of ALS cases, medical care can only be provided after apparition of the first symptoms. The earliest symptom that we were able to identify in

hSOD1^{G93A} rats was a tendency of the animal to lose spontaneous motor activity, which occurs at post natal day 90. Thus, we considered day 90 as the beginning of behavioural ALS symptoms.

Several mechanisms are known to induce cell migration, but little is known regarding migration of stem cells in models of neurodegeneration. Degenerative tissues constitute “niches” for stem cells via the local secretion of attractive molecules. SCF, is a chemotaxic molecule expressed in response to a multitude of lesions such as traumatic brain injury, ischemia, hypoxia and HD (Zhang et al., 1999; Jin et al., 2002; Bantubungi et al., 2007) and is recognized to attract NSCs and MSCs (Sun et al., 2004; Erlandsson et al., 2004; Bantubungi et al., 2007). We initially demonstrated that the expression of SCF was specifically increased during ALS progression. But the real physiological role of such high SCF expression in the degenerating spinal cord of hSOD1^{G93A} rats is still enigmatic. SCF can act as a trophic factor for neurons in PD (Yasuhara et al., 2006), suggesting that SCF expression from surrounding glial cells in ALS could constitute a protective mechanism. In addition, as mentioned above, SCF acts as chemotaxic agent for stem cells and we demonstrated that endogenous neural stem/progenitor cells were recruited at the site of lesions, a recruitment confirmed by other laboratories (Chi et al., 2006; Juan et al., 2007; Chi et al., 2007). The finding that these neural stem/progenitor cells stained positive for the activated form of the SCF receptor, c-Kit, allowed us to propose that SCF is implicated in the recruitment of neural stem/progenitor cells.

However, the real benefit of recruited endogenous stem cells is not clearly demonstrated. Does endogenous mobilization of stem cells slow disease progression or should this be seen as an artifact caused by the intense degeneration of the spinal cord? Endogenous neurogenesis during adulthood is recognized to maintain neurological functions (Imayoshi et al., 2008) and suppression of endogenous stem cells proliferation in a model of brain injury increased neurodegeneration, suggesting that endogenous neurogenesis is beneficial under physiological and pathological conditions. However, several lines of evidence suggest that, in chronic neurodegeneration, endogenous stem/progenitors cells participate in astrogliosis (Lepore et al., 2008; Magnus et al., 2008). This could be detrimental for MNs as astrocytes are involved in ALS progression (Yamanaka et al., 2008). An important issue would be to determine the influence of such endogenous stem/progenitor cells in the CNS of ALS.

Nevertheless, SCF could promote the migration of exogenous MSCs in the spinal cord of hSOD1^{G93A} rats as it does in the brain of rat models of HD (Bantubungi et al., 2007). Once integrated in the CNS of hSOD1^{G93A} rats, MSCs stained positive for the activated form of c-Kit, suggesting that MSCs interacted with endogenous signals promoting their homing at the site of degeneration, such as endogenous NSCs. It is unlikely that the SCF/c-Kit pathway alone can induce such migration. A better knowledge of other signalling pathways (such as CXCR-4/stromal derived factor-1 (SDF-1))

interaction) should be useful to improve the migration of stem cells at the site of lesion.

5. Grafted MSCs generate astrocytes in the lumbar spinal cord and prolong survival of hSOD1G93A rats

While the majority of MSCs penetrating into the white matter exhibited an undifferentiated phenotype, cells migrating into the grey matter differentiated massively into astrocytes, at the proximity of motor neuron cell bodies (Lamina IX). This corroborates those *in vivo* studies that evidenced the acquisition of an astrocyte phenotype by MSCs grafted into the CNS (Azizi et al., 1998; Kopen et al., 1999). Thus, molecules and/or cell contacts necessary for MSCs to adopt an astroglial phenotype are more likely to be detected near the cell body of degenerating MNs. It has been already proposed that tissue injuries could facilitate the (trans)differentiation and integration of adult stem cells. This is consistent with the region specific differentiation of MSCs in our model. Nevertheless, soluble factors and/or cell contacts inducing astroglial differentiation of MSCs during neurodegeneration are still unknown.

Of note, overall astrocyte activation, commonly observed in ALS was not modified in grafted rats, implying the participation of MSCs-derived astrocytes in the gliosis process. In mutant hSOD1^{G93A} mice, astrogliosis occurs at early stages, when loss of neuromuscular

junction starts (Gould et al., 2006) which results from the proliferation and differentiation of glial progenitors and/or neural stem cells rather than proliferation of resident astrocytes (Lepore et al., 2008; Magnus et al., 2008). This suggests that MSCs could be recruited to this “gliogenic” niche.

Another unexpected observation was the high percentage of chimerisation of the astroglial population. Indeed, MSCs transdifferentiated into astrocytes represent one third of the total astrocyte number in lamina IX, where MN cell bodies are localized. In fact, MSCs are injected just before retraction of MNs from muscles, giving them the possibility to reach the grey matter when glial progenitors and/or neural stem cells are instructed to differentiate into astrocytes. Massive chimerisation of a glial population has been already described in a model of hypomyelination. Windrem and colleagues implanted human glial progenitors in shiverer mice in which oligodendrocytes do not produce myelin. The injections resulted in the massive invasion of the white matter by human oligodendrocytes (Windrem et al., 2008). A possible explanation is that glial progenitors were implanted at post natal day 1, when oligodendrocytes interact with neurons in order to produce myelin around axons. This finding suggests, again, that environment is critical for stem cells differentiation. With the objective to treat ALS, injection of wild-type HSCs into irradiated hSOD1^{G93A} mice resulted in the chimerisation of 30% of the microglial population in the spinal cord (Corti et al., 2004).

The integration of MSCs in the lumbar spinal cord resulted in a significant increase in the lifespan of hSOD1^{G93A} rats. More precisely, the treatment strongly prolonged the duration of the disease from amyotrophy onset. As mentioned earlier, severity of ALS progression (at least, from amyotrophy onset) is related to toxicity of surrounding glial cells, astrocytes (Nagai et al., 2007; Di Giorgio et al., 2007; Yamanaka et al., 2008) and microglia (Beers et al., 2006; Boillee et al., 2006b). Considering the intense chimerisation of the astroglial population, we studied how substitution of resident astrocytes carrying mutated SOD1 could protect MNs in ALS.

6. Mechanisms of neuroprotection from MSCs via transdifferentiation into astrocytes

In vivo, the deletion of the mutated human SOD1 gene specifically in astrocytes prolongs survival in hSOD1^{G85R} mice after disease onset (Yamanaka et al., 2008). These astrocytes are characterized for the secretion of toxic factor(s) which selectively kill MNs *in vitro* (Di Giorgio et al., 2007; Nishitoh et al., 2008). A possible explanation could be the reduced production of this toxic factor(s) around MNs but this hypothesis can not be investigated as it is still unidentified.

Another toxic mechanism related to astrocytes and shared by fALS and sALS is the loss of GLT-1 and the subsequent toxic accumulation of glutamate close to MNs. As discussed above, MNs are very vulnerable against high concentrations of glutamate which lead to

excitotoxicity. Nevertheless, chimerization of astrocytes with MSCs did not increase the detection of GLT-1 in the lumbar spinal cord, suggesting that neuroprotection was not mediated by a decreased excitotoxicity. The loss of GLT-1 in endogenous astrocytes is not clear and could result from pathologic mechanisms arising from either astrocytes and/or MNs. Caspase-3 is activated as early as the presymptomatic stage in hSOD1^{G93A} mice (Wengenack et al., 2004) and is able to cleave the carboxy-terminal domain of GLT-1, resulting in the impairment of glutamate uptake (Boston-Howes et al., 2006). However, this publication also demonstrated that activation of caspase-3 was related to the presence of mutated SOD1, and it was not the case for MSCs derived-astrocytes. Moreover, selective suppression of the human mutated SOD1 gene in astrocytes did not counteract the loss of GLT-1 in the spinal cord of hSOD1^{G85R} mice (Yamanaka et al., 2008), suggesting that *in vivo*, the loss of GLT-1 is partially mediated by extracellular factors. Worth mentioning, ROS produced in suffering MNs were shown to diffuse to nearby astrocytes and disrupt glutamate transporters (Rao et al., 2003; Rao and Weiss, 2004). Accordingly, selective blockade of ionotropic glutamate receptors in hSOD1^{G93A} rats efficiently prevents ROS release from MNs and thereby preserves GLT-1 expression in astrocytes (Yin et al., 2007). Consistent with the emerging model that grafted cells could undergo host-to-graft disease transmission (Li et al., 2008; Kordower et al., 2008) it is likely that astrocytes derived from MSCs would be exposed to a deleterious influence of the toxic environment, resulting in the loss of GLT-1. A hypothetical treatment based on the restoration

of glutamate capture would combine stem cells and drugs limiting oxidative stress and production of ROS in order to protect GLT-1 at the surface of stem cells. Interestingly, human NSCs transplanted into the spinal cord of hSOD1^{G93A} rats lose also the expression of GLT-1, confirming this hypothesis (Suzuki et al., 2007).

It is likely that protection of MNs observed in our experiment is strongly related to the decreased inflammation in the lumbar spinal cord. Inflammation is mainly generated by microglial cells in the CNS which are implicated in ALS progression (see introduction, ALS: cellular environment move in as deadly neighbors; Microglia, when ALS turns into immune disease). Implantation of MSCs and chimerisation of the astroglial population in the ventral horn drastically reduce their activation, and more precisely their proliferation and the expression of proinflammatory mediators (NOX-2 and COX-2) which are toxic for MNs. The elegant work of Yamanaka and colleagues demonstrated the close interaction between astrocytes and microglia carrying mutant SOD1. Indeed, genetic manipulation reducing expression of mutant SOD1 in astrocytes leads to substantially delayed microglial activation (Yamanaka et al., 2008), a mechanism which fits perfectly with our results. Hence, substitution of a subset of mutant astrocytes by wild-type MSCs-derived astrocytes could decrease the secretion of factor(s) involved in microglial activation or increase the production of anti-inflammatory mediators which could be down regulated in transgenic astrocytes (this mechanisms is well described in ALS for an unknown protein produced by astrocytes and implicated in the regulation of GluR2

expression in MNs (Van Damme et al., 2007)). The two explanations are plausible. For instance, mutant astrocytes (and MNs) secrete misfolded hSOD1 via the chromatogranin pathway which can inflame the CNS (Urushitani et al., 2006) and non-mutant astrocytes are characterized to regulate microglial activation (Kostianovsky et al., 2008).

7. Other potential mechanisms of neuroprotection by MSCs

In addition, other beneficial effects could arise from MSCs in our experimental paradigm (see introduction, adult stem cell therapy for neurological disorders: benefit beyond neuronal replacement?). MSCs are considered as “biopump” which produce a range of trophic factors for neurons. For instance, NSCs have been shown to be beneficial in hSOD1^{G93A} rodents by ensuring the secretion of trophic factors (Xu et al., 2006; Corti et al., 2007). Moreover, MSCs can stimulate angiogenesis (Borlongan et al., 2004; Kasper et al., 2007) which is impaired in hSOD1^{G93A} mice (Zhong et al., 2008). In addition, it is proposed that HSCs could modulate inflammation in hSOD1^{G93A} mice via microglia substitution (Corti et al., 2004; Beers et al., 2006). However, this mechanisms is unlikely involved in our results with MSCs as we were not able to find the formers expressing microglial markers after implantation in the CNS of hSOD1^{G93A} rats. Worth mentioning, undifferentiated MSCs are remarkable for their potent immunomodulatory capacities (Ren et al., 2008). Very recently,

Other potential mechanisms of neuroprotection by MSCs

intraspinal injection of human MSCs in hSOD1^{G93A} mice has been shown to delay death of MNs in hSOD1^{G93A} mice (Vercelli et al., 2008). The major hallmark was the reduced astrogliosis and microgliosis, a mechanism of neuroprotection which seems at variance with our study.

A critical question is to know why astrogliosis was found to be reduced with a limited differentiation of human MSCs into astrocytes (<1%) in the study of Vercelli and colleagues whereas rat MSCs were shown to actively participate to astrogliosis in our study. Several explanations can be proposed. The signals implicated in the glial differentiation of MSCs may differ between rats and humans. Moreover, intraspinal injected human MSCs migrated rarely into the ventral grey matter in the study from Vercelli and colleagues, where we identified the mostly active gliogenic niche. Hence, the effects of human MSCs could arise from soluble factors but the target is unknown. Are glial cells the direct target of soluble factors from human MSCs or does the protection of MNs (likely via the secretion of trophic factors) indirectly delay neuroinflammation? This question is also pertinent for our study. Finally, these human MSCs have been injected at presymptomatic stage directly in the lumbar spinal cord, when the cellular environment in the CNS is totally different from symptomatic stage. Therefore, the timing and the localization of the graft appear critical in the outcome of cell therapy.

8. Rodents vs humans: limitation of our study

It is worth mentioning that up to now, the large majority of studies demonstrating the potential benefits brought by adult stem cells grafts have been conducted with animal models of diseases. This inevitably raises the question of the adequacy of these models and to the possibility to transpose these experimental conclusions into clinical perspectives. Such limitation is indeed largely documented in neuropharmacology as several promising drug candidates were proven ineffective when tested in human clinical trials. It is unlikely that the full complexity of ageing-related or traumatic-related human brain disorders could be reproduced in rodents. Indeed, monkeys and other primates with human-like nervous systems should certainly constitute more appropriate animal models for human neurological disorders (Redmond, Jr. et al., 2007; Yang et al., 2008). Nevertheless, the successful stem cell treatments in rodents constitute the first step in the development of human therapies, and available experimental data certainly offer substantial promises for future clinical applications in regenerative medicine.

9. Can we apply MSCs in clinical trials for ALS patients?

Adult stem cell therapy represents a great challenge for the treatment of ALS (and other neurodegenerative diseases), exerting a variety of biological activities that would efficiently face various harmful

processes of degenerative disorders (see introduction, adult stem cells for neurodegenerative disorders: benefit beyond neuronal replacement?). Obviously, although encouraging results have been obtained in animal models, the use of adult stem cells in clinical trials should be considered with caution. Indeed, the self-renewal property of adult stem cells which allows large *in vitro* expansion is likely to favour spontaneous transformation and generate cancers (Marx, 2003; Rubio et al., 2005; Tolar et al., 2007). In addition, the control of stem cell plasticity should also receive particular attention. Thus, even though it is suggested that signals from the tissue instruct the fate of grafted stem cells, several examples indicate that stem cells could follow their intrinsic specifications. Indeed, MSCs commonly characterised by their canonical differentiation potential into osteocytes, chondrocytes and adipocytes were found to cause ossification when grafted in the infarcted heart (Breitbach et al., 2007).

However, in the view of the work of Mazzini and colleagues, implantation of autologous MSCs in the spinal cord of ALS patients appeared to be safe (Mazzini et al., 2006), but was not a proof-of-concept that MSCs is beneficial in clinical trial for ALS patients. This pilot study, in which ALS patients received a single intraspinal MSCs injection, is difficult to interpret because of the small number of patients (nine), the lack of control patients and the variations in the stage of the disease (from 6 to 96 months since the diagnostic, amyotrophic lateral sclerosis functional rating scale between 13 and 34 and forced vital capacity between 25 and 108%) in addition to

Conclusion – discussion

large differences in the amount of grafted cells (from $7 \cdot 10^6$ to $152 \cdot 10^6$ cells) (Badayan and Cudkowicz, 2008).

Hence, numerous issues must be resolved in order to translate cell therapy in human clinical trials (selection of patients, stem cell origin, culture conditions, number and route of injection etc...) and to confirm their potential where many drugs failed to translate to patients. Nevertheless, preclinical experiments just reveal the amazing potential of stem cell therapies to treat neurodegenerative disorders, and further support the use of stem cells in future transplantation programs.

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