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Role of non-neuronal cells in Amyotrophic Lateral Sclerosis (ALS): transgenic neuron-glia-co-cultures as *in vitro* model for the evaluation of novel therapeutic strategies

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Therapeutic potential of Glucagon-like peptide-1 in primary motor neuron cultures derived from wild type and ALS-transgenic mice

Content

Summary

Hui Sun

Role of non-neuronal cells in ALS: transgenic neuron-glia-co-cultures as *in vitro* model for the evaluation of novel therapeutic strategies

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder affecting motor neurons in the primary motor cortex, brainstem and spinal cord, ultimately leading to death within 3-5 years due to respiratory failure. To date, there is no efficient neuroprotective treatment option. Mutations in the Cu/Zn –superoxide dismutase (SOD1) gene underlie a proportion of familial cases of amyotrophic lateral sclerosis (fALS); overexpression of mutant SOD1 created the most commonly used and best characterized ALS mouse model.

Motor neuron death in ALS is non-cell autonomous, i.e. it depends on the interaction of neurons with surrounding glial cells. In the first study, a motor neuron/astrocyte co-culture system, consisting of either non-transgenic or SOD1G93A transgenic motor neurons and astrocytes, respectively, was established as in vitro model. In this in vitro system, N-ac-GLP-1, an analogue of the endogenous insulinotropic peptide Glucagon-like peptide-1 (GLP-1) was assessed for its neuroprotective potential. We detected neuroprotective effects of N-ac-GLP-1 which could be attributed to an attenuation of intracellular calcium transients. Not only due to these antiexcitotoxic capacities but also with respect to the increasing knowledge about metabolic deficits in ALS which could be positively influenced by N-ac-GLP-1, this compound represents an interesting novel candidate for further in vivo evaluation in ALS.

In the second study, the protective effects of mesenchymal stromal cells (MSC) on motor neurons (derived from both non-transgenic and mutant SOD1G93A transgenic mice), NSC-34 cells and astrocytes (derived again from both non-transgenic and mutant SOD1G93A transgenic mice) were analysed in vitro. Attempting to dissect the molecular mechanisms, we found that MSC conditioned medium induced astrocytic mRNA expression of the

neurotrophic factors GNDF and CNTF and reduced expression of proinflammatory cytokines. These data suggest that MSC are capable to generate a more protective environment for degenerating motor neurons.

Zusammenfassung

Hui Sun

Die Rolle nicht-neuronaler Zellen bei der Amyotrophen Lateralsklerose (ALS): transgene Neuron-Glia-Ko-Kulturen als in vitro-Modell zur Evaluation neuer Theapiestrategien

Amyotrophe Lateralsklerose (ALS) ist eine schwerwiegende neurodegenerative Erkrankung, die zu rasch progredienter Degeneration motorischer Nervenzellen im primär-motorischen Kortex, Hirnstamm und Rückenmark führt. Die Patienten entwickeln fortschreitende Paresen und Muskelatrophien und versterben in der Regel 3 bis 5 Jahre nach Erkrankungsbeginn an Atemlähmung. Eine wirksame neuroprotektive Behandlung gibt es bisher nicht.

Bei einem Teil der an familiärer ALS erkrankten Patienten lassen sich Mutationen im für das Enzym Superoxid-Dismutase 1 (SOD1) codierenden Gen identifizieren; Überexpression der mutierten SOD1 führte zur Entwicklung des nach wie vor am meisten verwendeten und am besten charakterisierten ALS- Mausmodells.

Der Untergang motorischer Neurone bei der ALS ist nicht-zellautonom, d.h. abhängig von der Interaktion mit umgebenden Gliazellen. Im Rahmen der vorgelegten Arbeit wurde ein Zellkultursystem, bestehend aus nicht-transgenen oder G93ASOD1 transgenen Motoneuronen, jeweils ko-kultiviert mit entweder nicht-transgenen oder G93ASOD1 transgenen Astrozyten als ALS- in vitro –Modell etabliert. In diesem Modell wurde das neuroprotektive Potenzial von N-ac-GLP-1, einem Analogs des endogenen insulinotropen Peptids Glucagon-like peptide-1(GLP-1) getestet. Die beobachteten protektiven Effekte konnten einer Abschwächung intrazellulärer Kalzium-Transienten zugeordnet werden. Nicht nur aufgrund dieser in vitro gemessenen antiexzitotoxischen Wirkung, sondern auch im Hinblick auf das wachsende Wissen um metabolische Defizite bei der ALS, die durch N-ac-GLP-1 positiv beeinflusst werden könnten, stellt diese Substanz einen interessanten Kandidaten für weitere in vivo-Evaluation dar.

In der zweiten Studie wurde der protektive Effekt mesenchymaler Stromazellen (MSC) auf Motoneurone (isoliert aus sowohl nicht-transgenen als auch SOD1G93A transgenen Mäusen), NSC-34-Zellen und Astrozyten (wiederum isoliert aus nicht-trasngenen und SOD1G93A transgenen Mäusen) in vitro analysiert. Auf der Suche nach den molekularen Mechanismen fanden wir eine durch Behandlung mit MSC-konditioniertem Medium induzierte Hochregulation der mRNA-Expression der Wachstumsfaktoren GDNF and CNTF und eine Reduktion der Expression proinflammatorischer Zytokine in Astrozyten. Diese Ergebnisse sprechen dafür, dass MSC für eine neuroprotektive Zelltherapie bei ALS geeignet sind.

General introduction

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is one of the most common adult-onset neurodegenerative diseases, characterized by progressive and relatively selective degeneration of upper and lower motor neurons, leading to progressive atrophy and weakness of skeletal muscle failure (ROWLAND u. SHNEIDER 2001). The clinical features of ALS include a combination of both upper and lower motor neuron signs and symptoms. ALS occurs in both sporadic (sALS) and familial forms (fALS), with an incidence of 1-2 per 100,000 individuals. Familial ALS occurs 5-10% of cases and predominantly has an autosomal dominant inheritance. In 20% of familial cases, there are mutations in superoxide dismutase-1 (SOD1) gene on chromosome 21 (ROSEN 1993). Mutations in the gene are thought to cause disease through a toxic gain of function rather than causing impairment of the antioxidant function of the SOD1 enzyme (SHAW 2005).

Recent breakthroughs in genetics have led to a much broader knowledge of genetic causes of fALS. Among the most frequent ones are mutations in the genes coding for the RNA-binding proteins TDP-43 and FUS, and as a hexanucleotide repeat expansion of the C9orf72 gene which codes for a protein without known function (AL-CHALABI et al. 2012).

Despite these advances, the definitive pathogenic mechanisms in ALS remain unclear. Among the factors that are thought to play an essential role are chronic excitotoxicity, metabolic disturbances affecting lipid and glucose metabolism, and mitochondrial dysfunction (PASINELLI u. BROWN 2006). Classical excitotoxicity refers to neuronal degeneration induced by overstimulation of glutamate receptors (VAN DEN BOSCH et al. 2006). The potential excitotoxins include AMPA (α-amino-hydroxy-5-methylisoxasole-4 propionic acid) and kainate. The excitotoxic hypothesis has led to the identification of riluzole, the antiglutamate agent, as the first licensed disease-modifying treatment to slow the progression of ALS (BENSIMON et al. 1994b).

SOD1G93A ALS mouse model

Due to the clinical and pathological similarities of human fALS and sALS, the knowledge of ALS mechanisms has been enhanced by studies using hemizygous transgenic rodent animal models overexpressing different SOD1-mutations (GURNEY 1997). These animals develop a motor neuron disease which both clinically and neuropathologically mimics human ALS. One of the most commonly used models is the SOD1G93A mouse model. These mice overexpress a mutant allele of the human SOD1 gene carrying the Gly93 → Ala substitution, leading to motor impairment associated with progressive loss of motor neurons in the ventral horn of the spinal cord. Dependent on the copy number of the mutant gene, gait impairments first occur around 110 days of age, the average lifetime of these animals is 130 days (GURNEY 1995; JULIEN u. KRIZ 2006).

Despite recent discoveries of other ALS related gene mutations, the mouse model based on overexpression of mutant SOD1 still remains the best characterized model to date and most closely mimics human ALS both by phenotype and pathophysiology. It can therefore still be considered as gold standard for preclinical *in vivo* studies even though translation of results from drug testing studies in this model to clinical trials in human ALS patients has not resulted in the discovery of more efficient neuroprotective therapies (LUDOLPH et al. 2010).

Glial cells as intrinsic components of non-cell-autonomous motor neuron degeneration

Initially based on groundbreaking studies in mutant SOD1 mouse models, it has now been widely accepted that motor neuron degeneration in ALS is non-cell-autonomous and that astrocytes and microglia are key contributors to disease pathogenesis in ALS (LOBSIGER u. CLEVELAND 2007; NAGAI et al. 2007). It was first shown in chimeric mice that non-transgenic motor neurons surrounded by mutant SOD1 transgenic glial cells were more vulnerable while on the other hand, wild-type glial cells exerted protective effects (CLEMENT

et al. 2003). Expression of mutant SOD1 in motor neurons affects disease onset, whereas mutant SOD1 in microglia contributes to the propagation of disease at the late stage (BOILLEE et al. 2006). In addition to the recognition of ALS-affected glial cells as contributors to disease progression, these data have also generated the rationale for cellular therapies aiming to generate a protective environment by administration of healthy non-neuronal cells which could provide essential support to neighbouring motor neurons.

Primary motor neuron cultures as *in vitro* model

While preclinical trials in animal models are time consuming and make it sometimes difficult to precisely elucidate mechanisms of action of an experimental treatment, *in vitro* models have the advantage of greater transparency and less complexity, sometimes allowing for more rapid assessment of the therapeutic potential as well as of underlying mechanisms of a novel therapeutic approach prior to animal studies. The first aim of this thesis project therefore was to develop an ALS in vitro model consisting of motor neurons isolated from either mutant SOD1G93A-overexpressing mice or their non-transgenic littermates. In addition to motor neuron monocultures, co-cultures of motor neurons and astrocytes have been established. This enabled comparison of different co-culture combinations (non-transgenic or SOD1G93A motor neurons cultured together with non-transgenic or SOD1G93A astrocytes) to take into account the partially non-cell autonomous character of motor neuron death in ALS (Figure 1).

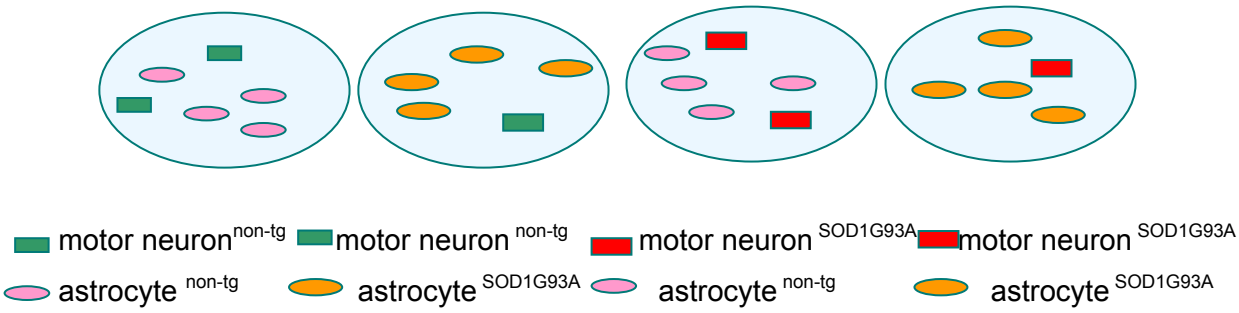

Figure 1: Co-culture system of neuronal/ glial cells derived from ALS-transgenic and non-transgenic mice

This model was then subsequently used in the first study to evaluate a novel pharmacological treatment approach, an agonist of the GLP-1 receptor, and in the second study to assess the effects and potential mechanisms of action of cell therapy with mesenchymal stromal cells.

Glucagon-like peptide-1 (GLP-1) as potential novel pharmacological treatment option in ALS

ALS is associated with several defects in energy metabolism, including weight loss, hypermetabolism, and hyperlipidaemia. Most of these abnormalities correlate with the time of survival, and there is evidence that disturbed energy metabolism plays a negative role in the pathogenic process (DUPUIS et al. 2011). Several studies showed that transgenic ALS mice suffer from a dramatic defect in energy homeostasis (DUPUIS et al. 2004) and bioenergetic defects are among the initial symptoms of SOD1G93A mice (BROWNE et al. 2006). Poor energy metabolism may lead to reduced ATP production. As a result, degenerative neurons are not properly adapted to energy requirements.

Glucagon-like peptide-1 (GLP-1) is an endogenous insulinotropic peptide that controls plasma glucose levels via its action on the pancreas; specifically, via the G-protein coupled GLP-1 receptor (GLP-1R). The peptide regulates energy metabolism by stimulating insulin synthesis and secretion from pancreatic ß-cells (KIM et al. 2010). This hormone is an important modulator of metabolic function. In light of this insulinotropic actions, several drugs that can mimic the biological functions of GLP-1 as treatment options for type 2 diabetes (T2DM) have attracted considerable attention (LOVSHIN u. DRUCKER 2009). GLP-1 receptors were found to be present in neurons of many different brain regions such as the cerebral cortex, hippocampus, and cerebellum (CAMPOS et al. 1994). The brain is a highly insulin sensitive organ and T2DM is known as a risk factor for many neurodegenerative diseases, including Alzheimer's Disease (AD) and Parkinson's Disease (PD) in which Exendin-4 (Ex-4), a GLP-1R agonist, is already being assessed as a new clinical treatment

strategy (HUANG et al. 2012). As glucose intolerance and insulin resistance have been linked to ALS (PRADAT et al. 2010), and in light of the neurotrophic and protective actions of GLP-1R activation in diverse cellular and animal models of neurodegenerative disease, we hypothesized that GLP-1 and its analogues may provide neuroprotective actions in ALS. To test this hypothesis, we aimed to investigate the therapeutic potential of N-acetyl-GLP-1 (7-34) amide (N-ac-GLP-1), a long acting, N-terminally acetylated, C-terminally truncated analogue of GLP-1 (JOHN et al. 2008) in both motor neuron monoculture and motor neuron/astrocyte co-culture conditions (Hui Sun et al, 2012, Publication I).

Mesenchymal Stromal Cells (MSC) for cell therapy of ALS

Based on the above mentioned evidence for non-cell autonomous motor neuron death in ALS, adult stem/ progenitor cells are currently investigated regarding their potential to generate a protective environment and provide trophic support for motor neurons in ALS.

Mesenchymal stromal cells (MSC), originally identified by Friedenstein and colleagues, are multipotential stromal precursor cells that can be readily isolated from the bone marrow and expanded *in vitro* as undifferentiated, plastic adherent cells (FRIEDENSTEIN et al. 1974). They can differentiate into cells of the mesodermal lineage such as osteoblasts, chondrocytes, and adipocytes (DELORME et al. 2006).

As opposed to embryonic stem and progenitor cells, MSC are are barely immunogenic, ethically inoffensive and easily available. They represent a promising tool for cell-based therapy by offering not only the possibility of cellular replacement, but also of targeted gene expression and neurotrophic factor release (ZHAO et al. 2007). Recently, several groups have studied MSC regarding treatment of neurodegenerative diseases, particularly of those that are fatal and difficult to treat, such as Huntington's disease and ALS (OLSON et al. 2012; UCCELLI et al. 2012). Regenerative approaches using MSC include delivery via intravenous, intraparenchymal or intrathecal injection (ZHAO et al. 2007; VERCELLI et al. 2008). MSC are known to secrete a variety of cytokines and growth factors that have both

paracrine and autocrine activities for damaged tissues (WILKINS et al. 2009). Several groups have demonstrated that intraparenchymal delivery of human MSC is safe and can delay loss of motor neurons in rodent models of ALS (VERCELLI et al. 2008). The feasibility and safety of intraspinal MSC injection has already been assessed in first human clinical trials (MAZZINI et al. 2012). A resent study showed that MSC exert beneficial effects on activated microglial cells in altering their phenotype and reactivity towards a more neuroprotective phenotype via release of the cytokine CXCL1 and interaction with microglial CXCL1 receptors (GIUNTI et al. 2012).

Secretion of growth factors is one of several possibilities how stem cells could influence the affected tissue in a positive way. It was shown that several growth factors such as glial cell line derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and ciliary neurotrophic factor (CNTF) can delay the progression of symptoms in ALS mice (GIESS et al. 2002; NAGANO et al. 2005; PASTOR et al. 2012; YANPALLEWAR et al. 2012).

The role of proinflammatory cytokines in exacerbating pathological and clinical features of ALS has already been described in vitro and in vivo (MOSLEY u. GENDELMAN 2010). One study showed that intracerebroventricular administration of human skeletal muscle-derived stem cells with MSC-like characteristics ameliorated clinical outcome in the wobbler mouse ALS model, attributable to stimulation of expression of anti-inflammatory cytokines (OPAL et al. 2000; CANZI et al. 2012). Another recent study showed that adult MSC-derived astrocytes, grafted into the central nervous system, abolished the target excessive inflammation in ALS mouse models (BOUCHERIE et al. 2009).

In our study, we analysed the protective effects of MSC co-culture and of MSC conditioned medium on primary motor neurons from both non-transgenic and SOD1G93A transgenic embryos (E13/14) against staurosporine induced apoptosis. In parallel to primary motor

neurons, we also analysed NSC-34 cells, a motor-neuron-like cell line, produced by fusion of motor-neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma (CASHMAN et al. 1992) and non-transgenic and mutant SOD1G93A transgenic astrocytes. Induction of growth factor expression as well as modulation of astrocyte activation was evaluated (Manuscript Ⅱ).

Manuscript I

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Therapeutic potential of N-acetyl-Glucagon-like peptide-1 in primary motor neuron cultures derived from non-transgenic and SOD1-G93A ALS mice

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of motor neurons in the motor cortex, brain stem and spinal cord. In the present study, we established an ALS in vitro model of purified embryonic motor neurons, derived from non-transgenic and mutant SOD1-G93A transgenic mice, the most commonly used ALS animal model. Motor neurons were cultured together with either non-transgenic or mutant SOD1-G93A astrocyte feeder layers. Cell viability following exposure to kainate (KA) as excitotoxic stimulus was assessed by immunocytochemistry and calcium imaging. We then examined the neuroprotective effects of N-acetyl-GLP-1 (7-34) amide (N-ac-GLP-1), a long acting, N-terminally acetylated, C-terminally truncated analogue of Glucagon-like peptide-1 (GLP-1). GLP-1 has initially been studied as a treatment for type II diabetes based on its function as insulin secretagogue. We detected neuroprotective effects of N-ac-GLP-1 in our in vitro system, which could be attributed to an attenuation of intracellular calcium transients. Not

only due to these antiexcitotoxic capacities but also with respect to the increasing knowledge about metabolic deficits in ALS, which could be positively influenced by N-ac-GLP-1, this compound represents an interesting novel candidate for further in vivo evaluation in ALS.

The text of the original publication can be found here: Cell Mol Neurobiol. 2013 Apr; 33(3):347-57.

Manuscript II

Submitted to Stem Cells

Therapeutic potential of mensenchymal stem cells in Amyotrophic Lateral Sclerosis (ALS) - In vitro evidence from primary motor neuron cultures, NSC-34 cells, astrocytes and microglia

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Abstract

Previous studies showed that administration of human mesenchymal stromal cells (MSC) improve functional outcome in the SOD1G93A mouse model of amyotrophic lateral sclerosis (ALS) as well as in models of other neurological disorders. Accumulating evidence suggests that the effects of MSC treatment are mainly based on the release of soluble factors in a paracrine fashion rather than on differentiation into neuronal/glial cells and integration into the central nervous system. We have now investigated the effect of the interaction between MSC and motor neurons (derived from both non-transgenic and mutant SOD1G93A transgenic mice), NSC-34 cells and astrocytes (derived again from both non-transgenic and mutant SOD1G93A transgenic mice) *in vitro*. Further we attempted to dissect the cellular and molecular mechanisms of this crosstalk. In primary motor neurons, NSC-34 cells and astrocytes, MSC conditioned medium (MSC CM) attenuated staurosporine (STS) - induced apoptosis in a concentration-dependent manner. Using specific inhibitors we could demonstrate that the MAPK/Erk1/2 and PI3K/Akt pathways are involved in the protection by MSC CM. Studying MSC CM-induced expression of neurotrophic factors in astrocytes and NSC-34 cells, we found that glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) gene expression in astrocytes were significantly enhanced by MSC CM, with differential responses of non-transgenic and mutant astrocytes. Expression of Vascular Endothelial Growth Factor (VEGF) in NSC-34 cells was significantly upregulated upon MSC CM-treatment. To analyse the immunomodulatory properties of MSC CM, we evaluated its effects on astrocyte-activation under lipopolysaccharide (LPS) treatment. MSC CM significantly reduced the expression of the cytokines TNFα and IL-6 and iNOS both in transgenic and non-transgenic astrocytes. The gene expression of CX3CL1 was also upregulated in mutant SOD1G93A transgenic astrocytes by MSC CM treatment.

Our data demonstrate that MSC CM modulates motor neuronal and astrocytic response to apoptosis and inflammation. MSC therefore represent an interesting candidate for further preclinical and clinical evaluation in ALS.

Keywords
Amyotrophic lateral sclerosis, Astrocytes, Mesenchymal stem-cells Conditioned medium, Growth factors, LPS, Motor neurons, NSC-34 cells, Staurosporine

Abbreviations

ALS	Amyotrophic lateral sclerosis
CM	Conditioned medium
CNTF	Ciliary neurotrophic factor
COX2	Cyclooxygenase - 2
GDNF	Glial cell line-derived neurotrophic factor
Erk	Extracellular signal-regulated kinase
FGF2	Fibroblast growth factor
iNOS	Inducible nitric oxide synthase
IGF	Insulin - like growth factor
IL-6	Interleukin - 6
IL-10	Interleukin - 10
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated extracellular kinase
MN	Motor neuron
MSCs	Mesenchymal stromal cells
NGF	Nerve growth factor
PI3K	Phosphoinositide 3-kinase
STS	Staurosporine
TNF-α	Tumor necrosis factor - α

VEGF Vascular endothelial growth factor

CX3CL1 Fractalkine

CX3CR1 Fractalkine receptor

Introduction

Cellular therapy is currently being investigated as a novel therapeutic option for the treatment of neurodegenerative disorders, including Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS). While in some diseases such as Parkinson's disease, cell replacement seems to be an option worth further exploration, in others such as ALS, cell therapy research rather focuses on the generation of a protective environment for degenerating neurons. This approach is supported by recent evidence that motor neuron death in ALS is non-cell autonomous and that non-neuronal cells can exert protective effects *in vitro* and *in vivo* [1, 2].

The rationale for the use of adult stem cells as a treatment for neurodegenerative diseases such as ALS comes from the notion that they are capable to provide the host tissue with growth factors or modulate the host immune system [3, 4]. Evidence from preclinical studies suggested that mesenchymal stromal cells (MSC), a subset of adult progenitor cells, can differentiate into neuronal-like cells [5]. Their neuroprotective effects, however, seem to be mainly based on anti-inflammatory and immunomodulatory activities [6]. They therefore do not require full engraftment of MSC following transplantation into the CNS, but rely on the capacity of MSC to release neuroprotective and anti-inflammatory molecules, resulting in the induction of a neuroprotective microenvironment [7, 8]. Intraspinal injection of human MSC delayed the onset of astrogliosis and microglia activation, motor neuron cell death, and the impairment of motor behaviour in transgenic ALS mice [9-11]. Another study has similarly shown protective effects of intravenous infusion of MSC in an ALS mouse model, indicating that the most appropriate route of administration with respect to both safety and efficacy still remains to be defined [11]. Bone-marrow-derived MSC have already been used in ALS patients

in a clinical phase I - trial and few adverse effects have been observed which is in favour of further clinical evaluation of this approach [12-14].

Considering that motor neurons are the primary structurally and functionally impaired cells in ALS, in the present study we generated primary motor neurons derived from non-transgenic and mutant SOD1G93A transgenic ALS mice, the most commonly used and best characterized ALS animal model. In order to clarify if the beneficial effects of MSC therapy are dependent on direct cell contact or if MSC mediate neuroprotection in an indirect way, embryonic primary motor neurons were either co-cultured on MSC feeder layers or incubated with MSC conditioned medium (CM). Using staurosporine (STS) as a toxic stimulus, we tested whether MSC-co-culture or MSC CM could protect motor neurons against apoptotic stress. We further assessed the protective effects of MSC CM against STS-induced apoptosis using the motor-neuron-like cell line, NSC-34 cells, produced by fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells, as *in vitro* motor neuron model. These cells express many of the morphological and physiological properties of primary motor neurons [15] and have the advantage to overcome the low yields and limited purity of primary motor neuron preparations. A breakthrough in ALS research was the discovery that non cell-autonomous processes due to functional dysregulation of surrounding non-neuronal cells, i.e. microglia and astrocytes, contribute to motor neuron death [1, 16]. Several studies showed that astrocytes are specific contributors to spinal motor neuron degeneration in mutant SOD1-linked ALS and that they exert toxicity on motor neurons via release of soluble factors [2, 17]. We therefore further analysed the protective effects of MSC CM pre-treatment in primary astrocyte cultures derived from either SOD1G93A or non-transgenic mice to determine whether astrocytes from mutant animals respond differently to MSC CM, attempted to clarify whether the MAPKK/ Erk1/2 or PI3K/Akt signalling pathways which have been extensively studies in neurons and can influence cell death and survival, are involved in the protective effects [18, 19].

Cytokines and growth factors are key mediators of central nervous system (CNS) networks. Cytokines and chemokines are multifunctional proteins that have been shown to be involved not only in autoimmune diseases of the CNS such as multiple sclerosis (MS), but also in neurodegenerative diseases such as Parkinson's [20] and Alzheimer's disease [21]. In ALS, proinflammatory mediators like tumor necrosis factor-α (TNF-α), and interleukin-1 beta (IL-1ß) have been shown to play a role in the disease pathogenesis [22-24]. Protective effects of growth factors, such as CNTF (ciliary neurotrophic factor), GDNF (glial cell line-derived neurotrophic factor), IGF-1 (insulin-like growth factor 1), FGF2 (basic fibroblast growth factor 2) and VEGF (vascular endothelial growth factor) [25-29] have been shown in rodent models of ALS. We therefore studied whether MSC CM could induce growth factor expression in astrocytes and NSC-34 cells, and modify LPS-induced production of pro-inflammatory cytokines. As MSC were previously reported to modify expression of the neuroprotective chemokine fractalkine (CX3CL1) in glial cells lines [30], we also measured mRNA expression of CX3CL1 and its receptor (CX3CR1) in astrocytes and microglia.

Materials and Methods

Primary motor neuron culture

Isolation and *in vitro* cultivation of motor neurons was performed by dissection of lumbar ventral spinal cords from individual mouse embryos (gestational age: E13/14) as previously described [31]. G93A transgenic familial ALS mice (high copy number; B6SJLTg ((SOD1-G93A)1Gur/J) [32] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). These mice over-express the human mutant SOD1 allele containing the Gly93→Ala (G93A) substitution. We maintained the transgenic G93A hemizygotes by mating transgenic males with B6SJLF1/J hybrid females. Transgenic offspring was genotyped by PCR assay of DNA obtained from the cerebellum of the embryos. After tissue dissociation, motor neurons were enriched by a p75NTR-antibody- (Abcam, Cambridge, UK) – based immunopanning technique. Culture medium (Neurobasal medium, Gibco Invitrogen, Darmstadt, Germany) with 2% horse serum, 2% B27-supplement (Invitrogen, Darmstadt, Germany); 0.5 mM

glutamax-I; 5 ng/ml rHuBDNF and 5 ng/ml rHuCNTF (both from Peprotech, Hamburg, Germany) was added to the resulting pellet. Highly enriched motor neurons were seeded on glass coverslips, pre-incubated first with polyornithin (diluted 1:1000, Sigma, Steinheim, Germany) and laminin (2.5µg/ml, Invitrogen, Steinheim, Germany) or on a mouse MSC feeder layer at a ratio of 1: 4. The average density of motor neurons was 2.0×10^4 cells cm^{-2}. All treatment described below were performed after 7 days *in vitro* (DIV 7). The purity of primary motor neuron cultures was determined by the ratio of SMI32 positive cells to DAPI stained nuclei, resulting in approximately 80% motor neurons [33, 34].

Microglia and astrocyte culture

Primary cultures were obtained from one day old non-transgenic and mutant SOD1G93A transgenic mice. Cerebra were dissected and meninges removed in HBSS/Hepes buffer. Brains were chopped with a razor blade. The brain pieces were put into 15ml tube and were then centrifuged for 5 min at 3000 rpm at RT. For dissociation the pellet was incubated with trypsin (0.1%) at 37 ºC for 20-30 min, under shaking. DNase (0.001%) was added and the tubes were turned upside down several times. The tubes were centrifuged at 3500 rpm for 5 min at RT. The supernatant was discarded and the red blood cells were removed with a pasteur pipette. The remaining pellet was triturated through a flame-narrowed glass pipette until a single cell suspension was obtained. The cells were seeded in DMEM (4.5 g/L Glucose, [+] L-Glutamine, [-] Pyruvate), 10%FBS, 1% Penicillin/Streptomycin onto poly-L-lysine (diluted 1:1000, Sigma, Steinheim, Germany) coated flasks. The next day, the culture medium was removed, cells were washed with PBS and new culture medium was added. Medium was changed every second day. On day 10-13, tissue culture flasks were closed tightly with parafilm and shaken for 30min at 180 rpm at 37 ºC on an orbital shaker-incubator (Edmund Bühler, Hechingen, Germany) and plated on culture dishes (Nunc, Roskilde, Denmark). Microglial purity was more than 95% as determined by CD11b (AbD Serotec, Kidlington, UK) immunoreactivity. Medium was replaced with 12ml DMEM/10%FBS and left

in the incubator for further shaking (isolation of astrocytes). On day 14, tissue culture flasks were closed tightly with parafilm and shaken for 16 h at 170 rpm overnight at 37 ºC. The next day, medium with AraC (100 µM, Sigma-Aldrich, Steinheim, Germany) was added to each flask. The flasks were incubated at 37 ºC for 72 h. After washing with PBS, cells were trypsinised, counted and plated at a density of 3×10^4 cells cm^{-2} for RNA extraction. For immunocytochemistry, cells were grown on poly-L-Lysine coated coverslips in 24 well plates at a density of 1×10^4 cells cm^{-2}.

Staurosporine-induced toxicity

Highly enriched motor neurons derived from either non-transgenic or mutant SOD1G93A transgenic ALS mice (non-tg MN/G93A MN) were seeded on glass coverslips either pre-incubated with polyornithin (diluted 1:1000, Sigma, Steinheim, Germany) and laminin (2.5 µg/ml, Invitrogen, Steinheim, Germany) or covered with a mouse MSC feeder layer. The ratio of MSC to motor neurons was 1: 4. The average density of motor neurons was 2.0×10^4 cells cm^{-2}. NSC-34 cells and astrocytes were both seeded on poly-L-lysine (diluted 1:1000, Sigma, Steinheim, Germany) coated glass coverslips. The average density of NSC-34 cells and astrocytes was 2.0×10^4 cells per cm^2.

After 7 DIV, monocultures of non-transgenic motor neurons were incubated for 24 h with different concentrations of staurosporine (STS) (0.1, 0.2, and 0.5 µM, respectively) to test for concentration dependent neurotoxic effects. NSC-34 cells and astrocytes were incubated for 24 h with different concentrations of STS (NSC-34 cells: 0.03, 0.1, 0.2, 0.3, 0.5 and 1 µM; Astrocytes: 0.1, 0.2, 0.5 and 1µM, respectively) to test for concentration dependent neurotoxic effects using the MTT assay.

Conditioned medium and cell treatments

MSC derived from bone marrow of C57BL/6 mice were obtained from Gibco (Karlsruhe, Germany). Cells were cultured in D-MEM/F-12 medium with GlutaMAX™-I (Gibco, Karlsruhe,

Germany) supplemented with 10% MSC-Qualified fetal bovine serum (FBS, Gibco, Karlsruhe, Germany) and 1% Penicillin/Streptomycin (Sigma–Aldrich, Steinheim, Germany). MSC were seeded at a density of 3.0×10^3 per cm^2. After rinsing with PBS, Neurobasal medium supplemented with 1% B27, 1% Penicillin/Streptomycin, 0.5 mM L-glutamine, and 2% FBS was added. After 24 h, supernatants from MSC cultures were collected, and centrifuged at 3000 rpm for 5 min to remove remaining cells. Debris was removed by rinsing the supernatant through a 0.22 µm filter and designated as conditioned medium (CM) for primary motor neurons. Aliquots of CM were frozen at - 80ºC. In order to get conditioned medium for NSC-34 cells, DMEM (low glucose 1g/L; GmbH, Aidenbach, Germany) was used instead of Neurobasal medium. Conditioned medium for astrocytes was obtained by growing MSC in DMEM (4.5 g/L Glucose, [+] L-Glutamine, [-] Pyruvate), 10% FBS, 1% Penicillin/Streptomycin.

CM was added at different concentrations ranging from 20% to 100% (V/V) in different culture medium either 4 h before induction of apoptosis or simultaneously together with the proapoptotic stimulus. Apoptosis was induced by staurosporine (STS), based on dose-finding experiments in each cell type (Fig.1)(0.1µM for primary motor neurons; 0.03 µM for NSC-34 cells; 1 µM for astrocytes; Sigma, Steinheim, Germany) for 24 h.

Microglia were co-cultured for 24 hours in the presence or absence of MSC at 1:1 ratio (3×10^5 cells) in a six - well plate in a transwell system (BD FALCON Cell Culture Inserts, BD Biosciences, San Diego, CA).

Immunocytochemistry for assessment of survival of motor neurons, NSC-34 cells and astrocytes

Cells were fixed with 4% paraformaldehyde (PFA). Motor neurons were stained over night with an antibody against SMI32 (1:1000; monoclonal, Cambridge, UK) which is specific for motor neurons derived from embryonic spinal cord. MSC were identified with CD44 (1:50; monoclonal, BD Biosciences, Heidelberg, Germany). NSC-34 cells were stained over night with an antibody against ß-III tubulin (1:140, Abcam, Cambridge, UK) and astrocytes with an

antibody against glial fibrillary acidic protein (GFAP; 1:300; Sigma, polyclonal, Dako Cytomation, Glostrup, Denmark). Appropriate Alexa Fluor 555 or Alexa Fluor 488 coupled secondary goat anti-mouse or goat anti-rabbit antibodies (Invitrogen, Darmstadt, Germany) were applied for 45min at RT. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen, Darmstadt, Germany). Motor neuron and astrocyte survival was quantified by cell counting in 5 visual fields of each coverslip in a total of eight different preparations for both non-transgenic and transgenic cells, as previously described [33, 34]

MTT cell viability assay

Cell viability of NSC-34 cells and astrocytes after STS treatment was assessed via the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte-trazolium bromide) assay (Sigma, Steinheim, Germany), which measures the ability of cells to reduce MTT to formazan. After acid isopropanol extraction, formazan absorbance was quantified at 570nm with a reference wavelength of 630 nm.

Blocking experiment

The PI3-K inhibitor Ly294002 and the MEK-1 inhibitor PD98059 (both from Biotrend, Cologne, Germany) were applied to both the NSC-34 cells and astroglial cultures at a final concentration of 10 μM 1 h prior to incubation with CM, hence 5 h prior to induction of apoptosis. The inhibitors were left in the medium during the whole pre-incubation period and were present after induction of apoptosis until the evaluation of apoptotic cells 24 h later.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Astrocytes and NSC-34 cells were harvested and total RNA was extracted according to the manufacture's protocol using RNeasy Micro Kit (Qiagen GmbH, Düsseldorf, Germany). Reverse transcription of 1500 ng total RNA per reaction was carried out using oligo-dT primer and Superscript ‖ reverse transcriptase (Invitrogen, Darmstadt, Germany). The

genetic expression of growth factors and cytokines were quantified using the TaqMan method with the following assays synthesized by Lifetech (Life Technologies; Applied Biosystems): GDNF (Mm00599849_m1), CNTF (Mm00446373_m1), VEGF (Mm00437304_m1), NGF (Mm00443039_m1), IGF (Mm00439560_m1), TNFα (Mm99999068_m1), IL-6 (Mm00446190_m1), iNOS (Mm00440502_m1), COX2 (Mm03294838_g1) and IL-10 (Mm00439614_m1), CX3CL1 (Mm00436454_m1), CX3CR1 (Mm00438354_m1). Hprt1 (Mm 00446968_m1) was used as reference gene for astrocytes. Gapdh (Mm99999915_g1) was used as reference gene for NSC-34 cells. Corresponding to mRNA, cDNA was used at a concentration of 25 ng/µL. qRT-PCR was performed with cDNA from 50 ng total RNA and TaqMan®Fast Universal Master Mix (Applied Biosystems) on a StepOnePlus instrument (Applied Biosystems) under the following standard conditions: 95 ºC for 20 s, followed by 40 cycles of 95 ºC for 1 s and 60 ºC for 20 s. The relative gene expression was calculated via the comparative Ct method as previously described by K. Livak (Applied Biosystems User Bulletin #2, 2001). Ct values were normalized to Hrtp1 and used to calculate the relative gene expression using the $2^{-\Delta\Delta Ct}$ method [28].

Statistical Analysis

All results were expressed as mean ± SEM. GraphPad Prism 3.0 software was used for statistical evaluation. Comparisons between different conditions were performed using one-way ANOVA and two-way ANOVA with Bonferroni post-test.

Results

Staurosporine (STS) induces apoptosis in primary motor neurons, NSC-34 cells and astrocytes

STS induced cytotoxicity was used to model in vitro motor neuron damage, because apoptotic motor neuron death is of major relevance in ALS. To induce apoptosis, DIV 7 non-transgenic motor neuron monocultures were exposed to STS in concentrations ranging from 0.1 to 0.5 µM for 24 h (Fig. 1A). A significant reduction in motor neuron number was observed following incubation with 0.1 and 0.5 µM STS and significant dose-dependency of

STS-toxicity was observed between 0.1 and 0.5 µM, 0.1 µM and 0.2 µM. In light of these results, 0.1 µM STS was chosen for further experiments with primary motor neurons.

In NSC-34 cells, a significant decrease in cell viability was observed following incubation with 0.03 µM, 0.1 µM, 0.2 µM, 0.3µM, 0.5 µM and 1 µM STS for 24 h. No dose dependent effect of STS toxicity on NSC-34 cells could be observed. 0.03 µM STS was sufficient to reduce cell viability by about 50% (Fig. 1B). Thus, 0.03 µM STS was chosen for the further experiments with NSC-34 cells.

In astrocyte monocultures, significant reduction of cell viability was only observed following incubation with 1 µM STS, as determined by MTT assay. Therefore, 1 µM STS was used for further experiments with astrocyte cultures (Fig. 1C).

MSC-conditioned medium (CM) protects primary motor neurons, NSC-34 cells and astrocytes against staurosporine toxicity in a concentration dependent manner

Our study aimed to further investigate the cellular interactions between MSC and motor neurons in vitro. In order to clarify whether the protective effects of MSC are attributable to direct cell interaction or rather to MSC-released factors which mediate neuroprotection in an indirect manner, mouse primary non-transgenic and mutant SOD1G93A transgenic motor neurons were either cultured in co-culture with MSC or incubated with MSC conditioned medium (MSC CM) prior to the STS-induced apoptotic insult. In motor neuron-MSC co-cultures, a slight but not significant protective effect of MSC against STS-induced toxicity was observed in both non-transgenic and transgenic motor neurons (Fig. 2A). STS-sensitivity of SOD1G93A transgenic motor neurons was significantly greater than of non-transgenic motor neurons (Fig. 2C). As shown in Fig. 2C, when the culture medium was substituted with MSC CM to a quantity of 40%, STS-induced apoptosis was significantly attenuated. In non-transgenic motor neuron cultures the maximum protective effect was already reached with 20% CM, whereas in transgenic cells 40% CM resulted in the highest percentage of surviving motor neurons. Interestingly, CM concentrations higher than 40% did not result in any

protective effect (Fig. 2C). Protective effects of MSC CM were only observed when it was added 4h before STS while administration of STS and MSC CM at the same time point did not result in significant neuroprotection (not shown).

Similar neuroprotective effects were observed with MSC CM in NSC-34 cells (Fig. 3) and astrocytes (Fig. 4). In NSC-34 cells, MSC CM significantly reduced STS-induced apoptosis with a maximum protective effect at 40-50% CM (Fig. 3). STS-induced apoptosis in astrocytes was most attenuated by 30% CM (Fig. 4). These results demonstrate that MSC CM protects SOD1G93A and non-transgenic primary motor neurons, NSC-34 cells and astrocytes against STS-induced apoptosis in a concentration dependent manner.

Potential involvement of MAPK/Erk1/2 and PI3K/Akt in neuroprotective effects of MSC CM in NSC-34 cells and astrocytes

The MAPK/Erk1/2 and PI3-K/Akt pathways are important signalling cascades that can mediate differentiation, proliferation, growth and survival of neurons and astrocytes (Gao, et al., 2005). In order to evaluate a potential functional role of these pathways in the anti-apoptotic effect of MSC CM, they were blocked by specific inhibitors. Pre-incubation with PD98059, a specific inhibitor of MEK that blocks the activation of the MAPK/Erk1/2 pathway, abolished the significant MSC CM-mediated neuroprotection after STS induced apoptosis in both NSC-34 cells and astroglial cultures (Fig. 5). Likewise, the protective effect of MSC CM against STS toxicity was reduced and no longer significant after pre-incubation with LY294002 (Fig. 5). Altogether, these findings point towards a functional involvement of these survival signalling pathways in the protective effect of MSC CM towards apoptotic cell death in NSC-34 cells and astrocytes.

GDNF and CNTF expression is upregulated by MSC conditioned medium in astrocytes, VEGF expression is upregulated by MSC CM in NSC-34 cells

To determine if co-incubation with MSC CM could result in self-mediated protection and survival of astrocytes and NSC-34 cells as well as in neuroprotection due to increased astrocytic and NSC-34 cells release of neurotrophic factors, gene expression of VEGF, IGF, NGF, FGF2, GDNF and CNTF was measured in both non-transgenic and mutant SOD1G93A astrocytes and NSC-34 cells. The presence of MSC CM significantly upregulated mRNA levels of GDNF in both non-transgenic and SOD1G93A transgenic astrocytes but the increase was significantly higher in mutant SOD1G93A astrocytes in comparison to non-transgenic astrocytes (Fig. 6A). CNTF mRNA expression was significantly upregulated upon MSC CM exposure in non transgenic astrocytes while this effect was not significant in SOD1G93A astrocytes (Fig. 6B). VEGF mRNA expression was significantly upregulated upon MSC CM exposure in NSC-34 cells (Fig. 6C). Quantification of gene expression of IGF, NGF and FGF2 did not result in significant differences (not shown). This indicates that protective effects of both non-transgenic and SOD1G93A transgenic astrocytes and NSC-34 cells may be enhanced by MSC CM.

MSC CM influences astrocytic gene expression

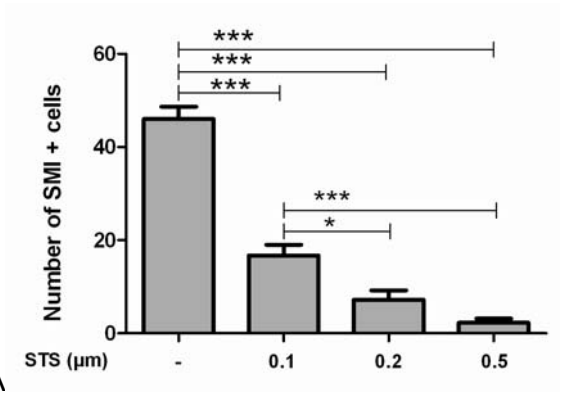
Using lipopolysaccharide (LPS)-induced activation of astrocytes, we wanted to find out if functional adaptations to inflammation were influenced by MSC CM. The pro-inflammatory cytokines TNFα and IL-6, as well as the pro-inflammatory enzyme iNOS are typically upregulated under inflammatory conditions and take part in a pro-inflammatory immune response [36, 37]. LPS-induced upregulation of TNFα, IL-6 and iNOS was significantly higher in SOD1G93A transgenic than in non-transgenic astrocytes. In cells that had been exposed to MSC CM, the LPS-induced expression of TNFα, IL-6 and iNOS was significantly decreased in both non-transgenic and SOD1G93A transgenic astrocytes, but the decrease was significantly greater in SOD1G93A transgenic astrocytes compared to non-transgenic ones, while expression of COX-2 and IL-10 was not modified (Fig. 7 A-C).

MSC increase expression of neuroprotective molecules in astrocytes and microglia

We analyzed the effect of MSC on mRNA expression of the neuroprotective chemokine CX3CL1 in astrocytes and its receptor CX3CR1 in microglia. MSC CM significantly upregulated CX3CL1 mRNA in SOD1G93A transgenic astrocytes after LPS-treatment (Fig. 7 D). The mRNA expression of CX3CR1 was significantly upregulated by co-culture with MSC in SOD1G93A transgenic microglia, and slightly but not significantly upregulated in non-transgenic microglia (Fig. 7 D-E).

Figures

Fig. 1



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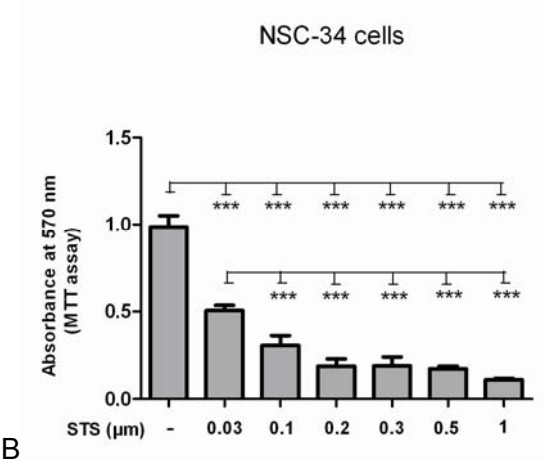

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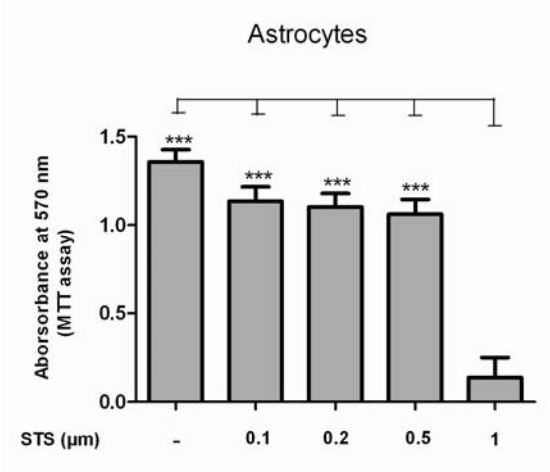


C

Fig. 1: Assessment of Staurosporine-induced toxicity in motor neurons, NSC-34 cells and astrocytes.

Dose dependent neurotoxic effect of STS on non-transgenic motor neurons cultured in monoculture as quantified by immunocytochemistry at DIV 7 (n=8, Fig. 1A). Significant toxic effect of different concentrations of STS on viability of NSC-34 cells (Fig. 1B) and non-transgenic astrocytes (Fig. 1C) as detected by MTT assay. Values represent means ± SEM, *** $p < 0.001$, * $p< 0.05$. One-way ANOVA with Bonferroni post-test.

Fig. 2

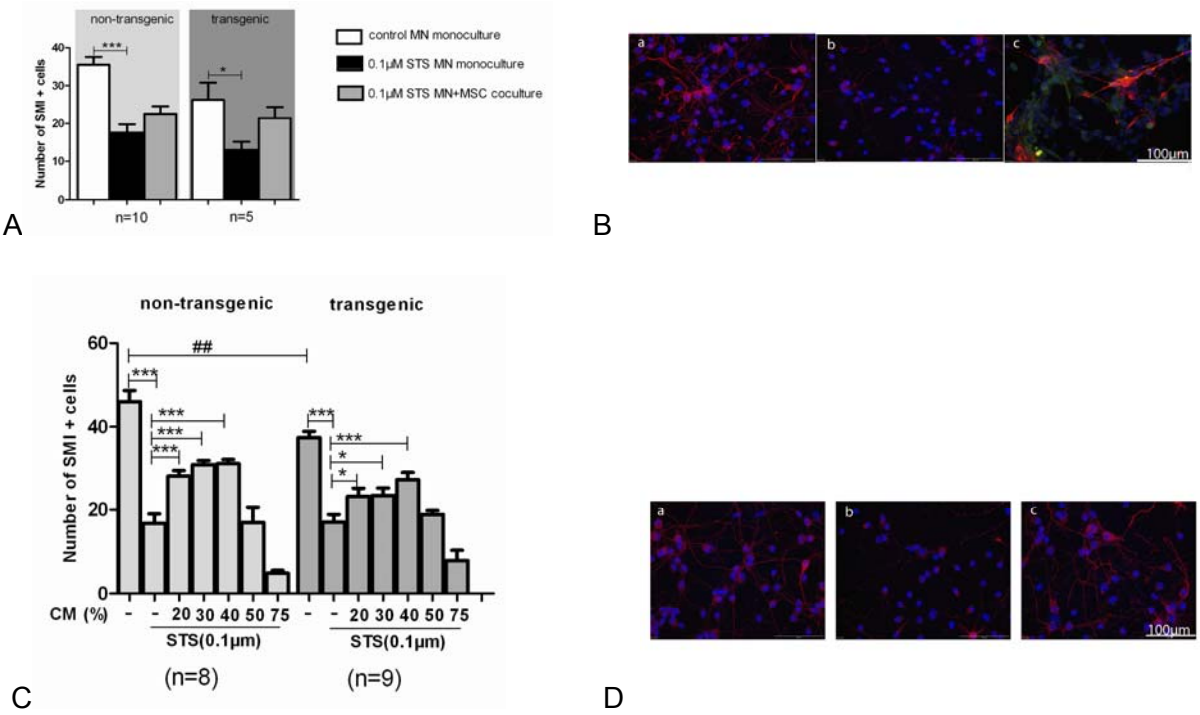


Fig. 2: Protective effects of MSC- co-culture and of MSC conditioned medium against STS toxicity in primary motor neuron monocultures.

A: The co-cultivation of motor neurons on MSCs showed a slight but not significant protective effect against STS toxicity, with greater STS-sensitivity of SOD1G93A motor neurons. Values represent means ± SEM, ***p < 0.001, * p < 0.05, two-way ANOVA with Bonferroni post-test.

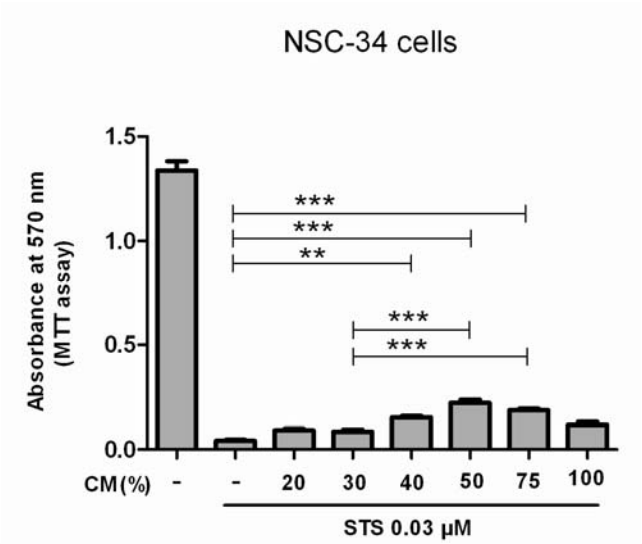
B: Photographs show representative motor neuron cultures at DIV 7 (B). a: SOD1G93A transgenic motor neuron monoculture without any treatment. b: Reduction of SMI 32 positive motor neurons following exposure to 0.1µM STS. c: Increase in motor neuron survival due to co-cultivation on MSC. Motor neurons were stained by an antibody against SMI 32 (red). MSC were immunopositive for CD44 (green). Stained nuclei of cultured cells appear in blue (DAPI). Scale bar 100 µm.

C: STS-induced apoptosis was attenuated best by 40% dilution of CM, whereas at concentrations higher than 50% CM did not mediate neuroprotection. SOD1G93A motor

neurons were significantly more sensitive to STS than non-transgenic motor neurons. Values represent means ± SEM, ***p < 0.001, **/## p < 0.01, * p < 0.05, two-way ANOVA with Bonferroni post-test.

D: Immunostainings revealed the apoptotic morphology of motor neurons after STS treatment (b) and the protective effect of CM (c).

Fig. 3



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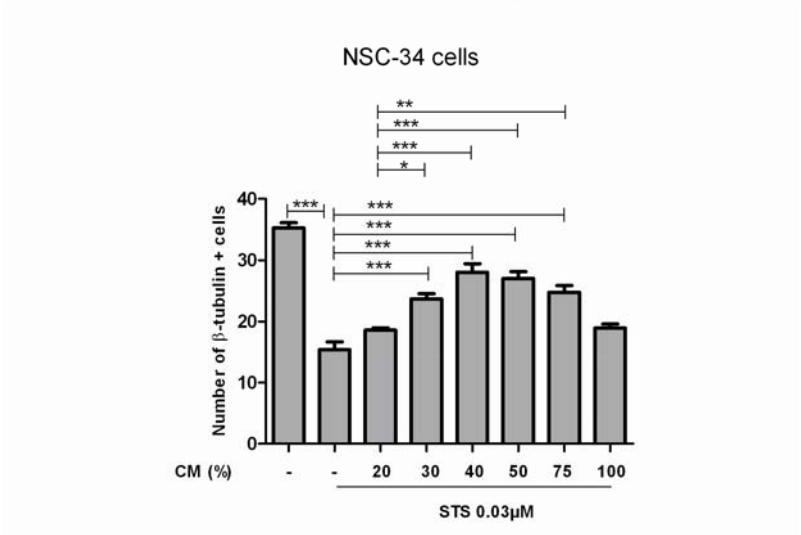

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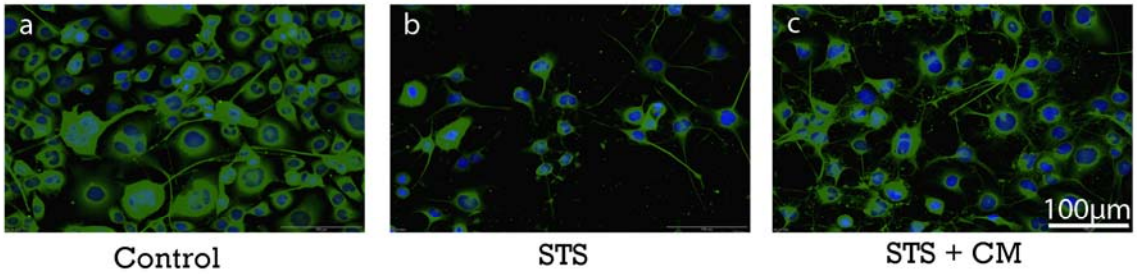


Fig. 3: Protective effects of MSC-conditioned medium against STS toxicity in NSC-34 cells

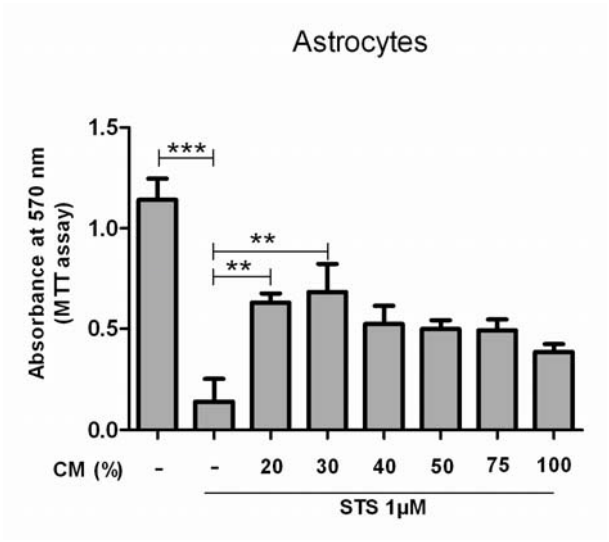
NSC-34 cells were incubated with DMEM conditioned by MSC (CM) starting 4 h before exposure to STS (0.03µM).

A: STS-induced apoptosis was attenuated best by a 50% dilution of CM, as shown by MTT assay.

B: Quantification of cell death by immunocytochemical analysis similarly revealed most neuroprotection at a 10 - 50% dilution of MSC CM. Values represent means ± SEM, ***p < 0.001, ** p < 0.01, * p < 0.05, one-way ANOVA with Bonferroni post-test.

C: Immunostainings showed a reduction of cell number due to induction of apoptosis by STS, as well as the protective effect of CM. a: NSC-34 cell monoculture without any treatment. b: Reduction of ß-III tubulin positive NSC-34 cells following exposure to 0.03 µM STS. c: Increase in NSC-34 cell survival due to CM treatment. NSC-34 cells were stained by an antibody against ß-III tubulin (green). Stained nuclei of cultured cells appear in blue (DAPI). Scale bar 100 µm.

Fig. 4



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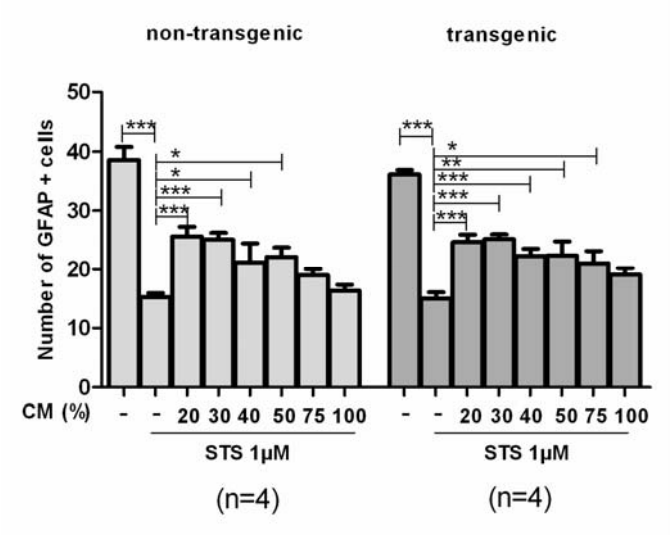

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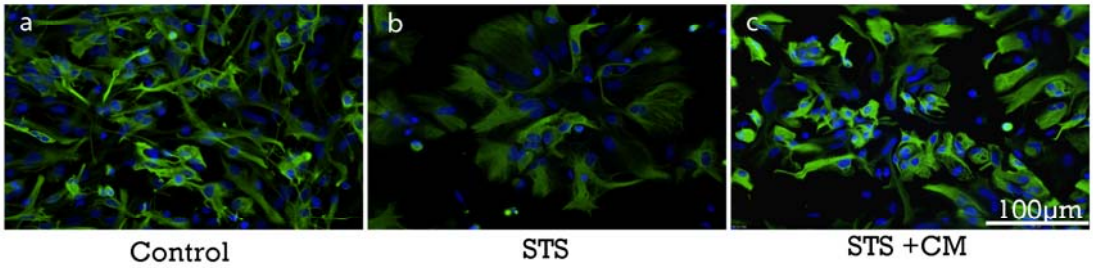

C

Fig.4: Influence of MSC conditioned medium on astrocytes under STS treatment.

Astrocyte cultures were examined at high magnification by fluorescence microscopy after GFAP staining. Cultures were incubated for 48 h in conditioned medium, 1 µM STS was added during the last 24 h.

A: MTT assay showed significantly increased survival of non transgenic astrocytes in presence of 20% and 30% CM.

B: Immunocytochemical quantification of astrocyte survival showed most efficient attenuation of STS-induced apoptosis with a 30% dilution of CM, whereas concentrations higher than 50% CM did not mediate significant protective effects. STS-sensitivity of non-transgenic and SOD1G93A transgenic astrocytes did not significantly differ. Values represent means ± SEM, ***p < 0.001, **p < 0.01, *p < 0.05, two-way ANOVA with Bonferroni post-test (Fig. 4B); one-way ANOVA with Bonferroni post-test (Fig. 4A).

C: Immunostainings revealed STS-induced apoptosis of astrocytes (a as compared to b) and a protective effect of MSC CM (c).

Fig. 5

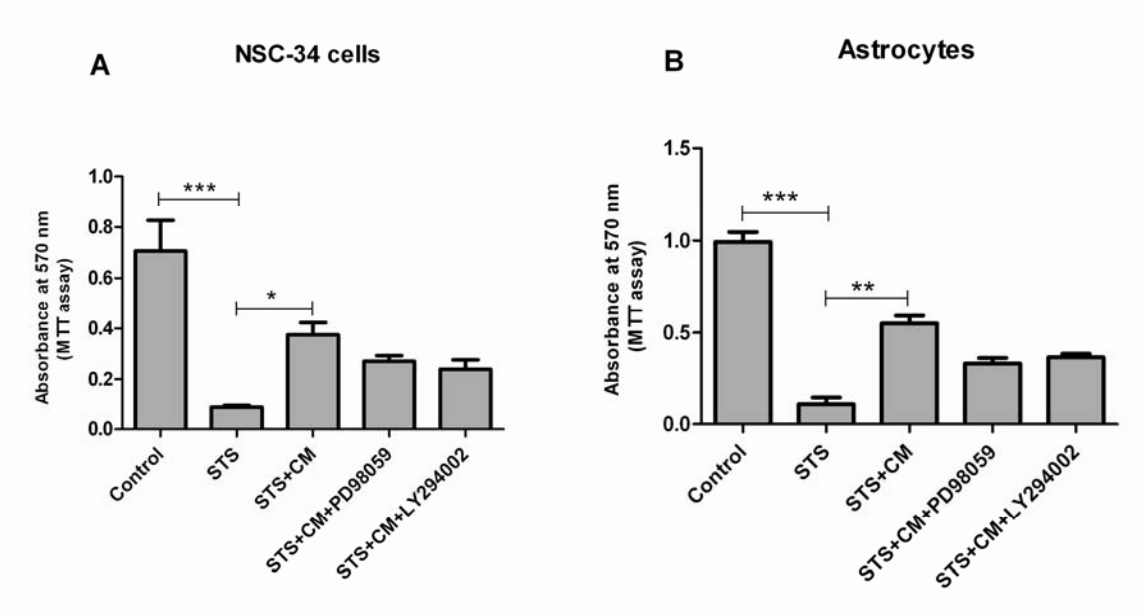

Fig. 5: Involvement of the MAPK/Erk1/ 2 and PI3-K/Akt pathways in MSC CM-mediated protection.

10 µM MAPK/Erk1,/2 inhibitor PD98059 and 10 µM PI3-K/Akt inhibitor LY294002 were applied to both NSC-34 cells (A) and astrocytes (B) 1 h before CM co-incubation and 5 h before induction of apoptosis by STS (0.03 µM for NSC-34 cells, 1 µM for astrocytes). This pre-incubation with PD98059 and LY294002 lowered the protective effect of CM against STS-induced apoptosis resulting in a no longer significant increase in cell survival as compared to STS-exposure. Values represent means ± SEM, ***p < 0.001, **p < 0.01, * p < 0.05, one-way ANOVA with Bonferroni post-test.

Fig. 6

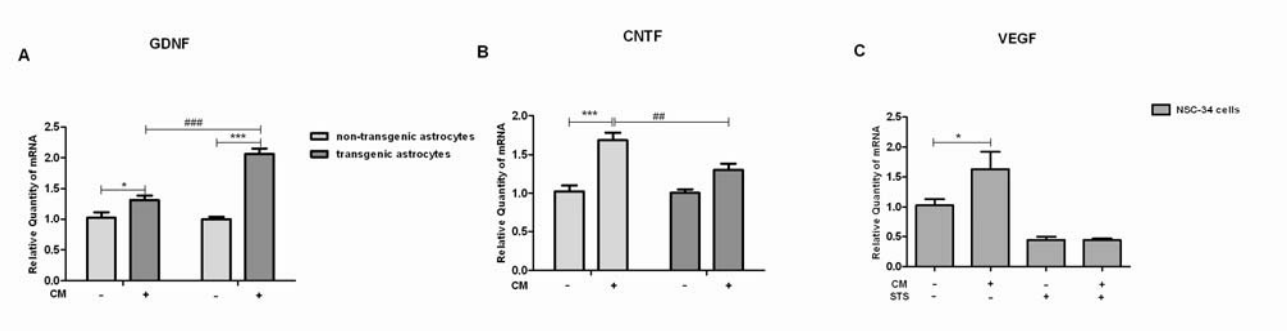

Fig. 6: MSC CM differentially induces expression of GDNF and CNTF in both non-transgenic and SOD1G93A transgenic astrocytes, and induces expression of VEGF in NSC-34 cells.

mRNA levels of GDNF in both non-transgenic and SOD1G93A transgenic astrocytes were both significantly up-regulated by MSC CM. The GDNF mRNA level upon incubation with 100% CM was significantly higher in SOD1G93A transgenic astrocytes compared to non-transgenic ones (A). The mRNA level of CNTF was significantly up-regulated in non-transgenic astrocytes and slightly but not significantly up-regulated in SOD1G93A transgenic astrocytes (B). The mRNA level of VEGF was significantly up-regulated in NSC-34 cells under MSC CM treatment (C). Values represent means ± SEM, ### / *** $p < 0.001$, ## $p < 0.01$, * $p < 0.05$, two-way and one-way ANOVA with Bonferroni post-test.

Fig. 7

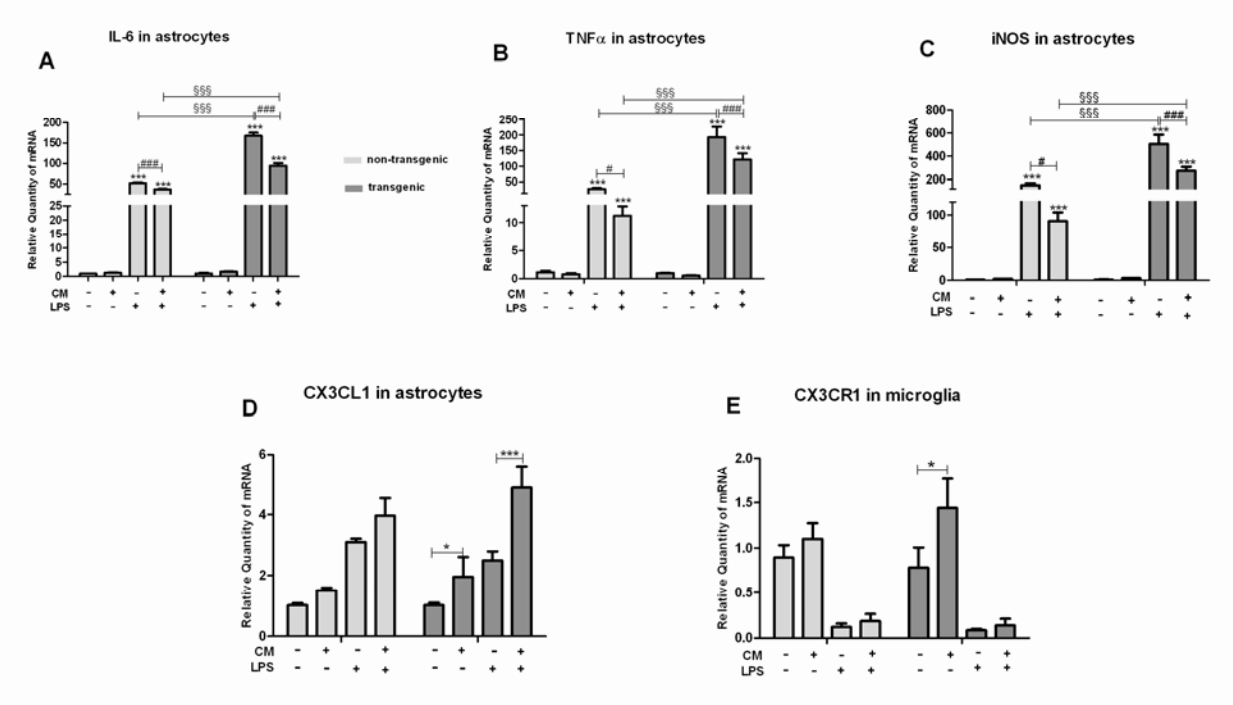

Fig. 7: Influence of MSC CM on astrocytic and microglial gene expression.

The relative mRNA expression of the pro-inflammatory cytokines TNFα, IL-6, the pro-inflammatory enzyme iNOS, and the neuroprotective chemokine CX3CL1 was examined in non-transgenic and SOD1G93A transgenic astrocyte cultures incubated for 28h in 100% MSC CM or regular medium without CM and with or without LPS stimulation (500 ng/mL, added during the last 4 h) (A-D). The relative mRNA expression of the corresponding receptor CX3CR1 was analysed in non-transgenic and SOD1G93A transgenic microglia cultures co-cultured with or without MSC in a transwell system (E). LPS-induced upregulation of the pro-inflammatory cytokines IL-6 and TNFα and of the proinflammatory enzyme iNOS was significantly higher in transgenic than in non-transgenic astrocytes and was significantly attenuated by MSC CM (A-C). MSC CM significantly upregulated CX3CL1 expression in SOD1G93A transgenic astrocytes, while the MSC CM induced increase in CX3CL1 in non-transgenic astrocytes was not statistically significant. MSC significantly increased the expression of CX3CR1 in transgenic microglial cells, and slightly but not significantly upregulated it also in non-transgenic ones. Values represent means ± SEM, §§§ / ### / ***p < 0.001, # / *p < 0.05, two-way ANOVA with Bonferroni post-test.

Discussion

The present study demonstrates that MSC have protective effects on primary motor neurons, NSC-34 cells and astrocytes against STS-induced apoptosis. In comparison MSC CM was even more efficient than co-culture of motor neurons together with MSC, indicating that the effects are mediated in a paracine manner via release of protective factors by MSC, that exert their effects across cell type barriers, which is typical for growth factors and cytokines. Use of MSC CM probably resulted in higher concentrations of MSC-secreted factors than the co-culture of MSC and motor neurons. While protective effects of MSC CM had already been demonstrated in rodent hippocampal neurons [7] and astrocytes [35], in the present study, we showed for the first time that MSC CM has protective effects on primary non-transgenic and SOD1G93A transgenic motor neurons (with greater sensitivity but similar rescue effects for the latter ones), on NSC-34 cells, a cell line with motor neuron characteristics, and on primary astrocytes derived from either non-transgenic or SOD1G93A mice. It is important to note that MSC CM exerted protection in a concentration-dependent manner, depending on the cell type investigated. Significant protective effects were observed at dilutions of 20-75%. At concentrations higher than 75%, however, the protective effect of MSC CM was diminished in all investigated cell types. This observation is in accord with one previous study where a similar concentration dependency of the protective effects of MSC CM was observed in rat hippocampal neurons [7]. As already discussed there, this could render clinical application of MSC in neurological disorders problematic, given that too high concentrations of MSC may result in accelerated neuroapoptosis and increased tissue damage [7]. First clinical studies have however shown that transplantation of MSC in patients with ALS is a clinically feasible and relatively safe procedure and induces immediate immunomodulatory effects [13, 38].

To gain further insights into the mechanisms underlying the protective effects of MSC CM on motor neurons, NSC-34 cells and astrocytes against STS induced apoptosis we examined involvement of both the MAPK/Erk1/2 and PI3K/Akt pathways. They represent the most

prominent survival signalling cascades that can be stimulated in neurons and astrocytes by a wide variety of growth factors or cytokines [35, 39]. Whether these pathways were involved in the protective effects of MSC transplantation in the previous *in vivo* studies in ALS mice [9, 11] has not yet been studied. Here, we demonstrated that activation of the MAPK/Erk1/2 and PI3K/Akt pathways is involved in the protective effects of MSC CM in motor neurons and astrocytes as CM-mediated protection against STS - induced apoptosis was attenuated by the MEK-1 inhibitor PD98059 and the inhibitor of PI3-K LY294002 in NSC-34 cells and astrocytes. This observation is in line with one previous study which showed that MSC CM activated phosphorylation of MAPK/Erk and/or PI3K/Akt in primary rat DRG neurons [7, 8, 35].

Our study provides the first experimental evidence for an influence of MSC CM not only on non-transgenic but also on mutant SOD1G93A transgenic astrocytes. Gene expression of neurotrophic factors was differentially regulated depending on the genotype of the astrocytes: while MSC CM induced GDNF expression was significantly higher in SOD1G93A transgenic astrocytes, CNTF mRNA expression was significantly increased upon MSC CM incubation only in non-transgenic astrocytes. This highlights the notion that disease-specific differences and interactions in neurotrophic factor expression profiles may be relevant in ALS pathogenesis [28]. We also observed that exposure of NSC-34 cells to MSC CM caused significant induction of VEGF expression which has also been implied in ALS pathophysiology and neuroprotection [40, 41]. These data further suggest that MSC induced protection of mutant transgenic astrocytes and motor neurons involve regulation of neurotrophic factor production and subsequent effects on both astrocytes themselves and motor neurons. This is in accord with a previous study which found higher GDNF immunoreactivity in the spinal cord of the motor neuron disease mouse model after intraspinal MSC injection [42]. Beneficial effects of intra-muscular injection of MSC that were genetically modified to release increased levels of GDNF have also been reported in a rat model of ALS [42, 43]. One study showed that daily applications of CNTF protected fast twitch and fast fatigable (FF) axons from synaptic vesicle loss and increased axonal resistance in

ALS mice [44]. These growth factors could also be involved in activation of MAPK/Erk1/2 and PI3K/Akt survival signalling pathways. It has been well established that GDNF promotes differentiation and survival in neurons by acting on receptor tyrosine kinases (Trk), and downstream activation of PI3-K/Akt and MAPK pathways [45, 46]. One study also showed that the effect of CNTF on the viability of muscle progenitor cells (MPCs) was mediated via the PI3-Akt pathway [47].

Besides the influence on neurotrophic factor gene expression levels, we also analysed the effects of MSC CM on astrocytes, pre-incubated with MSC CM before induction of inflammation by LPS. mRNA expression of the cytokines TNF alpha and IL-6 as well as of iNOS was pronouncedly induced by LPS as expected, with more increased reactivity of SOD1G93A transgenic astrocytes, and significantly down regulated by pre-incubation with MSC CM. Several studies have similarly described an altered expression of inflammatory molecules upon MSC treatment [48-50]. One must therefore conclude that this down regulation of pro-inflammatory factors is mediated via factors released by MSC. This modulation of inflammatory cytokines and enzymes certainly contributes to the therapeutic benefit of MSC administration which has been demonstrated *in vivo* in models of ALS, inflammatory and autoimmune disease [6, 9, 11, 48, 51, 52].

The CX3CL1 – CX3CR1 axis may play an important role in immunoregulation in several neurodegenerative diseases such as PD, AD and ALS [53-56]. In our study, first, we observed that MSC CM increased astrocytic CX3CL1 expression upon LPS treatment. Second, we demonstrated that MSC can induce the expression of the corresponding receptor CX3CR1 in SOD1G93A transgenic microglia. This was in accord with a recent study which showed that MSC exert beneficial effects on activated microglial cells of the N9 cell line in altering their phenotype and reactivity towards a more neuroprotective phenotype via release of the chemokine CX3CL1 and interaction with microglial CX3CL1 receptors (CX3CR1) [30]. Our results indicate that MSC could provide neuroprotective effects in ALS not only by direct release of CX3CL1 but also by stimulation of astrocytic CX3CL1 secretion and microglial

CX3CR1 expression. Protective effects of exogenous CX3CL1 administration has largely been shown in animal models of neuroinflammation [57, 58] but plasma levels of soluble CX3CL1 were also positively correlated with disease severity and progression in human PD patients, indicating that CX3CL1 also plays a role in chronic neurodegeneration [59]. Another study showed that enhancing CX3CR1 expression protects against microglial neurotoxicity [56].

In summary, our data indicate that MSC CM exerts a protective role against *in vitro* induced apoptosis in different cell types (primary motor neurons, NSC-34 cells and astrocytes) and maintains its protective potential in motor neurons and astrocytes carrying the ALS-causing SOD1G93A mutation. This function may involve the activation of both MAPK/Erk1/2 and PI3K/Akt pathways. The regulation of astrocytic neurotrophic factor expression and secretion certainly contributes to MSC-mediated neuroprotection. In this context, related to our observation of differentially induced GDNF and CNTF-upregulation in non-transgenic and SOD1G93A astrocytes, it is crucial to highlight and further characterize disease-related differences in basal and inducible growth factor expression levels. Last, our data confirm previously described functional modulation of astrocytes and microglia by MSC CM *in vitro*, again with distinct reactions of SOD1G93A glial cells. Future studies will need to more precisely define potential disease-related alterations in the reactivity to MSC CM and in the modulation of motor neuron-astrocyte-microglia crosstalk *in vitro* and *in vivo*. Our results support the potential of MSC to contribute to a more protective environment for degenerating neurons in ALS via regulation of growth factor, cytokine and chemokine secretion and therefore to be further evaluated as novel therapeutic approach for the treatment of ALS.

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General discussion

Most ALS patients die within 3-5 years after diagnosis due to respiratory failure (BRUIJN et al. 2004). Efficient neuroprotective pharmacological treatments have not been developed so far. Only the glutamate antagonist Riluzole has shown marginal therapeutic potential with an average prolongation of survival by 3-4 months (BENSIMON et al. 1994a). As pharmacotherapy of ALS has largely failed so far, it is urgent and important to find a new drug or a new therapy for ALS patients which more efficiently targets chronic neurodegeneration. In our first study, we evaluated the potential impact of N-ac-GLP-1 against KA induced excitotoxicity in both non-transgenic and SOD1G93A transgenic motor neurons. In our second study, mechanisms of action of cell therapy using mesenchymal stromal cells (MSC) were analysed *in vitro*.

To address these issues, an ALS primary cell culture system including motor neurons and astrocytes was established. This in vitro model represents a promising tool to study disease mechanisms but also novel therapeutic approaches for ALS therapy in vitro. Using this primary culture system, we tested GLP-1 treatment not only in ALS motor neuron monocultures, but also in motor neuron-astrocyte cocultures. In the second study, the influence of MSC CM on both non-transgenic and SOD1G93A transgenic motor neurons and astrocytes was assessed. In this culture system, distinct ALS pathomechanims can be addressed. Kainate was used to mimic excitotoxicity, staurosporine for apoptosis and LPS to model astrocyte activation. Toxicity experiments, cell viability assays, immunocytochemistry and calcium imaging experiments could easily be performed in this primary culture system.

In our first study, we co-cultured motor neurons and astrocytes from both non-transgenic and SOD1G93A transgenic astrocytes. We found significant effects of the co-culture condition on the survival of motor neurons: in our motor neuron/astrocyte co-culture system, motor neuron viability was reduced and KA-induced neuronal death was potentiated in presence of SOD1G93A transgenic astrocytes as compared to non-transgenic astrocytes. Our co-culture

system therefore presents a valuable in vitro tool to screen for novel neuroprotective compounds in ALS.

GLP-1 R expression has been described throughout the brain, including the hypothalamus, cortex, hippocampus, striatum, substania nigra and brain stem (ALVAREZ et al. 2005; KORNER et al. 2007; HAMILTON u. HOLSCHER 2009). In our study, we also found that GLP-1 receptors expressed on motor neurons, providing an avenue for direct signalling. We evaluated the potential impact of N-ac-GLP-1 against KA induced toxicity and found an independent significant neuroprotective effect of 100 nM N-ac-GLP-1. To further assess this mechanism in our culture system, we performed calcium imaging experiments. After KA application, 10 nM N-ac-GLP-1 was applied for 70 s. This led to a decrease of Ca^{2+} influx which then returned to baseline when the application of N-ac-GLP-1 together with KA was stopped. The precise mechanism how GLP-1 antagonizes pro-apoptotic stimuli in diverse cell types is unclear, but, based on previous studies it is most likely that the inhibitory effects of N-ac-GLP-1 on excitotoxicity and calcium transients are not directly mediated by interaction of N-ac-GLP-1 with ion channels but by activation of cAMP and subsequent activation of down-stream kinases and transcription factors (PERRY et al. 2002).

Systemic *in vivo* evaluation of N-ac-GLP-1 may be required to understand whether, in addition to the direct antiexcitotoxic potential shown in vitro, it can also be beneficial via counteracting the well known metabolic disturbances in ALS.

In the second study, MSC conditioned medium was found to have significant effects against staurosporine-induced apoptosis in non-transgenic and SOD1G93A motor neurons and astrocytes and also in NSC-34 cells (a hybrid cell line produced by the fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma), as shown both by immunocytocemical analysis and by the MTT cell viability assay.

Using specific inhibitors we found evidence for involvement of the PI3-K/Akt and MAPK/Erk 1/2-pathways into this protective effect. GDNF and CNTF gene expression in both non-

transgenic and SOD1G93A transgenic astrocytes was significantly increased by the presence of MSC conditioned medium. This is in accord with several in vivo studys which provide evidence for GDNF as contributor to the protective effects of MSC (SUZUKI et al. 2008; PASTOR et al. 2012) and in general of the motor neuron protective effects of GDNF and CNTF (PUN et al. 2006; SUZUKI et al. 2008). It remains noteworthy that non-transgenic and SOD1G93A astrocytes showed differences in their response to MSC CM: MSC CM induced GDNF expression was significantly higher in SOD1G93A transgenic astrocytes, CNTF mRNA expression was significantly increased upon MSC CM incubation only in non-transgenic astrocytes. These possibly disease/ genotype -specific differences certainly will require further elucidation.

In addition to induction of growth factor expression, we observed modulation of astrocytic cytokines/ enzymes by MSC CM. These effects of MSC CM indicate the release of protective factors which act across cell type barriers, which is typical for growth factors and cytokines. Further *in vitro* studies involving enlarged cytokine profiles, gene expression studies in NSC-34 cells transfeced with either wild-type or mutant SOD1 (as primary motor neurons do not provide sufficient material for quantitative real time PCR studies) and further comparison of the differential responses of non-transgenic versus SOD1G93A transgenic astrocytes and microglial cells will help to more precisely delineate the mechanisms of action of MSC in ALS.

In conclusion, our findings demonstrate that primary cell cultures from both non-transgenic and SOD1G93A transgenic mice can be used as *in vitro* tool for the evaluation of ALS mechanisms and novel therapeutic approaches. In the first study, we identified N-ac-GLP-1 as potential drug candidate for the treatment of neurodegenerative disease. In the second study, we have shown that MSC CM protected non-transgenic and SOD1G93A-transgenic

motor neurons and astrocytes as well as NSC-34 motor neuron-like cells against apoptotic cell death via modulation of astrocytic growth factor and cytokine expression.

Further *in vitro* and in *vivo* studies are required to clarify more precisely the molecular interactions and mechanisms in the context of chronic motor neuron degeneration.

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Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled “Role of non-neuronal cells in Amyotrophic Lateral Sclerosis (ALS): transgenic neuron-glia-co-cultures as *in vitro* model for the evaluation of novel therapeutic strategies”.
No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions:
Institute of Neurology
Hannover Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

[date], signature

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