Studies on the therapeutic potential of adult stem cells in the G93A animal model of amyotrophic lateral sclerosis (ALS)

Dissertation

Submitted in partial fulfillment of the requirements for the degree

Doctor rerum naturalium

– Dr. rer. nat. –

At the centre for Systems Neuroscience Hannover
Awarded by the University of Veterinary Medicine Hannover

by

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Hannover 2011
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Date of final exam: 08.04.2011

Parts of this thesis are published or submitted in following journals:

**Knippenberg S**, Thau N, Dengler R, Petri S. Significance of behavioural tests in a transgenic mouse model of amyotrophic lateral sclerosis (ALS). Behav Brain Res 213:82-87. (published)


Results of this dissertation were presented in form of posters or presentations at following congresses:

7th European ALS Congress, Research Workshop and Young Investigators Meeting (Torino, 2009):
**Intraspinal transplantation of adult stem cells in a G93A ALS transgenic mouse model**

20th International Symposium on ALS/MND (Berlin, 2009):
**Intraspinal transplantation of adult stem cells improves motor function and survival in the G93A ALS transgenic mouse model**
8th European ALS Consortium Young Investigators Meeting (London, 2010):
Significance of behavioural tests in a transgenic mouse model of amyotrophic lateral sclerosis

9th Göttingen Meeting of the German Neuroscience Society (Göttingen, 2011):
Studies on the therapeutic potential of adult stem cells in the G93A animal model of amyotrophic lateral sclerosis (ALS)

9th Meeting of the European Network for the Cure of Amyotrophic Lateral Sclerosis (Hannover, 2011):
Therapeutic potential of encapsulated human mesenchymal stem cells producing Glucagon-like-peptide 1 (GLP-1) in the SOD1 (G93A) mouse model
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1. Introduction

First described in 1869 by Jean-Martin Charcot, Amyotrophic Lateral Sclerosis (ALS) is a progressive, fatal neurodegenerative disease with marginal therapeutic options (Mulder et al., 1986). Primary hallmark of the disease is the death of motor neurons in brain and spinal cord resulting in muscular weakness and atrophy, progressive paralysis and ultimately death due to failure of respiratory muscles within 3 to 5 years after diagnosis (Hirano 1991; Rosen et al., 1993; Bradley 1995). With an incidence of 0.31 to 3.2 per 100,000 /year and prevalence of 0.8 to 8.5 per 100,000, ALS occurs predominantly after the fifth decade (Haverkamp et al., 1995; Vazquez et al., 2008). Besides age, sex seems to be a risk factor too, as several epidemiological studies show a predominance of male patients at younger age (Chio et al., 1991; Haverkamp et al., 1995; Traynor et al., 1999).

Most of the cases are sporadic (sALS) but approximately 10% are familial (fALS), whereat these forms are clinically indistinguishable. While the exact mechanisms of ALS remain elusive, about 20% of fALS are linked to mutations in the gene coding for superoxide dismutase 1 (SOD1) (Rosen et al., 1993). This protein acts as a free radical scavenger by catalyzing the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen. To date more than 100 SOD1 mutations have been identified in fALS patients but the exact mechanism of SOD1-mediated pathogenesis is still not fully understood (Lee et al., 1996; Andersen 2000). SOD1 knockout mice do not develop motor neuron disease, suggesting that SOD1 is not necessary for normal motor neuron function. Therefore the SOD1 mutations are thought to not lead to a loss of function but to a gain of neurotoxic properties of the protein (Reaume et al., 1996). Studies in transgenic SOD1 mouse models suggest complex pathways of
motor neuron death, including formation of protein aggregates, defects in axonal transport, oxidative damage, excitotoxicity and mitochondrial dysfunction (Julien 2001; Rowland et al., 2001). Current findings indicate that the mutant SOD1 has to be expressed not only in neuronal but also in non-neuronal cells to induce the disease, suggesting that interactions between motor neurons and their neighbourhood can greatly influence degeneration of motor neurons. Excitotoxicity due to astrocyte dysfunction and inflammatory processes following microglial activation apparently play a crucial role in the pathogenesis (Clement et al., 2003; Pehar et al., 2005; Di Giorgio et al., 2007).

Due to the clinical and pathological similarities to human fALS and sALS, the knowledge of ALS mechanisms has been enhanced by studies using hemizygous transgenic rodent animal models with different SOD1-mutations (Gurney 1997). These animals develop a motor neuron disease which both clinically and neuropathologically mimics human ALS. One of the most common models is the G93A mouse model. These mice overexpress a mutant allele of the human SOD1-gene carrying the Gly\(^93\) → 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 expected lifetime of these animals is 130 days of age (Gurney et al., 1994; Julien et al., 2006).

Because of the extensive use of this mouse model for preclinical assessment of neuroprotective compounds, an objective and reliable quantification of disease onset and progression is required. Otherwise false positive treatment effects could be observed as a result of inappropriate motor function tests (Barneoud et al., 1997).
Cell death in G93A mice already starts before paralysis becomes visible (Bruijn et al., 2004). Therefore most sensitive behavioural tests are required for early detection of first changes. One of the most common tests of locomotor function is the rotarod test which documents how long the animals are able to run on a rotating cylinder (Barneoud et al., 1997; Vercelli et al., 2008). Another appropriate test to detect motor impairment is the analysis of footprint patterns to measure step length and other parameters (Klapdor et al., 1997; Garbuzova-Davis et al., 2002).

To determine the most sensitive and appropriate methods for the detection of disease onset and monitoring of disease progression, in a first study we compared wild-type and transgenic G93A-SOD1 mice in several behaviour tests (scoring general condition, weighing, rotarod and footprint analyses).

Because of the complex pathophysiology of ALS, development of effective pharmacologic therapies is challenging. To date Riluzole is the only FDA-approved treatment for ALS, but its effect on progression and survival is generously described as modest (Gurney et al., 1998; Bruijn et al., 2004). Recent studies have demonstrated stem cell transplantation as a potential treatment for a variety of neurological diseases. Stem cells are undifferentiated cells that have the capacity of self-renewal and are able to differentiate into multiple mature cell types. They can be isolated from a range of different tissues, including blastocyst-stage embryos and foetal and adult tissue. Embryonic stem (ES) cells have the ability to differentiate into all cell lineages and after transplantation in the adult central nervous system (CNS) they can adopt a cellular fate that is appropriate to the transplanted region (Nagy et al., 1993; Wang et al., 2007). Even though they typically proliferate in an
undifferentiated state for long time, there is a high risk of tumor formation after transplantation of ES cells (Bjorklund et al., 2002).

Adult stem cells still exhibit the property of self-renewal and multi-potentiality, and differentiation is apparently not restricted to derivatives of the host tissue. Fewer ethical and technical problems as well as low risk for tumor formation make adult stem cells like mesenchymal (MSCs) and hematopoietic stem cells (HSCs) promising candidates in the search for novel therapeutic agents. Both bone marrow and umbilical cord blood are sources for MSCs and HSCs. Compared to bone marrow derived stem cells, cells from human umbilical cord blood are less immunogenic and are easily available (Dasari et al., 2007). Several studies indicated the neuroprotective potential of human umbilical cord blood cells (hUCBCs) in animal models of neurodegenerative disorders (Ende et al., 2000; Chen et al., 2007; Garbuzova-Davis et al., 2009). Preliminary clinical trials with hematopoietic stem cells have already been performed but further preclinical and clinical evaluation is mandatory (Janson et al., 2001; Silani et al., 2003; Mazzini et al., 2009). Therefore several more preclinical studies need to be done to let stem cell treatment become a reality.

There are two different ways of stem cell therapy in neurological diseases, dependent on the specific pathology: Direct replacement of lost or degenerating neurons or transplantation of stem cells due to their neuroprotective effects, e.g. release of neurotrophic factors. In neurodegenerative disorders like Parkinson’s disease (PD), direct cell replacement may be effective. PD leads to the degeneration of nigrostriatal dopaminergic neurons. After intrastriatal transplantation of human fetal mesencephalic tissue, grafted dopaminergic neurons were able to survive and
reinnervate the striatum followed by a normalization of striatal dopamine release (Kordower et al., 1995; Piccini et al., 1999). These studies showed that grafted dopaminergic neurons can become functionally integrated into neuronal circuitries in the brain (Lindvall et al., 2004). ALS, on the other hand, causes widespread degeneration of upper and lower motor neurons in the primary motor cortex, brain stem and spinal cord as well as dysfunction of other cell types like glial cells and muscles (Hall et al., 1998; Barbeito et al., 2004; Cassina et al., 2005). While direct cell replacement seems suitable when neurodegeneration affects one specific cell type and is restricted to one brain region such as in PD, this goal is much more challenging in diseases like ALS. Besides the need to differentiate into motor neurons, recognize and connect to the host tissue, grafted cells would have to extend large axonal processes to reinnervate skeletal muscles over long distances. Furthermore, it is known by now that ALS does not only affect the motor system. Intense activation of astrocytes and microglia is observed in both ALS patients and transgenic ALS mouse models, whereby it is still uncertain whether this activation is a cause of neuronal loss or is secondary to neuronal loss (Hall et al., 1998; Barbeito et al., 2004). As mentioned above, evidence for neuronal-glial interactions in ALS pathogenesis was shown in several studies. The “neighbourhood” of motor neurons plays a crucial role for their survival. In chimeric mice harbouring SOD1 mutant motor neurons and wild-type non-neuronal cells, survival of motor neurons was significantly prolonged when they were surrounded by wild-type glial cells (Clement et al., 2003). The fact that healthy glial cell can protect degenerating motor neurons argues for the use of stem cells in order to generate such protective environment rather than to replace dead motor neurons.
Thereby secretion of growth factors or other trophic factors is one of several possibilities how stem cells could influence the affected tissue in a positive way. It was shown that growth factors such as insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF) can delay the progression of symptoms in ALS mice (Azzouz et al., 2004; Dobrowolny et al., 2005). Furthermore, glial cell-derived neurotrophic factor (GDNF), one of the most potent neurotrophic factors, is produced in glial cells of the CNS and peripheral nervous system (PNS). Stem cells could be both a source of glial replacement and trophic factor delivery (Klein et al., 2005).

Not only the cell type best suited for transplantation but also the best way of cell administration needs further evaluation. Intravenous administration of hematopoietic stem cells in mutant SOD1 mice delayed onset of symptoms and death and showed benefits in hind-limb extension and gait but the donor cells were also detected in several other organs such as spleen, kidneys, liver, lungs and heart (Ende et al., 2000; Sanberg et al., 2005).

To directly improve the non-neuronal environment of motor neurons, intraspinal delivery seems more appropriate because of the disability of stem cells and growth factors to cross the blood-brain-barrier (Nayak et al., 2006). Bringing stem cells directly to the affected tissue enables them not only to unfold their therapeutic potential by secretion of neurotrophic factors but also possibly by differentiation into glial cells. As previously reported, intraspinal surgery can be safely performed in ALS patients (Mazzini et al., 2003) thus considered to be the most effective method for transplantation in order to reduce the amount of injected cells and to avoid bringing stem cells into other organs.
In a second study we therefore established a method of intraspinal injection of human umbilical cord blood cells (hUCBCs) in the G93A mouse model of ALS. We compared two different treatment time points to investigate whether early treatment at the presymptomatic stage (day 40) is more efficient than treatment after symptom onset (day 90). Survival time of G93A-ALS transgenic mice transplanted with either hUCBCs or vehicle at both treatment time points was measured and behavioural assessment (scoring of general condition, weighing and motor function tests) was continuously performed starting at week 11. Additional animals were sacrificed at day 110 to test the effect of stem cells on survival of motor neurons, inflammation or secretion of growth factors.
2. Manuscript I

Published in Behavioural Brain Research November 2010

Significance of behavioural tests in a transgenic mouse model of amyotrophic lateral sclerosis (ALS)

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Preface- about this manuscript

The G93A mouse model of amyotrophic lateral sclerosis is widely used in preclinical trials. For the interpretation of these preclinical trials, it is important to assess neurological function as sensitively as possible. In the first part of the dissertation, five different parameters were compared with respect to their significance to detect symptom onset and to monitor disease progression in transgenic G93A ALS mice. Rotarod testing and step length analyses detected first deficits and are therefore the most effective methods to detect symptom onset and potential treatment induced improvements.

The text of the original publication can be found here: Behavioural Brain Research, Volume 213(1), Pages 82-87.
3. Manuscript II

Intraspinal injection of human umbilical cord blood-derived cells is neuroprotective in a transgenic mouse model of amyotrophic lateral sclerosis

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3.1 Abstract

Background: Amyotrophic Lateral Sclerosis (ALS) is characterized by progressive degeneration of motor neurons in the spinal cord, brain stem and motor cortex and has only marginal therapeutic options. Adult stem cells have recently come into the focus of neurological research. While replacement of motor neurons by stem cells currently appears not feasible, there is evidence that non-neuronal cells can be neuroprotective.

Objective: Therefore, we evaluated the effects of direct intraspinal administration of human umbilical cord blood cells in a G93A transgenic mouse model of ALS before (day 40) and after symptom onset (day 90).

Methods: Treatment effects were assessed by survival analysis, behavioral tests, histological and biochemical analyses.

Results: Treatment at early stages increased survival, led to significant improvements in motor performance and significantly reduced motor neuron loss and astrogliosis in the spinal cord. Interestingly females tended to respond better to treatment than males.

Conclusion: This study confirms the neuroprotective potential of human umbilical cord blood cells and encourages further investigations.
3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult onset motor neuron disorder leading to progressive degeneration of motor neurons in brain and spinal cord. Most patients die within 3-5 years after diagnosis due to respiratory failure [1]. Approximately 5 – 10% of ALS cases are familial. About 20% of these familial cases are due to dominantly inherited mutations in the gene coding for superoxide dismutase 1 (SOD1) [2]. Based on this discovery, a transgenic mouse model was generated by over-expression of a mutant form of SOD1 carrying the Gly$_{93}$$\rightarrow$Ala (G93A) substitution. In this model, progressive death of motor neurons occurs in the ventral horn of the spinal cord, and the mice develop a phenotype mimicking human ALS [3].

So far riluzole is the only drug with marginal therapeutic efficacy, attributed to its anti-glutamatergic properties [4], while a large variety of other supposedly neuroprotective compounds failed to show significant effects in ALS patients. Therefore, current research focuses more on experimental treatment approaches including cellular therapy. While direct replacement of lost motor neurons does not appear feasible to date, recent evidence suggests that non-neuronal cells can unfold neuroprotective capacities in ALS spinal cord. Motor neuron degeneration in ALS must be considered a non cell-autonomous process. In chimeric mice composed of mixtures of wild-type cells and cells expressing human mutant SOD1, wild-type non-neuronal cells prolonged survival of mutated motor neurons (Clement et al., 2003). In vitro studies in motor neuron- astrocyte co-cultures similarly showed that glial cells with an ALS genotype are toxic for motor neurons while wild-type glial cells are neuroprotective
The nature of these effects is not fully understood although secretion of growth factors such as IGF 1 or GDNF was assumed to play a role [8]. Following the idea of neuroprotection rather than motor neuron replacement, several studies showed beneficial effects of stem and progenitor cells in ALS animal models. Human neural stem cells (hNSCs) reduced motor neuron loss and increased survival in SOD1 rats due to increased levels of GDNF and BDNF after intraspinal transplantation [9]. Intraspinal injection of human neuron-like cells (hNT neurons) and of lineage-restricted astrocyte precursor cells into the spinal cord of transgenic ALS mice delayed onset and improved motor function [10, 11].

Human umbilical cord blood cells (hUCBCs) represent promising candidates for cell therapy because of their differentiation potential and availability. The procedure of hUCBC collection is easy without risks for mother or baby, and use of these cells is associated with few ethical issues [12]. Intravenous administration of hUCBCs in G93A-mice has previously been shown to delay symptom onset and death and to improve motor performance, probably due to systemic effects rather than a direct impact on degenerating motor neurons [13]. Intrathecal administration of hUCBCs, on the other hand, did not significantly influence onset and survival in transgenic ALS mice [14]. Due to the blood-brain-barrier which is not compromised in chronic neurodegenerative diseases such as ALS, local administration of cell therapy appears to be the most efficient administration mode.

We injected hUCBCs intraspinally in a G93A transgenic mouse model of ALS. Different injection time points were chosen to verify whether treatment before symptom onset (d40) was more efficient than treatment after symptom onset (d90).
Survival time of G93A-ALS transgenic mice transplanted with either hUCBCs or vehicle (sodium chloride) at both time points was recorded. The locomotor activity was determined weekly (rotarod and footprint analyses) as well as changes in weight. Moreover, mice were monitored daily for general condition. Additional animals of all treatment groups were sacrificed at day 110 (d110) to determine whether the treatment had an effect on motor neuron loss, inflammation or secretion of growth factors.

3.3 Material and methods

Animals
All experiments were carried out in accordance with the internationally accepted principles in the care and use of experimental animals and were approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the local government. G93A transgenic familial ALS mice (high copy number; B6SJLTg (SOD1-G93A)1Gur/J) (Gurney et al. 1994) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). These mice over-express the human mutant SOD1 allele containing the Gly$_{93}$ $\rightarrow$ 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 tail tissue. Mice were housed under controlled conditions with free access to food and water. Animals of the same sex were kept in groups of up to five animals in Makrolon cages type II (UNO, Zevenaar, Netherlands). Males were kept solitary in the same cage type only when they were also used for breeding.
Isolation of CD34+ cells from human umbilical cord blood

Isolation of CD34+ cells was performed by magnetic bead-associated cell sorting (MACS) from human umbilical cord blood. Thus, cord blood was obtained from healthy pregnant woman with non-complicated pregnancies at spontaneous term-deliveries (38-40 weeks of gestation) or by Cesarean section after obtaining informed written consent, respectively, as approved by the Institutional Review Board, project #3037 on June 17th, 2006. The cord blood was immediately transferred into EDTA-monovettes on ice and separated via Ficoll density gradient centrifugation. Following removal of the lymphocyte interphase, further separation was performed via the CD34+-associated MACS technology. The isolated population was analyzed for an appropriate CD34+ hematopoietic stem cell enrichment by flow cytometry. Thereafter, the separated primary stem cell population was cultured and expanded in the presence of a distinct growth medium (IMDM, 10% FCS, 1% Pen/Strep, SCF, IL-3, IL-6, Flt-3) at 5% CO2 and constant 37°C for 7 up to 14 days with a medium change every third day. Before surgery, the expanded cells (further termed hUCBCs) were washed twice with a 0.9% (w/v) sodium chloride solution, and resuspended at a density of 100,000 cells/µl. In order to guarantee a sufficient bulk of cells we used a mean cell concentration based on earlier studies [15]; [16].

Surgery

Starting 3 days before surgery, animals received cyclosporin (25mg/kg/day) for immunosuppression which was continued throughout the study. For surgery, animals were anesthetized by a combination of ketamine (0.1ml/100g, 100mg/kg), rompune (0.01ml/100g, 2mg/kg) and midazolame (0.05ml/100g, 0.5mg/kg), prepared under
sterile conditions with 0.9% sodium chloride. Anesthesia was administered intraperitoneally, adjusted to the body weight (0.1ml/10g). Duration of anesthesia was up to 60 minutes, which was sufficient for the surgery. Depth of anesthesia was controlled via the toe-and eyelid-reflex.

An area of 3 x 2 cm on the back of the animals was shaved and disinfected. Eye ointment protected the eyes against dehydration. Animals were fixed in ventral position by tape and skin was disclosed longitudinally. Laminectomy was performed at vertebral bodies Th12/L1 with sharp scissors to expose the spinal cord at level L1-L4. For better visibility, the backbone adjacent to the exposed spinal cord was fixed with a clamp, which again was fixed in a stereotactic frame.

A Hamilton syringe with an elongated glass capillary on top (50-80µm diameter) was used for injection. The syringe was filled with 0.9% sodium chloride before 2µl stem cell solution was charged. 100,000 cells per side in a volume of 1µl were administered bilaterally into the lumbar region of the spinal cord. 0.9% sodium chloride was used for vehicle injections. The syringe was clamped into the arm of the stereotactic frame and inserted 1mm in ventral direction from the dorsal surface of the spinal cord, followed by slow injection of the cells over a period of three minutes. After a period of additional two minutes the syringe then was removed slowly to prevent reflux of inserted cells.

After wound closure, animals received a single dose of carprofene (5mg/kg subcutaneous) and metamizole via drinking water (200mg/kg/day) for 3 postoperative days for analgesia.
Animals were treated with either hUCBC solution or 0.9% sodium chloride (NaCl) as vehicle (table 1). Equal numbers of animals of each litter were attributed randomly to either hUCBC or vehicle treatment. All treatment groups were gender mixed, half males and half females. 30 animals per treatment group received injections at the presymptomatic stage (d40) or at the symptomatic stage (d90), respectively. 10 animals of each group were sacrificed at d110 for immunohistochemistry and protein analyses (western blot). The remaining 20 animals of each group were monitored for behavioral and survival analyses (table 1).

Table 1: Experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>G93A+vehicle</th>
<th>G93A+vehicle</th>
<th>G93A+hUCBC</th>
<th>G93A+hUCBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>D40</td>
<td>18</td>
<td>18</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Survival</td>
<td>(7♀/11♂)</td>
<td>(8♀/10♂)</td>
<td>(10♀/10♂)</td>
<td>(10♀/10♂)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>5 (3♀/2♂)</td>
<td>5 (3♀/2♂)</td>
<td>5 (3♀/2♂)</td>
<td>5 (3♀/2♂)</td>
</tr>
<tr>
<td>Protein analysis</td>
<td>5 (2♀/3♂)</td>
<td>5 (2♀/3♂)</td>
<td>5 (2♀/3♂)</td>
<td>5 (2♀/3♂)</td>
</tr>
</tbody>
</table>

Detection of transplanted cells via eGFP-Labeling (6 animals)

To assess the survival of transplanted hUCBCs in the spinal cord of mice over time, cells were transduced with a lentiviral vector encoding enhanced green fluorescent protein (RRL.PPT.SF.eGFP.pre) [17]. Thawed viral supernatants were bound to RetroNectin (Takara Bio, Japan) coated plates before cells were added to conduct infection. After four days of particle exposure eGFP expression was determined by
flow cytometry (FACScalibur, Becton-Dickinson, Heidelberg, Germany) and cells were transplanted resolved in sodium chloride at a density of 100,000 cells/µl. 6 wild-type animals were injected with these eGFP-labeled hUCBCs and sacrificed either at day 1, week 1, 2, 3, 6 or 9 after surgery. Spinal cords were removed for detection of eGFP+ cells by fluorescence microscopy.

Behavioral assessments and survival
After surgery, experimenters were blinded to treatment groups so that animals were objectively monitored throughout the whole period of behavioral assessment.

General condition
For weekly assessment of general condition from d100 (week 15) as previously described [18], we used a behavioral score system based on the score developed by Vercelli et al. [15] from 1 to 5 defined as follows:
5: healthy without any symptoms of paralysis,
4: slight signs of destabilized gait and paralysis of the hind limbs,
3: obvious paralysis and destabilized gait,
2: fully developed paralysis of the hind limbs, animals only crawl on the forelimbs,
1: fully developed paralysis of the hind limbs, animals predominantly lie on the side and/or are not able to straighten up after turning them on the back or lost more than 20% of their starting weight.
When animals reached a score of 2, macerated food was given daily for easy access of food and hydration. Reaching a score of 1, the animals were euthanized.

Evaluation of onset and survival
Day of onset was set as the first day the animals reached a score of 4 in the daily monitoring. Animals were killed when they reached a score of 1 and this age (in days) was recorded as survival time.

**Weight**

We recorded the weight of the animals weekly, beginning at 10 weeks of age, using a normal digital balance ranging up to 800g in 0.1g steps. From week 11 we weighed them twice a week, from week 16 they were weighed three times a week as previously described [18].

**Rotarod**

Beginning at 11 weeks of age, we analyzed motor function using a rotarod apparatus from IITC (IITC Life Science Inc. California). After an adaptation period of 5 days the test was performed weekly. Mice had to remain on the rotating cylinders for up to 180 s with an increasing speed up to 18 rpm. Rotarod test was performed as previously described [18]

**Footprint analyses**

Footprint analyses for step length and runtime were performed weekly, starting the same week as rotarod tests performance [18]. The hind feet were dipped into black fingerprint and animals were placed on a gangway covered with conventional masking tape. Footprints were analyzed with respect to the step length using the FOOTPRINTS software (Version 1.22 by K. Klapdor and B. Dulfer [19]). In addition, the time animals needed to run along the track (50cm) was measured.
Histological evaluation

At d110, five animals of each group (see table 1) were sacrificed by an overdose of the same anesthetics (ketamine (0.1ml/100g, 100mg/kg), rompune (0.01ml/100g, 2mg/kg) and midazolame (0.05ml/100g, 0.5mg/kg)) used for the surgeries. After transcardial perfusion with 25ml 4% paraformaldehyde (PFA) in PBS the lumbar part of the spinal cord was removed. Postfixation for 1h in 4% PFA was followed by cryopreservation in 30% sucrose for several days. After embedding in cryo protection compound (Sakura Finetek Germany GmbH, Staufen, Germany), tissue was stored at -80°C and sections of 12µm were cut on a cryostat. 120 to 160 sections, depending on the size of the removed spinal cord, were distributed onto 20 object slides.

In addition to the hUCBC or vehicle treated animals, five wild-type mice and five untreated G93A mice were sacrificed at d40 and d90, respectively, to assess motor neuron loss and astrocytosis at the respective surgery time points.

Motor neuron survival

One slide containing 6-8 spinal cord sections of each animal was stained with 0.2% Thionin, dehydrated in graded ethanols and xylene (Mallinckrodt Baker B.V., Deventer, Netherlands) and coverslipped with Eukitt quick hardening mounting medium (Sigma-Aldrich, Steinheim, Germany). At 20x magnification in an Olympus BX61 microscope, cells in the ventral horn region with a diameter >200µm² were defined as motor neurons, according to Chen et al., who determined α-motor neurons as cells with cell body areas ranging from 200 to 1100µm² [20] and counted, using cell* software (Olympus, Hamburg, Germany). Only intact ventral horn regions
were used for counting so that, depending on the quality of the section, 5 to 14 ventral horn regions were counted for motor neuron numbers and the mean was used for statistical evaluation.

**Immunohistochemistry**

For immunohistochemical staining, slides were immersed in PBS with 1mM Tris-HCl, blocked with peroxidase block (DakoCytomation, Glostrup, Denmark) for 10-15min and 5% goat serum in PBS with 0.3% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 30 min to 1 h followed by overnight incubation at 4°C with primary antibodies specific for GFAP (1:6000; polyclonal rabbit anti-glial fibrillary acidic protein; DakoCytomation, Glostrup, Denmark) diluted in 1% goat serum in PBS with 0.006% Triton X-100 or for 2 h at room temperature with primary antibodies specific for HSP70 (1:500; monoclonal mouse anti heat shock protein 70; Invitrogen, Darmstadt, Germany) diluted in 5% goat serum in PBS with 0.3% Triton X-100.

Secondary HRP antibody anti-rabbit or anti-mouse respectively (EnVision+System-HRP (AEC+); DakoCytomation, Glostrup, Denmark) was added for 30min, followed by 20-25min incubation in chromogene substrate (EnVision+System-HRP (AEC); Dako-Cytomation, Glostrup, Denmark). Slides were covered with Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).

For quantitative analyses of astrocytosis and heat shock response, the percentage of stained area of the ventral horn was determined using the phase analysis tool of cell* software in pictures taken at 20x magnification by an Olympus Bx61 microscope.

To determine the differentiation of transplanted eGFP labeled cells, slides were immersed in PBS with 1mM Tris-HCl, blocked with peroxidase block
(DakoCytomation, Glostrup, Denmark) for 10min and 5% goat serum in PBS with 0.3% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 1 h followed by overnight incubation at 4°C with primary antibodies specific for GFAP (1:50; polyclonal rabbit anti-glial fibrillary acidic protein; DakoCytomation, Glostrup, Denmark), MBP (1:100; polyclonal rabbit anti-myelin basic protein; Millipore, Bedford, USA), Iba1 (1:100; monoclonal mouse anti-ionized calcium-binding adaptor molecule 1; Abcam, Cambridge, UK) and APC (1:300; monoclonal mouse anti-adenomatous polyposis Coli; Merck, Darmstadt, Germany) diluted in 1% goat serum in PBS with 0.006% Triton X-100. Secondary anti-rabbit or anti-mouse Alexa Fluor 555 antibody (1:500; anti-IgG (H+L); Invitrogen, Darmstadt, Germany) was added for 45min. Slides were then counterstained with the fluorescent DNA dye DAPI (10mg/ml; Invitrogen, Darmstadt, Germany).

**Western blot analysis**

At d110, five animals of each group were sacrificed by cervical dislocation. Spinal cords were removed, quick-frozen in liquid nitrogen and stored at -80°C. For determination of protein concentration tissues were homogenized in Tris-HCl lysis buffer. Concentrations were determined using Protein Assay Reagent A, B and S (BioRad Laboratories, Hercules, USA) according to the manufacturer’s instructions. Protein homogenates (80µg of protein) were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA) by standard procedures. Membranes were blocked with tris buffered saline containing 5% conventional dry milk (Sucofin, Zeven, Germany) and then incubated overnight at
4°C with each of the following primary antibodies: BDNF (1:300; polyclonal rabbit; 
abcam, Cambridge, UK), CNTF (1.300; monoclonal mouse; Millipore, Bedford, USA), 
GDNF (1:300; polyclonal rabbit; abcam, Cambridge UK), IGF-1 (1:300; monoclonal 
mouse; upstate, USA), VEGF (1:300; monoclonal mouse; abcam, Cambridge, UK) 
and beta-actin (1:1000; abcam, Cambridge, UK) in tris buffered saline containing 5% 
milk powder (for GDNF and beta-actin) or only tris buffered saline (for BDNF, CNTF, 
IGF-1 and VEGF). After washing, membranes were incubated for 1 h at room 
temperature with suitable horseradish peroxidase-conjugated secondary antibodies 
(1:1000; R&D System, Minneapolis, USA) respectively. After reacting with 
chemiluminescent substrate (BDNF, CNTF, GDNF, IGF-1, VEGF: SuperSignal West 
Femto Maximum Sensitivity Substrate; Thermo Scientific, Rockford, USA; beta-actin: 
ECL kit; Amersham, Buckingham, UK), signals were detected by a 
Chemoluminescence Imager (Intas Science Instruments, Göttingen, Germany). 
Membranes were stripped with Glycine-HCl and reprobed up to three times. 
Quantification was done by densitometry using Image J software. Values were 
normalized to beta-actin.

Statistics

Behavioral data were analyzed by two-way ANOVA in order to evaluate time 
evolution of the different parameters. In case of significant parameter interaction (p< 
0.05), comparison with a Bonferroni posthoc test was performed. Survival was 
analyzed by the Gehan- Breslow- Wilcoxon test with a significance level of p< 0.05. 
Mean survival, histological and western blot data were analyzed by unpaired t-test. 
Histological data for Nissl and GFAP staining were normalized to Wt values at d40
and d90. Data from visible symptoms at d110 of d40 treated animals were compared with Fisher’s exact test. All data are presented as mean ± SEM and significance level was set as p< 0.05.

3.4 Results

**Intraspinal injection of hUCBCs was safe and well tolerable**

The procedure of intraspinal transplantation was generally well tolerated. Within few hours postoperatively animals recovered and were able to straighten up on their hindlimbs. No motor impairment was visible after surgery. Only one animal had to be euthanatized due to persisting paralysis following surgery.

**eGFP-labeled hUCBCs could be detected in the ventral horn up to 9 weeks after transplantation**

eGFP labeled hUCBCs were detected up to 9 weeks after intraspinal transplantation. One day after surgery hUCBCs were visible in the branch canal (Fig. 1A). After one and two weeks, eGFP+ cells showed a round cell body. At later time points (week 3, 6 and 9 after surgery), eGFP+ cells were still detectable in the ventral horn, now displaying a more elongate phenotype with the eGFP signal mostly localized in the dendrites (Fig. 1B). Staining with antibodies against astrocyte-, microglia- and oligodendrocyte-markers showed only occasional cross-immunoreactivity with oligodendrocyte-markers but no co-localization for the majority of transplanted hUCBCs (data not shown).
Figure 1. GFP labeled hUCBCs were detectable up to 9 weeks after transplantation in wild type mice. One day after surgery, eGFP+ cells (green) with a round cell body were visible in the branch canal (A). After 9 weeks cells displayed a more elongate phenotype with the eGFP signal mostly localized in the dendrites (B). The square top left in (B) shows an enhanced section the picture. Cell nuclei were visualized by DAPI staining (blue). Scale bars, 200 µm.

Presymptomatic hUCBC treatment improved locomotor activity and survival

General condition

Animals injected with hUCBCs at d40 showed a slighter decrease in the general condition compared to vehicle treated animals throughout the whole test duration (Fig. 2A). This shift of decrease in general condition was 5 days on average.

In female mice treated with hUCBCs at d40, differences were more obvious (Fig. 2B). hUCBC-treated females showed the first decrease in general condition 15 days later
than vehicle-treated females. Differences to these controls became significant from d116 to 126 (week 17 to 19; Two-way ANOVA, p<0.05). In animals injected at d90, only marginal but not significant hUCBC-induced changes were observed (Fig 2C & D).

**Figure 2.** Effects of hUCBC treatment on general condition. Presymptomatic treatment with hUCBCs ("hUCBC") improved general condition compared to vehicle-treatment ("NaCl") in mixed gender groups (A) and females only (B). General condition of day 90 treated mixed gender groups (C) and females only (D) was marginally altered. A: Data are mean ± SE of 18 animals per treatment group (NaCl: 8♂/10♀; hUCBC: 7♂/11♀). B: Data are mean ± SEM of 10 (NaCl) and 11 (hUCBC) treated females. C: Data are mean ± SE of 20 animals per treatment group (NaCl: 10♂/10♀; hUCBC: 10♂/10♀). D: Data are mean ± SEM of 10 NaCl- and 10 hUCBC-treated females. (*) p < 0.05. A & B: two-way ANOVA, followed by Bonferroni post-test.
**Survival**

While treatment with hUCBCs at the symptomatic stage only led to a small and not significant increase in survival (Fig. 3D – F), average survival time of d40 hUCBC-treated animals was significantly prolonged as compared to vehicle-treated mice (134 vs. 128 days; unpaired t-test, p<0.05) (Fig. 3A & B). Survival time of d40 hUCBC treated females was even prolonged for 12 days on average compared to vehicle-treated females (137 vs. 125 days; unpaired t-test, p<0.05 and Gehan-Breslow-Wilcoxon test, p<0.05) (Fig. 3A & C).

**Figure 3.** Effects of hUCBC treatment on survival. Presymptomatic treatment with hUCBCs ("hUCBC") prolonged survival compared to vehicle-treatment ("NaCl") in mixed gender groups (A & B) and females only (A & C). Survival of day 90 treated mixed gender groups (D & E) and females only (D & F) was not altered. A (all) & B: Data are mean ± SE of 18 animals per treatment group (NaCl: 8♂/10♀; hUCBC: 7♂/11♀). A (♀) & C: Data are mean ± SEM of 10 (NaCl) and 11 (hUCBC) treated females. D (all) & E: Data are mean ± SE of 20 animals per treatment group (NaCl: 10♂/10♀; hUCBC: 10♂/10♀). (D (♀) & F) Data are mean ± SEM of 10 NaCl- and 10 hUCBC-treated females. (*) p < 0.05. A & D: unpaired t-test. B, C, E & F: Kaplan-Meyer analysis, followed by Gehan-Breslow-Wilcoxon test.
**Weight**

Treatment with hUCBCs at d90 had no effect on weight loss. Animals treated at d40 showed less weight loss compared to controls (Data not shown). Differences to vehicle-treated animals were significant in week 17 (Two-way ANOVA, p<0.05). No gender differences appeared in the treatment groups at both time points.

**Rotarod**

Almost all animals learned to run on the rotating cylinders and the best value was around 96-97% for all groups. Treatment at d90 did not induce changes in the performance of gender mixed groups (Data not shown). Treatment at the presymptomatic stage on the other hand led to a delayed deterioration of rotarod performance in hUCBC-treated mice compared to vehicle-treated controls (Fig. 4A). Significant differences to controls occurred in week 17 (Two-way ANOVA, p<0.01) and 0% of performance was reached one week later than in control animals.

When female G93A mice were analyzed separately, decrease of performance was milder in hUCBC treated mice than in controls until week 17 (Fig. 4B) and became significantly different in the same week (Two-way ANOVA, p<0.001). There was a general shift of deterioration of approximately two weeks.

**Footprint analyses – step length**

In mice injected at d90, vehicle-treated controls showed the first decrease in step length at week 17, while hUCBC-treated animals started to worsen one week later. At week 19 the difference turned out to be significant (Two-way ANOVA, p<0.01) (Data not shown). While step length was not measureable at week 22 in d90 vehicle
treated animals, hUCBC-treated mice still reached at least 7% of their maximum value.

d40 vehicle-injected animals decreased in step length already in week 15, animals treated with hUCBCs one week later (Fig. 4C). A significant difference appeared at week 17 when controls reached only two-thirds of the length of hUCBC treated animals (Two-Way-ANOVA, p<0.01). While deterioration of performance was delayed by one week by hUCBC treatment, for the last 4 weeks of observation, no differences between the groups were visible any more.

In females treated with hUCBCs at d40, decrease in footprint length started 3 weeks later (week 17 vs. 14) than in the vehicle-control group and significances to control females appeared in week 17 and 18 (Two-way ANOVA, p<0.01 & p<0.05) (Fig. 4D). The time point when no step lengths were measurable any more occurred two weeks later in hUCBC treated females than in vehicle-treated females.

**Footprint analyses – runtime**

First increase of runtime along the footprint track occurred at week 17 in d90 vehicle-treated mice and at week 18 in hUCBC-treated animals (Data not shown). At week 19 the difference between these groups became significant (Two-way ANOVA, p<0.001) and remained significant in week 20 (Two-way ANOVA, p<0.05).

Treatment with hUCBCs at the presymptomatic stage delayed deterioration by two weeks compared to vehicle-controls (week 18 vs. 16) (Fig. 4E). Significant differences between hUCBC treatment and control mice were visible in week 18 (Two-way ANOVA, p<0.01). Similar differences were visible when female mice were analyzed separately (Fig. 4F).
Figure 4. Effects of presymptomatic hUCBC treatment on rotarod performance and footprint analyses. Presymptomatic hUCBC-treatment (“hUCBC”) improved rotarod performance (A & B), step length (C & D) and runtime (E & F) compared to vehicle-treatment (“NaCl”) in mixed gender groups (A, C & E) and females only (B, D & F). A, C & E: Data are mean ± SE of 18 animals per treatment group (NaCl: 8♂/10♀; hUCBC: 7♂/11♀). B, D & F: Data are mean ± SEM of 10 (NaCl) and 11 (hUCBC) treated females. (*) p < 0.05; (**) p < 0.01; (***) p < 0.001, two-way ANOVA, followed by Bonferroni post-test.
Reduction of motor neuron loss and astrocytosis by presymptomatic hUCBC treatment

At the time point of euthanasia for histological assessment (d110), 8 out of 10 control animals already showed visible paralysis while only 2 of 10 animals from the hUCBC-treated group showed apparent motor impairment (Fishers exact test, p< 0.05) (Fig. 5).

![Symptoms at day 110 in presymptomatic treated groups. 8 (4♂/4♀) out of 10 control animals (5♂/5♀) ("NaCl") showed visible paralysis while in hUCBC treated animals ("hUCBC") symptoms occurred only in 2 (1♂/1♀) of 10 animals (5♂/5♀). (*) p < 0.05. Fisher exact test.](image)

Motor neuron survival

When comparing untreated G93A and wild-type mice at d40, there was no difference in motor neuron numbers. Motor neuron loss became more evident at 90 days of age: approximately 33% reduction of motor neurons was detected in spinal cord of G93A mice compared to wild-type animals (Fig. 6A).
After treatment with hUCBCs at d90, motor neuron loss was only slightly reduced at d110. While in vehicle treated controls motor neuron numbers were reduced to one third of the value of wild-type mice at d90, animals treated with hUCBCs showed a less pronounced loss of motor neurons. In animals treated with hUCBCs at d40, on the other hand, motor neuron loss was ameliorated by nearly 50% compared to vehicle treated animals at d110 (Fig 6A; unpaired t-test, p<0.01; Fig 7A & B).

**Immunohistochemistry**

Astrocytosis in untreated G93A mice (as detected by GFAP immunohistochemistry) was already obvious in the presymptomatic stage (Fig. 6B; 50% increase as compared to age-matched wild type mice) and also in untreated early symptomatic G93A mice at d90 (33% increase).

Analysis of hUCBC- and vehicle-treated G93A mice revealed a decrease in astrocyte activation induced by hUCBC treatment both when treatment was done at d40 and d90. The difference was significant only in G93A mice treated with hUCBCs before symptom onset: this led to a reduction of astrocytosis by 40% in hUCBC-treated animals compared to vehicle treated controls (Fig 6B; unpaired t-test, p<0.05; Fig 7C & D).

In hUCBC-treated animals of both treatment time points there was a higher amount of HSP70 staining than in controls (Fig 8). Differences did not reach statistical significance.
Figure 6. Evaluation of motor neuron numbers (A) and astrocytosis (B) in ventral horn regions of lumbar spinal cord. Motor neuron numbers were not significantly different in wild types (“Wt”- dashed bars) and transgenic SOD1 mice (“G93A”- black bars) at day 40 but at day 90 motor neuron numbers decreased to 66% from wild types. At day 110 of age, animals treated with hUCBC at d40 (“hUCBC”- white bars) showed significantly higher motor neuron numbers than vehicle-treated controls (“NaCl”- grey bars). HUCBC treatment at the symptomatic stage led to a not significant reduction of motor neuron loss at day 110 (A). Astrocytosis was increased at the presymptomatic and symptomatic stage in transgenic SOD1 mice (“G93A”- black bars) compared to wild types (“Wt”- dashed bars). At day 110 of age, astrocytosis was reduced in animals treated with hUCBC at d40 and d90 (“hUCBC”- white bars).
bars) compared to controls ("NaCl"- grey bars). Differences were significant only between d40 treated
groups (B). Data are mean ± SEM of 5 animals (2♂/3♀) per group. Values were normalized to means
of wild types. (*) p < 0.05, p < 0.01, unpaired t-test.

Figure 7. Motor neuron survival and astrocytosis in ventral horn regions of lumbar spinal cord of day
40 treated animals. Treatment with hUCBCs at the presymptomatic stage caused reduction of motor
neuron degeneration (B) compared to controls (A). Astrocytosis was reduced in hUCBC treated
animals (D) compared to controls (C) as well. Scale bars, 200 µm.

Figure 8. Heat shock response in ventral horn regions of lumbar spinal cord of day 40 treated
animals. Quantification of HSP70 staining in NaCl and hUCBC-treated mice showed an increase in
hUCBC-treated animals which did not reach statistical significance (A). Treatment with hUCBCs at the
presymptomatic stage led to an increased heat shock response (C) compared to vehicle-treatment
(B). Data are mean ± SEM of 5 animals (2♂/3♀) per group, unpaired t-test.
Western blot analyses failed to detect significant differences in growth factor secretion

After normalization to beta-actin no significant differences in the levels of BDNF, CNTF, GDNF, IGF-1 and VEGF between controls and hUCBC treated animals at both time points were visible (Fig 9). For IGF-1, however, there was a small but not statistically significant increase in spinal cord homogenates of hUCBC treated animals at both time points, more obvious in the d90 treated mice.

Figure 9. Protein levels of growth factors in lumbar spinal cord of day 40 & 90 treated animals normalized to β-actin levels. Treatment with hUCBC (“hUCBC”- white bar) at d40 (A) and d90 (B) didn’t change amount of BDNF, CNTF, GDNF and VEGF compared to controls (“NaCl”- grey bar). IGF-1 levels were increased slightly in hUCBC treated animals at both time points. Analysis of IGF-1 levels of d40 treated groups showed bands at around 24kDa (C). A & B: Data are mean ± SEM of 5 animals (2♂/3♀) per group, unpaired t-test.
3.5 Discussion

Direct replacement of motor neurons by stem or progenitor cells does not present a realistic option for the treatment of ALS to date, despite of promising preliminary data from animal models of motor neuron diseases [21-23]. Protection of motor neurons by providing a supportive environment seems to be more realistic, especially with respect to the translation into clinical trials in human ALS patients [24]. Different types of adult stem cells and progenitor cells have already been tested in pre-clinical studies in ALS mice [25]. Different application modes were used, showing partially controversial results. Umbilical cord blood cells have previously been administered in mutant SOD1 mice both intravenously which increased lifespan and delayed progression [13], and intrathecally which did not show significant positive effects [14]. In our study, hUCBCs were injected for the first time directly into the ventral horn of the spinal cord, in order to overcome the blood-brain barrier. This technique has been used by other groups assessing cellular therapy in ALS mice as well: human neural precursor cells (hNPCs) releasing GDNF had positive effects on motor neuron loss in mutant SOD1 rats when injected intraspinally [26], intraspinal injection of mouse bone marrow stem cells (CD117+) in mice with manifest hind limb paralysis due to the muscle deficient mutation (mdf) led to improvements of motor function [16], injection of mesenchymal stem cells from human bone marrow (CD90/CD106+; CD29/CD44+; CD166/CD105+) at the presymptomatic stage increased survival, improved motor performance and reduced neuroinflammation in mutant SOD1 mice [15].
Intraspinal injection

Intraspinal injection of cells requires laminectomy. In a previous study in rats, it was shown that extensive laminectomy with removal of spinous processes and laminar bone caused an increase of hyperalgesia-response and spinal deformation as compared to minimal laminectomy [27]. Minimal laminectomy without removal of bony structures, however, is not feasible in mice. We therefore decided to perform extensive laminectomy as it was done in other studies before [15, 16]. Though we did not specially test animals for hyperalgesia, none of the operated mice displayed impairment of limb function or other obvious behavioral abnormalities after surgery. Spinal cords used for histological examination did not show apparent damage, neither macroscopically nor microscopically. We therefore conclude that dorsal laminectomy and intraspinal injection of cells into the ventral horn as described here is safe and well tolerable in mice.

Monitoring of eGFP-labeled hUCBCs

In a study assessing the fate of bone marrow-derived mesenchymal stem cells (MSCs) into adult rat brain, Coyne and colleagues showed almost complete reduction of GFP+ MSCs 2 weeks after implantation and transfer of donor labels to host macrophages [28]. In our study, however, detection of eGFP+ hUCBCs for up to 9 weeks after intraspinal injection in healthy animals was observed. This corresponds to results of Chen et al. who showed survival of hUCBCs for up to 5 weeks in rat brain [29]. In agreement with others, our experiments did not show hUCBC differentiation into astrocytes. Sanchez-Ramos et al. found only 2% of hUCBCs expressing GFAP four weeks after transplantation in the developing rat brain [30].
Transplantation of hUCBCs into the injured spinal cord of rats led to predominant differentiation into oligodendrocytes after two weeks and only some hUCBC-derived astrocytes as detected via GFAP staining [31]. Rare (<2%) GFAP or MAP2 positive grafts were found 10 weeks after transplantation of hMSCs into G93A transgenic mice [15] and in cell culture of cryopreserved hUCBCs without separation procedure only a marginal amount of cells expressed markers for astrocytes or oligodendrocytes [32]. Similar to these previous studies, we observed only minimal co-localization of eGFP+ cells and the oligodendrocyte-marker APC and no cross-immunoreactivity with markers for astrocytes and microglia (GFAP, MBP and Iba1). Further investigations on the fate of transplanted cells have to be done in the future to better understand the mechanisms underlying neuroprotection.

**Behavioral assessment and survival**

In the analyses of weight and general condition, only hUCBC treatment at the presymptomatic stage (d40) led to partially significant improvements compared to the vehicle treated animals. Disease onset as assessed by the general condition score was slightly delayed by early hUCBC treatment. Moreover, when sacrificed for histological assessment at 110 days of age, 80% of controls and only 20% of hUCBC treated animals showed symptoms at day 110 which further confirms the assumption of delayed onset by hUCBC-treatment.

In mice treated at d90 disease progression probably had already been too advanced so that hUCBCs could not develop positive effects on weight and general condition anymore. We could previously show that specific locomotor evaluation like the rotarod and footprint tests are most sensitive in detection of treatment-induced
changes in the disease course [18]. This is supported by the results of motor function assessments in the present study: Treatment at d90 did still induce significant improvements in footprint runtime in the hUCBC-treated group. HUCBC treatment at the presymptomatic stage even led to significantly better performances in all locomotor tests.

Results of behavior tests were in line with survival data: Lifespan was significantly prolonged by early (d40) hUCBC injection. It is noteworthy, however, that survival of female G93A mice was prolonged much more while male animals only showed marginal benefit, and that improvement in behavioral tests induced by d40 hUCBC treatment was much more obvious in females. Gender differences have previously been observed in preclinical studies in ALS mouse models: intraventricular administration of hMSCs, for example, affected only females [33] and intrathecal delivery of hMSCs showed improvements of motor function only in female ALS mice [14]. A generally longer lifespan of female G39A mice (independent of any treatment) compared to males has been reported by several groups [34, 35] but was not apparent in our animals [18]. Though there is no conclusive explanation for this phenomenon so far, neuroprotective effects of estrogen must be considered. Estradiol was shown to protect spinal cord cultures from glutamate induced neurotoxicity and estrogen replacement after ovariectomy had positive effects on motor performance and survival in SOD1 mice [36, 37]. Estrogen-dependent gender-specific differences in the neural environment might account for the differential response to hUCBC treatment.
Histological evaluation

Motor neuron counting and GFAP staining of untreated control transgenic and wild-type animals at the two different surgery time points showed that at the presymptomatic stage (d40) there were no differences between wild-type and transgenic mice regarding the number of motor neurons but an increase of astrocytosis in the transgenic animals. This is concordant with other studies showing that astrocytosis in G93A mice is triggered by motor neuron degeneration [38] and occurs prior to symptom onset [39]. From these observations one could speculate that at d40, spinal motor neurons in transgenic animals were already affected and therefore induced astrocytosis even though they were not yet significantly reduced in numbers.

Our histological data from untreated mice provide an explanation for the much more positive results of behavior tests, survival analyses and histological evaluation in G93A mice injected with hUCBCs at this early time point: The injected cells could unfold their neuroprotective potential because degeneration of motor neurons had not progressed yet. Intraspinal transplantation of hUCBCs seems to provide a neuroprotective environment in the spinal cord and to reduce the inflammatory response as it was reported for other adult stem cells as well [15, 40]. As astrocytosis was increased at day 40 but decreased at day 110, hUCBC treatment seems to even reverse the astrocytosis.

We could further show an increase in HSP70 levels in the spinal cord of hUCBC-treated mice. This protein and its co-inducers were already shown to exhibit neuroprotective effects *in vitro* and *in vivo* in mouse models of ALS [41, 42]. Motor neurons are not capable to upregulate HSP70 [43], but transplanted cells may
stimulate the endogenous stress response leading to increased expression of this protein. HSP70-related anti-inflammatory effects may have contributed to the protection of motor neurons in hUCBC-treated animals.

At d90, a time point when major motor neuron loss had already taken place, this could not be expected to be similarly reversible by hUCBC transplantation. Increased astrocytosis at d90 may have further reduced survival of transplanted cells. Consequently, treatment at d90 only led to slight reduction of astrocytosis and motor neuron loss and minor changes in behavior tests but did not show an effect on survival.

Because of the small group sizes we refrained from separating the groups in females and males. It would be interesting to see if hUCBCs had a greater effect on motor neuron survival and astrocytosis in females than in males.

**Growth factor expression**

Besides reduction of glial activation and subsequent motor neuron damage, secretion of growth factors is another presumed mechanism behind the neuroprotective capacities of stem and progenitor cells. By Western blot analysis of spinal cord homogenates of both groups, we did not detect significant changes in protein expression levels of BDNF, VEGF, GDNF and CNTF. For IGF-1, an increase which did not reach statistical significance was observed in G93A mice treated with hUCBCs at d40 and d90. A study assessing hUCBC effects in rats following spinal cord injury showed increased levels of VEGF and GDNF in the injured spinal cord [44]. The time interval between cell injection and protein analysis in this study was, however, much shorter (7 days after spinal cord injury and cell injection) as opposed
to 20 and 70 days after transplantation, respectively. Although we showed the survival of hUCBCs up to 9 weeks (63 days) after injection in wild-type animals, injection in spinal cord of transgenic mice may lead to shorter survival times due to the inflammatory environment and therefore fail to increase growth factor production for such a long period. Furthermore, Western blot analysis of whole spinal cord homogenates may not be sensitive enough to detect protein expression changes which would be expected in the ventral horn only.

**Conclusion**

In summary, our findings demonstrate that intraspinal transplantation of hUCBCs is feasible in transgenic ALS mice. Cell injection before disease onset, at a time point when no major motor neuron loss had occurred, improved motor skills and prolonged survival of G93A mice. In line with several previous studies, females had a greater benefit of hUCBC treatment than males. Transplantation of hUCBCs reduced loss of motor neurons as well as astrocytosis and induced HSP70-expression, therefore demonstrating anti-inflammatory mechanisms of action of hUCBCs, while a significant increase of growth factors was not proven. These apparent neuroprotective effects warrant further investigations into the properties and therapeutic potential of hUCBCs. Because of the lack of a substantial effect of hUCBC injection after symptom onset on survival which is essential for the therapy of ALS patients, further studies will aim to better determine the fate and differentiation of transplanted cells and to compare different injection schemes with respect to time points, dose-dependency, height of the spinal cord and possibly repeated injections in the same animal over time. Another promising approach is
potentiation of the neuroprotective potential by genetic manipulation of the cells in order to induce secretion of growth factors or other neuroprotective factors. Our study supports intraspinal injection of hUCBCs as a promising candidate for the treatment of ALS.

Acknowledgements

We thank C. Kassebaum for help with the surgeries, C. Hotopp-Hergesell for genotyping of the mice, M. Thren for hUCBC isolation and culture, C. Baum for the supply of reagents and resources, T. Neumann for eGFP labelling of hUCBC and A. Niesel for technical support.

This study was supported by a grant from Deutsche Gesellschaft für Muskelkranke to RH and SP and by a grant from Deutsche Forschungsgemeinschaft (Pe 924/2-2) to SP.

3.6 References


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4. Comprehensive Discussion

The G93A transgenic ALS mouse model is widely used in preclinical trials, wherefore quantification of symptom onset and disease progression has to be performed as sensitively as possible in order to detect treatment effects. One novel therapeutic approach in ALS beyond classical pharmacotherapy is transplantation of adult stem cell in order to protect motor neurons from degeneration.

Within this thesis, different behavioural tests were compared with respect to their significance to detect symptom onset and progression. In a second study, we used the same mouse model to assess the effect of an intraspinal injection of hUCBCs on survival, locomotor skills, motor neuron loss and inflammation in the spinal cord.

Although the animal model used here is the most common model for ALS, there are disadvantages that cause a problem for preclinical trials in these mice. Large variation in age of onset and severity appears in both human ALS patients (Camu et al., 1999) and SOD1 transgenic mice (Alexander et al., 2004) leading to high standard deviations in studies on mouse models, as shown in our studies as well. Another important point of critique is the fact that only 10% of all human ALS cases are familial and only 20% of these are due to mutations in the SOD1 protein. But the clinical similarity between familial and sporadic cases indicate that there is a final common pathway of motor neuron death (Gurney 1997), leading to the assumption that a treatment affecting this pathway will work in both familial and sporadic ALS. Recent evidence from neuropathological studies, however, brings up the question whether the SOD1 mouse model accurately reflects the pathology of human ALS cases accurately: Transactivation response DNA-binding protein (TDP-43), a DNA
and RNA-binding protein, has been identified as the key component of the ubiquitinated intracytoplasmic inclusions which are the neuropathological hallmark of ALS (Neumann et al., 2006). TDP-43 positive inclusions can be detected in brain and spinal cord tissue of sporadic and familial ALS patients but not in mutant SOD1-associated fALS (Mackenzie et al., 2007), and mutations in the TARDP gene coding for TDP-43 have been identified in familial and sporadic ALS cases (Sreedharan et al., 2008). Future mouse models based on these findings might be better suited to elucidate the pathophysiology of sporadic ALS. So far, however, the G93A SOD1 mice are still the most widely used animal model mimicking the disease in many aspects and having the benefits of relatively early onset and rapid disease progression (Gurney et al., 1994). When preclinical studies in G93A mice are done appropriately, they can still provide important information for future translation into clinical applications (Ludolph et al.,).

For that reason it is of particular importance to assess first symptoms and progression of motor neuron disease in this model as sensitively as possible, wherefore suitable tests for locomotor functions are required. This is essential for studies assessing novel treatments because otherwise side effects could be missed by using incorrect analyses or by deceptive performance of the tests. We therefore compared in the first study five different methods (scoring general condition, weight, rotarod, step length and runtime) to find the most appropriate tests for this mouse model.

A frequently used parameter in neurodegenerative animal models is the inspection of the animals' general condition because of its simplicity and the possibility of quick
assessment (Dasari et al., 2007; Habisch et al., 2007; Vercelli et al., 2008). A drawback is, however, that subtle decreases in muscle strength can easily be missed by a brief and too superficial inspection. This assumption is supported by our study where first abnormalities in the general condition score were visible not before week 16 and therefore later than in rotarod and footprint analyses.

Recording the body weight is another general tool to detect changes in animal condition. As recently shown, a disease-specific metabolic deficit occurs in transgenic ALS mice (Dupuis et al., 2004). Body weight is, however, a relatively fluctuating parameter. Minor weight loss cannot necessarily be attributed to motor neuron death but rather to variations in the time of day or to metabolic changes unrelated to motor neuron loss. This is reflected in our study where first changes in body weight appeared two to three after first decline in rotarod and footprint analyses. Loss of weight rather seems to be a consequence of advanced disease than directly related to early damage of motor neurons. Only in comparison to wild-type animals which gained weight over the time weight differences were detected earlier. Therefore recording body weight as well as scoring of general condition should be performed to monitor disease progression during later stages, especially in preclinical drug trials without wild-type controls.

To detect onset and early progression, more specific motor related tasks are necessary. Referring to several other studies that used the rotarod test in animal models of neurodegenerative diseases (Weydt et al., 2003; Ikeda et al., 2005; Vercelli et al., 2008), we performed this test to monitor motor function and coordination. Distinct alterations were detected at week 14, subtle changes were visible even earlier supporting the conclusion that rotarod test is the most sensitive
method for detecting first motor symptoms. To get results as precise as possible and to be certain that any decrease in performance is due to impaired motor function, appropriate training of the mice is essential in this test. Otherwise, variations in performance due to insufficient training can mimic disease-associated alterations. Another functional method is the analysis of footprint patterns. In addition to step length we measured the time needed to run along the track. Both analyses showed first abnormalities at week 15 but in recording runtime there appears the problem of misinterpreting the results if increased runtime results from different levels of fear or explorative behaviour of the mice instead of motor impairment. This problem will not occur within the step length analysis because only steps of moving mice were analyzed. A further advantage is that this test is less time consuming and stressful, and that no training is required. Alterations were detected almost as sensitively as by the rotarod test.

An observation made by several studies in this model is longer survival of female G93A SOD1 mice (Kirkinezos et al., 2003; Suzuki et al., 2007; Choi et al., 2008). This is not directly supported by the results of our study, in which the onset and survival times of both genders did not differ significantly even though disease progression was slightly slower in females. No significant gender differences were observed in motor performance tests either. Nevertheless these findings support the prediction that gender mixed groups are essential especially in preclinical drug trials to avoid gender specific effects.

To summarize, the data of the first study support the use of rotarod and footprint analyses as most appropriate to record the onset of symptoms as early as possible,
while weight and scoring general condition are additionally helpful for monitoring
disease progression in G93A SOD1 mice.

The second study within the thesis project evaluated the effects of intraspinal
transplantation of human umbilical cord blood cells in G93A SOD1 mice. Behavioural
testing for the detection of hUCBC induced changes in motor function was based on
the results of the first study.

HUCBCs were injected intraspinally to deliver the therapeutic agent directly to the
affected tissue. This technique was used in other studies before (Cabanes et al.,
2007; Vercelli et al., 2008) and appeared to be adequate in our study as well. Mice
quickly recovered from surgery and showed no behavioural abnormalities.
Laminectomy and intraspinal injection of stem cells was therefore shown to be safe
and well tolerable in mice. As demonstrated in studies in human patients before
(Mazzini et al., 2009) this technique can be used in clinical trials as well.
Although we could show survival of implanted hUCBCs in spinal cord of wild-type
mice for up to 9 weeks, almost no co-localisation of eGFP-labelled hUCBCs and
markers for microglia, astrocytes or oligodendrocytes was detected. Adequate
determination of the fate of transplanted cells is still challenging (Sanchez-Ramos
2002; Dasari et al., 2007; Vercelli et al., 2008) and further studies addressing this
issue are required.

As suggested by the results of our first study, analyses of weight and general
condition were not sensitive enough to detect major treatment-induced changes in
the disease course. Rotarod and footprint tests, on the other hand, revealed
significant improvements induced by hUCBC treatment before symptom onset, and
to a lesser extent, also in mice treated at the symptomatic stage. These results were
in line with survival data showing that animals treated with hUCBCs at the
presymptomatic stage lived significantly longer than vehicle treated mice.

We observed apparent gender differences regarding the benefit from stem cell
treatment, in line with several previous studies (Habisch et al., 2007; Morita et al.,
2008). Neuroprotective effects of estrogen may have played a role in this
phenomenon although a conclusive explanation is still missing (Nakamizo et al.,
2000; Choi et al., 2008). These results underline the importance of gender mixed
groups in therapy studies in the G93A SOD1 mouse model.

Several studies are dealing with the interactions between neurons and non-neuronal
cells and their role in ALS pathogenesis (Hall et al., 1998; Clement et al., 2003;
Barbeito et al., 2004). The observation that astrocitosis is already increased at
presymptomatic stages (Hall et al., 1998) in mutant SOD1 mice was confirmed by our
results while the amount of motor neurons was not altered at presymptomatic day 40.
These histological observations can explain the much more positive results of
animals injected early with hUCBCs. At this time point when no major motor neuron
loss had occurred yet, the injected cells could fully unfold their neuroprotective
potential, reduce astrocitosis and prolong motor neuron survival. This is in line with
the assumption that the cellular environment of motor neurons plays a crucial role in
ALS (Clement et al., 2003) and with other studies showing beneficial effects of adult
stem cells in neurodegenerative diseases (Nayak et al., 2006; Vercelli et al., 2008).

At day 90 apparent astrocitosis and motor neuron degeneration was obvious in our
mice, in line with studies in this model (Hall et al., 1998; Pehar et al., 2005). At this
time point motor neuron degeneration has already taken place and could not be
prevented any more which explains the marginal effects of hUCBC treatment at this time. Another assumed neuroprotective mechanism of stem cells is the secretion of growth factors or stimulation of endogenous growth factor production. In contrast to other studies (Chen et al., 2008), we did not observe a treatment-induced significant increase in the levels of neurotrophic factors possibly due to analysis of the whole spinal cord instead of the ventral horn region only.

In conclusion, our findings demonstrate that intraspinal injection of hUCBCs before disease onset improved motor skills and survival of SOD1 mice, and led to a significant reduction of motor neuron loss and astrocytosis, especially in females. Motor-specific tests such as rotarod and footprint analyses are necessary to detect treatment-induced improvements as sensitively as possible. Future studies are necessary to better elucidate the fate, differentiation and migration of transplanted cells. Attempts to improve the neuroprotective capacities of stem cells such as genetically induced overexpression of growth factors or other neuroprotective molecules require further evaluation (Mohajeri et al., 1999; Aebischer et al., 2001; Behrstock et al., 2004).

A promising candidate with therapeutic potential for the treatment of neurodegenerative disorders is glucagon-like peptide 1 (GLP-1) (Doyle et al., 2001). Neuroprotective and antiapoptotic effects of GLP-1 have previously been shown in vitro and in vivo (Doyle et al., 2001; Harmening et al., 2009; Heile et al., 2009). We are therefore currently investigating the therapeutic potential of encapsulated human mesenchymal stem cells producing GLP-1 in the G93A mouse model.
5. Summary

Sarah Knippenberg

Studies on the therapeutic potential of adult stem cells in the G93A animal model of amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease leading to death within 3-5 years. Of all cases 5-10% are familial, among these around 20% are caused by mutations in the Superoxide dismutase 1 (SOD1) gene. Transgenic mice expressing mutant forms of human SOD1 mimic clinical and histopathological aspects of human ALS. The most widely used model is the G93A SOD1 mouse model. For pre-clinical studies in this model, objective and sensitive quantification of disease-onset and progression is required. We thus tested wild-type and transgenic G93A SOD1 mice in several behavioural assessments in order to determine their sensitivity. Mice were tested for general condition, changes in weight, rotarod and footprints analyses.

Our results show that motor specific tasks such as rotarod and footprint analysis are most appropriate to record the onset of symptoms as early as possible. Weight and behaviour scores for general condition are additionally helpful in order to monitor disease progression. Differences between females and males, as they appeared in several of our tests, support the importance of gender mixed groups in pre-clinical trials.
Because Riluzole is the only FDA-approved treatment for ALS so far, further pre-clinical trials are necessary to develop novel therapies for ALS. Stem cells seem to be a promising tool in the treatment not only of ALS but of neurodegenerative disorders in general. Thereby stem cells from human umbilical cord blood have several advantages to other adult stem cells, for example their low immunogenicity and easy availability. Therefore we transplanted hUCBCs intraspinally in G93A mice, followed by monitoring of survival, motor function, quantification of motor neuron loss and astrocytosis. We could show that intraspinal transplantation of hUCBCs is feasible in transgenic ALS mice. Transplantation of hUCBCs before disease onset, at a time point when no major motor neuron loss had occurred, improved motor skills and prolonged survival of G93A mice. Thereby females responded better to the treatment than males. Histopathologically we demonstrated reduced loss of motor neurons and astrocytosis in hUCBC treated animals. In western blot analyses, no significantly increased production of growth factors could be shown.

The apparent neuroprotective effects warrant further investigations of the properties and therapeutic potential of hUCBCs. Fate and differentiation of transplanted cells need to be further elucidated. In addition, different injection schemes with respect to time points, height of the spinal cord and possibly repeated injections in the same animal over time have to be considered. Spinal injection of hUCBCs appears a promising candidate for the treatment of ALS.
6. Zusammenfassung

Sarah Knippenberg

Studien zum therapeutischen Potential von adulten Stammzellen im G93A Tiermodell der amyotrophen Lateralsklerose (ALS)


Unsere Ergebnisse zeigen, dass spezifische motorische Untersuchungen wie Rotarod und Footprint- Tests am besten geeignet sind, um den Beginn der ALS-Symptomatik so früh wie möglich zu bestimmen. Gewicht und Allgemeinzustand sind dagegen eher beim Beobachten des Krankheitsverlaufs nützlich. In vielen der
Untersuchungen traten geschlechtsspezifische Unterschiede auf, was die Wichtigkeit der Verwendung von gemischtgeschlechtlichen Gruppen in präklinischen Studien deutlich macht.


sollen verschiedene Injektionsschemata mit unterschiedlichen Behandlungszeitpunkten, Injektion auf unterschiedlichen Höhen des Rückenmarks und eventuell wiederholte Injektionen geprüft werden. Die intraspinale Injektion humaner Nabelschnurblutzellen erscheint als vielversprechender neuer Therapieansatz der ALS.
7. References


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Acknowledgements

First I want to thank Prof. Reinhard Dengler for giving my the opportunity to perform my PhD thesis in the Institute of Neurology, Hannover Medical School and Prof. Susanne Petri for being a great main supervisor, her continued support and scientific advise and for giving me the opportunity to visit several congresses.

Special thanks to my co-supervisors Prof. Thomas Brinker and Prof. Stephan Steinlechner for their support and scientific comments.

Further I want to thank Christiane Hotopp-Herrgesell and Carola Kassebaum for excellent technical assistance and especially to Carola for her help with the surgeries. Special thanks to Nadine Thau for being a great help during the whole animal experiments and for the nice times in Berlin and London.

Thanks to all PhD students and members of the Neurology for pleasurable working conditions during the last 3 years, especially the people of the “chicken coop” for having a funny time.

Thanks to all the people who were helpful in the background, especially Darius Moharregh-Khiabani and of course Andreas Niesel for helping me with so many minor and major problems.

Thanks to all PhD students started 2008 for being a great group.

Special thanks to my family and friends, especially Jens, for being there all the time motivating and calming me down. Without your support I would never had managed all this in such a short period and without getting crazy.
Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled „Studies on the therapeutic potential of adult stem cells in the G93A animal model of amyotrophic lateral sclerosis (ALS)‟.

No third party assistance has been enlisted.

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.

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Signature