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REVIEW ARTICLE
Cell therapy and stem cells in animal models of motor neuron disorders

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Abstract

Amyotrophic lateral sclerosis (ALS), spinal bulbar muscular atrophy (or Kennedy's disease), spinal muscular atrophy and spinal muscular atrophy with respiratory distress 1 are neurodegenerative disorders mainly affecting motor neurons and which currently lack effective therapies. Recent studies in animal models as well as primary and embryonic stem cell models of ALS, utilizing over-expression of mutated forms of Cu/Zn superoxide dismutase 1, have shown that motor neuron degeneration in these models is in part a non cell-autonomous event and that by providing genetically non-compromised supporting cells such as microglia or growth factor-excreting cells, onset can be delayed and survival increased. Using models of acute motor neuron injury it has been shown that embryonic stem cell-derived motor neurons implanted into the spinal cord can innervate muscle targets and improve functional recovery. Thus, a rationale exists for the development of cell therapies in motor neuron diseases aimed at either protecting and/or replacing lost motor neurons, interneurons as well as non-neuronal cells. This review evaluates approaches used in animal models of motor neuron disorders and their therapeutic relevance.

Introduction

To date, there is no treatment that can substantially prolong the life span of affected individuals for motor neuron diseases such as amyotrophic lateral sclerosis (ALS), spinal bulbar muscular atrophy (SBMA), spinal muscular atrophy (SMA) and spinal muscular atrophy with respiratory distress 1 (SMARD1). However, recent studies with delivery of growth factors such as IGF-1 and VEGF into the $SOD1^{G93A}$ animal model of ALS have given encouraging results in presymptomatic as well as symptomatic animals (Kaspar *et al*., 2003; Azzouz *et al*., 2004; Storkebaum *et al*., 2005). Although growth factor treatment holds considerable promise of either delaying onset of the disease (relevant for dominantly inherited degeneration of motor neurons) and/or the progression, this strategy does not involve restoring previously lost functions. Herein lies the hope with cell transplantation, which at its best could not only delay onset and progression of disease by providing trophic support, but could potentially restore already lost functions. Indeed, experiments using models of acute motor neuron death have shown that embryonic stem (ES)-cell-derived motor neurons implanted into the spinal cord can extend axonal processes to functionally innervate muscle targets (Gao *et al*., 2005; Deshpande *et al*., 2006; Xu *et al*., 2006). Non-cell autonomous contributions to motor neuron toxicity in models of ALS raise the question of whether transplanted healthy motor neurons would survive in a hostile environment with activated microglia and other inflammatory events (Clement *et al*., 2003; Beers *et al*., 2006; Boillee *et al*., 2006b; Kim *et al*., 2006). However, transplantation studies in Parkinson's disease and Huntington's disease (HD) have shown that transplanted dopamine neurons and striatal neurons can survive and function long term without being affected by disease (Piccini *et al*., 1999; Freeman *et al*., 2000; Mendez *et al*., 2005; Bachoud-Levi *et al*., 2006; Isacson, 2006). Furthermore, replacement of mutant Cu/Zn superoxide dismutase 1 (SOD1)-overexpressing microglia with normal microglia, which can be derived from ES cells (Tsuchiya *et al*., 2005) or by pharmacological suppression of microglial activation in models of ALS, can substantially delay progression of motor neuron disease (Kriz *et al*., 2002; Corti *et al*., 2004; Tsuchiya *et al*., 2005; Beers *et al*., 2006; Boillee *et al*., 2006b). Future therapies for motor neuron diseases could include a combination of strategies aimed at both neuroprotection of host motor neurons and cellular replacement of neurons and glia, possibly through the use of stem cells.

The potential use of cellular therapies in different motor neuron disorders

ALS, SBMA, SMA and SMARD1 are diseases characterized by degeneration of motor neurons, with resulting muscle wasting and paralysis. However, the subset of motor neurons that are affected differs between these diseases. In ALS, motor neurons in the spinal cord, brain stem and cortex die. In SBMA, degeneration is more

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restricted, with spinal and brain stem motor neurons dying, but not cortical motor neurons (Kennedy *et al*., 1968; Ringel *et al*., 1978), while in SMA and SMARD1, only spinal motor neurons are affected. In future cellular therapies aimed at replacing endogenous motor neurons, the location and number of implantation sites as well as the subtypes of motor neurons that need to be generated will thereby differ greatly between these diseases.

The clinical onset and rate of progression varies widely between the different motor neuron diseases. In ALS and SBMA, clinical symptoms typically initiate during mid-life. However, while ALS is usually fatal within 1–5 years (Yoshida *et al*., 1986), SBMA has a much slower progression and the vast majority of individuals have a normal life expectancy (http://www.ninds.nih.gov/disorders/kennedys/kennedys.htm). SMA, by contrast, is divided into four types based on severity and age of onset. Types I–III are childhood-onset SMAs, with type I being the most severe form with onset before 6 months of age and death by 2 years. Type II is intermediate in severity with onset before 18 months and patients never gain the ability to walk and type III is the mildest form of childhood SMA, with onset after 18 months and patients being able to walk (Munsat & Davies, 1992). Type IV is an adult SMA, where symptoms begin around age 35 years, and patients usually have a normal life span. SMARD1, which was previously often misdiagnosed as sudden infant death syndrome, presents within 1–6 months of age, with severe respiratory distress due to paralysis of the diaphragm (Mellins *et al*., 1974; Grohmann *et al*., 1999; Lamperti *et al*., 2003). The time of onset and the rate of progression of these diseases will be important determinants for selecting a suitable cell therapy. Types II and III SMAs with an early onset and slower progression could enable therapy aimed at transplanting stem cell-derived motor neurons for reconnection to the muscle (Wirth *et al*., 2006a), as the distances needed to traverse would be smaller in a child than an adult, and the time needed for the axon to reach muscle would therefore be sufficient. Patients with SBMA (or Kennedy's disease) (Poletti *et al*., 2005) have a slower progression (10–20 years) than most forms of ALS and are therefore also likely candidates, as are ALS patient with a slowly progressing disease.

Furthermore, these diseases have distinct genetic causes. SBMA is an X-linked disorder, caused by an expansion of CAG/glutamine repeats in the first exon of the androgen receptor gene (Kennedy *et al*., 1968; Ringel *et al*., 1978; La Spada *et al*., 1991, 1992). Pathogenesis in SBMA appears to be due to both a toxic gain-of-function of misfolded AR protein as well as a loss of function of AR protein (Thomas *et al*., 2006). ALS is dominantly inherited in 5–10% of patients (referred to as familial ALS, fALS), but in 90–95% of patients there is no apparent genetic linkage (referred to as sporadic ALS). Approximately 15–20% of fALS cases have been linked to mutations of SOD1 (Gaudette *et al*., 2000; Andersen, 2001) (for a continually updated list: http://www.alsod.org). SOD1 is not required for development or survival of motor neurons, but is necessary for the maintenance of normal neuromuscular junctions (NMJs) (Reaume *et al*., 1996; Flood *et al*., 1999; Shefner *et al*., 1999). Mutations of SOD1 in ALS are thought to result in a toxic gain-of-function of the protein (for a review see Boillee *et al*., 2006a). Both SMA and SMARD1 are autosomal recessive disorders. SMA is caused by mutations in the telomeric survival motor neuron gene (SMN1) (Bussaglia *et al*., 1995; Lefebvre *et al*., 1995; Parsons *et al*., 1996; Hahnen *et al*., 1997; Talbot *et al*., 1997). The level of the centromeric survival motor neuron gene (SMN2) is the main predictor of severity of the disease, with an increased copy number appearing partially to protect SMA patients (Vitali *et al*., 1999; Feldkotter *et al*., 2002; Wirth *et al*., 2006b). SMARD1 is instead caused by mutations in the immunoglobin μ-binding protein 2 (IGHMBP2) (Grohmann *et al*., 2001). While SMA and SMARD1 are likely cell-autonomous diseases caused by the loss of genes that are necessary for motor neuron survival, motor neuron death in ALS appears to involve non-cell-autonomous as well as cell-autonomous events (for a review see Boillee *et al*., 2006a). Consequently, replacement of mutant SOD1-expressing cells with wild-type non-neuronal cells can substantially increase the life span in animal models of ALS (Clement *et al*., 2003). Furthermore, specific replacement of mutant SOD1-expressing microglial cells with wild-type microglial cells, while not affecting onset of disease, can considerably increase the life span of the animals. Initiation of disease appears to be dependent on over-expression of mutant SOD1 within motor neurons and could be either a cell-autonomous event and/or dependent on cells other than microglia (Corti *et al*., 2004; Beers *et al*., 2006; Boillee *et al*., 2006b).

Neuronal death in HD, a polyglutamine expansion disease like SBMA, involves both cell-autonomous and non-cell-autonomous events (Ross, 2004; Gu *et al*., 2005), rendering it possible that SBMA is also due to a combination of these two.

Although the degeneration of motor neurons is the most prominent feature of these diseases, other cell types are also affected. For example, in ALS, spinal interneurons degenerate during disease progression (Oyanagi *et al*., 1989; Terao *et al*., 1994). Loss of spinal interneurons can in itself cause paraplegia (Kwak & Nakamura, 1995; Hadi *et al*., 2000; Marsala *et al*., 2004). In SBMA, dorsal root ganglia may degenerate, causing a mild distal sensory deficit (Barkhaus *et al*., 1982; Olney *et al*., 1991). SBMA patients also develop varying degrees of testicular atrophy, impaired fertility, gynecomastia and elevated androgen levels, all signs of androgen insensitivity (Dejager *et al*., 2002). In SMARD1, sensory and autonomic nervous system may be involved. These additional features of the diseases might also need to be targets of cellular therapy. For example, it is quite possible that an ALS patient will still have paralysis after receiving functional ES cell-derived motor neuron transplants due to loss of interneurons and therefore will need replacement also of these.

Stem cell-based therapies aimed at neuroprotection and cellular replacement

Cell-based therapy in motor neuron disorders can be used both with the aim of creating trophic support to preserve endogenous cells and for the replacement of host cells (Table 1). Delivery of glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1) or vascular endothelial growth factor (VEGF), preserve motor neurons in animal models of ALS and increase animal survival (Acsadi *et al*., 2002; Wang *et al*., 2002; Kaspar *et al*., 2003; Lambrechts *et al*., 2003; Azzouz *et al*., 2004; Zheng *et al*., 2004; Storkebaum *et al*., 2005) (Table 2). GDNF appears primarily to influence disease onset and not progression (Wang *et al*., 2002; Kaspar *et al*., 2003; Azzouz *et al*., 2004), making it an unlikely candidate for gene therapy in ALS (Table 2). However, IGF-1 and VEGF retain protective efficacy even when delivered after clinical onset of the disease, when 50% of the motor neurons are already lost, making these potential for use in patients (Kaspar *et al*., 2003; Storkebaum *et al*., 2005; Azzouz *et al*., 2004) (Table 2). Although viral delivery of growth factors appears to hold promise for future use in patients with motor neuron disease, careful considerations to avoid the risks associated with viral delivery need to be taken. First, the virus could be transported to other regions of the central nervous system (CNS) and potentially cause side-effects (Hsich *et al*., 2002). Secondly, virus-integration in proximity to oncogens could trigger malignant cell proliferation (Hacein-Bey-Abina *et al*., 2003). However, the use of

TABLE 1. Overview of cellular transplantations in animal models of motor neuron disorders

Animal model	Cell source	Injection sites and cell numbers	Cells identified post grafting	Effect on onset and/or survival	References
Presymptomatic $SOD1^{G93A}$ rat (P80)	Cortical hNSCs (22 w fetus), lenti-GDNF-infected, treated with CNTF	Bilateral lumbar SC injections, 2 sites, 1.2×10^5 cells/site	$Nestin^+$, 5% $GFAP^+$, no mature neurons	No effect	(Klein *et al*., 2005)
Presymptomatic $SOD1^{G93A}$ rats (P62)	Cervical sc hNSCs (8 w old fetus)	Bilateral lumbar SC injections, 4 sites, 50 000 cells/site	Mature neurons, BDNF and GDNF release from graft	Delay in onset (7 days) and increased average life span (11 days)	(Xu *et al*., 2006)
Adult rats with chronic, unilateral motor neuron deficiency (through sciatic axotomy)	K048 hNSCs (Svendsen *et al*., 1998), treated with Shh	Unilateral lumbar SC injection, 1 site, 1×10^5 cells	50% motor neurons, formation of NMJ with host muscle	Partial recovery from paralysis	(Gao *et al*., 2005)
5–7 w old rats with chronic, bilateral motor neuron deficiency (through sindbis virus)	mES cells treated with Shh and RA	Bilateral lumbar SC injections, 1 site, 6×10^4 cells	Motor neurons, forming NMJs with host muscle	Partial recovery from paralysis	(Deshpande *et al*., 2006)
Presymptomatic $SOD1^{G93A}$ mice (P70)	LeX+/CXCR4+ mNSCs from CBA-eGFP (Okabe *et al*., 1997) or Hb9-eGFP (Wichterle *et al*., 2002) mouse brains (6–8 w old) treated with bFGF, laminin, Shh and RA	Bilateral lumbar SC injections, 1 site, 1×10^4 cells	NF, MAP2, NeuN, Hb9, ChAT, O4 and GFAP + cells, VEGF and IGF release from grafts	Delay in onset (21 days) and increased average life span (22–23 days) (progression time the same). Delayed loss of lumbar motor neurons.	(Corti *et al*., 2007)
Presymptomatic nmd mice (P1-2)	$ALDH^{hi}SSC^{lo}$ mNSC from Thy1-YFP SC treated with Shh, RA, cAMP and NGF	Intrathecal delivery, 2×10^4 cells	MAP2, NF, TuJ1, NeuN, GFAP, Hb9 and ChAT + cells	Delayed onset and increased average life span (18–19 days). Decreased loss of motor neurons and preservation of large axons (ventral root)	(Corti *et al*., 2006)
Presymptomatic, irradiated $SOD1^{G93A}$ mice (8 w old)	hUCB cells	R.o. injection, $34.2–35 \times 10^6$ cells	hRNA identified in some animals, no analysis of cellular phenotypes	Delay in onset (22 days) and increased life span (21 days)	(Ende *et al*., 2000)
Presymptomatic, irradiated $SOD1^{G93A}$ mice (8 w old)	mBM cells	R.o. injection, 5×10^6 cells	No histological analysis performed	Delay in onset (7 days) and increased life span (12–13 days)	(Ende *et al*., 2000)
Presymptomatic, irradiated $SOD1^{G93A}$ mice (4 w old)	mBM cells from Thy1-YFP or CBA-eGFP or $SOD1^{G93A}$ mice	I.p. injection, 30×10^6 cells	Skeletal and heart muscle, microglia, neurons?	Delay in onset (14 days) and increased life span (12–13 days) of wild-type BMCs, no effect of $SOD1^{G93A}$ BMCs	(Corti *et al*., 2004)
Presymptomatic $SOD1^{G93A}$ mice (P55-65)	Sertoli cells (P17-18)	Unilateral lumbar SC injection, 1×10^5 cells	No cells identified 3 months post implantation	No effect	(Hemendinger *et al*., 2005)
Presymptomatic $SOD1^{G93A}$ mice (P56)	hNT cells	Bilateral lumbar SC injection, 1 site, 7.5×10^4 cells	hNuMa, but no effect on life span	Slight delay of onset,	(Willing *et al*., 2001)
Symptomatic $SOD1^{G93A}$ mice (P114)	hNT cells	Bilateral lumbar SC injection, 1 site, 7.5×10^4 cells/site	Cresyl violet	No effect on average survival	(Garbuzova-Davis *et al*., 2001)
Presymptomatic $SOD1^{G93A}$ mice (P53-54)	hNT cells	Bilateral lumbar SC injection, 1 site, 7.5×10^4 cells/site	Cresyl violet, hNuMa	No effect	(Garbuzova-Davis *et al*., 2002)
Presymptomatic $SOD1^{G93A}$ mice (P61)	hNT cells	Bilateral lumbar SC injection, 1–3 sites, 7.5×10^4 cells/site	hNuMa, hNF	No effect	(Garbuzova-Davis *et al*., 2006)

ChAT, choline acetyl transferase; GFAP, glial fibrillary acidic protein; Hb9, homeobox gene Hb9; hNF, human neurofilament; hNuMa, human nuclear matrix antigen; i.p., intraperitoneal; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclei; NF, neurofilament; nmd mouse, neuromuscular degeneration mouse; O4, oligodendrocyte marker O4; r.o., retro-ocular; SC, spinal cord.

TABLE 2. Overview of growth factor delivery in animal models of motor neuron disorders

Animal model	Factor	Route of delivery	Effect on onset and/or survival and motor neuron cell numbers	Refs
Presymptomatic (60-day-old) and symptomatic (P90) $SOD1^{G93A}$ mice	IGF-1	I.m. injections of AAV-IGF (hind limb quadriceps and intercostal)	Delay in onset (31 days) and increased survival (37 days) in 60-day-old animals and increased survival (22 days). Decreased loss of lumbar motor neurons, decreased caspase activation in motor neurons and delay of astroglial response in spinal cord.	(Kaspar *et al*., 2003)
Presymptomatic mnd (*m*/m) mice (3 month old)	IGF-I + GAGs	S.c. injections of IGF-1 (20 μg/kg/day) and GAGs (1 mg/kg/day) during 7 months	Attenuation of onset of neuromuscular deficits, evaluated by grip strength, holding time, isometric tension of EDL muscles and preservation of motor neurons innervating EDL muscles. IGF-1 alone or GAG alone did not have an effect in this study.	(Gorio *et al*., 1999)
Symptomatic wobbler mice (P21)	IGF-I	S.c. injections of IGF-I (20 μg/kg/day) for 3 weeks	Decreased decline in grip strength and reduced biceps muscle atrophy	(Vergani *et al*., 1997)
Symptomatic wobbler mice (P21)	GAG	S.c. injections of GAGs (20?g/kg/day) for 3 weeks	Decreased decline in grip strength and reduced biceps muscle atrophy	(Vergani *et al*., 1997)
Symptomatic wobbler mice (P21)	IGF-I + GAGs	S.c. injections of IGF-1 (20 μg/kg/day) and GAGs (1 mg/kg/day) for 6 weeks	Decreased decline in grip strength and reduced biceps muscle atrophy is correlated with preservation of motor neurons in triceps muscle. Effect of combination treatment with IGF-1 and GAGs was significantly higher than with the single drugs, even if IGF-I was used at 1 mg/kg/day.	(Vergani *et al*., 1999)
Presymptomatic $SOD1^{G93A}$ mice (∼P63)	GDNF	I.m. injections of AAV-GDNF (gastrocnemius and triceps brachii)	Delay in onset (13 days) and increased survival (17 days). Decreased loss of cervical and lumbar motor neurons.	(Wang *et al*., 2002)
Presymptomatic $SOD1^{G93A}$ mice (P5-7)	GDNF	I.m. injections of AVR-GDNF (anterior tibialis, gastrocnemius, quadriceps and paraspinal)	Slight delay in onset (7 days) and increase in survival (17 days). Delayed loss of lumbar motor neurons.	(Acsadi *et al*., 2002)
Presymptomatic (P60) and symptomatic (P90) $SOD1^{G93A}$ mice	GDNF	I.m. injections of AAV-GDNF (hind limb quadriceps and intercostal)	Delay in onset (16 days) and increased survival (11 days) in P60 animals and increased survival (7 days) in P90 animals	(Kaspar *et al*., 2003)
Presymptomatic $SOD1^{G93A}$ mice (∼P21)	GDNF	I.m. injections of EIAV-GDNF (gastrocnemius, diaphragm, intercostals, facial and tongue)	Slight increase in survival (6 days). No analysis of motor neuron numbers.	(Azzouz *et al*., 2004)
Presymptomatic $SOD1^{G93A}$ mice (∼P21)	VEGF	I.m. injections of EIAV-VEGF (gastrocnemius, diaphragm, intercostals, facial and tongue)	Delay in onset (28 days) and increased survival (38 days). Decreased loss of bulbar and lumbar motor neurons.	(Azzouz *et al*., 2004)
Presymptomatic $SOD1^{G93A}$ mice (P74)	VEGF	I.p. injection of VEGF (1 μg/kg) or 0.1 μg/kg) 1/week	The higher dose delayed onset (12 days) and increased survival (11 days). No analysis of motor neuron numbers.	(Zheng *et al*., 2004)
Presymptomatic (P60) and symptomatic (P85) $SOD1^{G93A}$ rat	VEGF	I.c.v. (Alzet pump) infusion of 0.2 mg/kg/day	Delay in onset (17 days) and increased survival (22 days) in P60 animals and prolonged survival (10 days) in P85 animals. Decreased loss of large motor neurons in cervical spinal cord.	(Storkebaum *et al*., 2005)
Symptomatic pmn mice (∼P21)	CNTF	I.p. injection of CNTF-secreting D3 cells	Increased survival. Preservation of motor neurons	(Sendtner *et al*., 1992)
Symptomatic wobbler mice (P21-28)	CNTF + BDNF	S.c. injection of alternating doses of CNTF (1 mg/kg) and BDNF (5 mg/kg) 3 times/week	Arrested disease progression by 1 month. Preservation of motor neurons	(Mitsumoto *et al*., 1994b)

TABLE 2. Continued

Animal model	Factor	Route of delivery	Effect on onset and/or survival and motor neuron cell numbers	Refs
Symptomatic wobbler mice (P21-28)	CNTF	S.c. injection of CNTF (1 mg/kg) for 4 weeks, 3 times/week	Disease progression attenuated (measurement done until 4th postnatal week), as measured by grip strength, paw position abnormalities, running time and bicep–tricep muscle twitch tension. Effect on motor neuron number was not evaluated.	(Mitsumoto *et al*., 1994a)
Symptomatic pmn mice (P16-20)	CNTF	S.c. implantation of encapsulated CNTF-excreting BHK fibroblasts	Increased the survival time by 40%. Preservation of motor neurons.	(Sagot *et al*., 1995)
Presymptomatic pmn mice (P1-5)	CNTF	AdCNTF: I.m. into P3-5 (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunc); I.v. into P2-3 (temporal vein); I.c.v. P1-2.	30% increase in mean life span in i.m and i.v. delivery, but no effect on life span in i.c.v. delivery. I/m and i.v. delivery increased the number of myelinated phrenic nerve fibers at P25, i.c.v. delivery did not.	(Haase *et al*., 1999)
Presymptomatic $SOD1^{G93A}$ mice (P22–41)	CNTF	Local application of CNTF to tibialis anterior muscle or osmotic minipump infusion into triceps surae muscle	Onset and survival not evaluated. Preservation of fast-fatigue-resistant motor neurons, pruning of axon branches, reduction of axonal neurofilament density and down-regulation of anti-apoptotic protein Bcl2a1–2.	(Pun *et al*., 2006)
Presymptomatic pmn mice (P3-5)	NT3	I.m. injection of AAV-NT3 (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunk)	50% increase in mean life span. Increase in the size of motor units and a 20–30% reduction in loss of myelinated axons in phrenic nerves at P25	(Haase *et al*., 1997)
Pmn	NT3 + CNTF	I.m. injection of AAV-NT3 and AAV-CNTF (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunk)	50% increase in mean life span. 30% reduction in loss of myelinated axons in phrenic nerves at P25 compared with AAV-NT3 treatment alone.	(Haase *et al*., 1997)
Presymptomatic pmn mice (P3-5)	CT-1	I.m. injection of AdCT-1 (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunk)	18% increase in mean survival. 50% reduction in loss of myelinated axons in phrenic nerve	(Bordet *et al*., 1999)
Presymptomatic pmn mice (P3-5)	CT-1 + GDNF	I.m. injection of AdCT-1 and AdGDNF (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunk)	18% increase in mean survival.	(Bordet *et al*., 1999)
Presymptomatic $SOD1^{G93A}$ mice (neonatal)	CT-1	I.m. injection of AdCT-1 (gastrocnemius, triceps brachii and the long dorsal muscles of the thorasic trunk)	Delay in onset of disease (27 days) and increase in survival (13 days). Decreased loss of CMAP amplitude in gastrocnemius muscle. Decreased muscle atrophy. 30% reduction in loss of myelinated axons in phrenic nerve at P130.	(Bordet *et al*., 2001)
Presymptomatic pmn mice (P6-7)	CT-1	5–40 μg CT-1 plasmid electroporation (gastrocnemius muscles)	10 μg CT-1 plasmid increased the mean survival with 26%. Decreased muscle atrophy at P25. Decreased loss of CMAP amplitude in gastrocnemius muscle. 30% reduction in loss of myelinated axons in phrenic nerve at P25. Notably, repeated electroporation with 10 μg CT-1 plasmid decreased mean life span below that of untreated pmn mice. Single electroporation of 40 μg of plasmid induced growth retardation and death.	(Lesbordes *et al*., 2002)

AAV, adeno-associated virus; Ad, adenovirus; BHK, baby hamster kidney; CNTF, ciliary neurotrophic factor; CMAP, compound muscle action potential, CT-1, cardiotrophin-1; EIAV, rabies-G pseudotyped lentiviral vector; GAGs, glycosaminoglycans; GDNF, glial cell line-derived neurotrophic factor; I.c.v., intracerebroventricular; IGF-1, insulin growth factor-1; I.m., intramuscular; I.p., intraperitoneal; NT-3, Neurotrophin-3; Pmn, progressive motor neuronopathy; S.c., subcutaneous; VEGF, vascular endothelial growth factor.

adeno associated virus or lenti viral vectors is unlikely to cause toxicity (Montini *et al*., 2006), with both vectors showing capacity for retrograde transport from the muscle to the spinal cord (Mazarakis *et al*., 2001; Kaspar *et al*., 2003; Azzouz *et al*., 2004; Storkebaum *et al*., 2005). However, the use of cellular transplants to deliver growth factors, either through the normal release from the transplanted cells or after *in vitro* manipulations of cells for over-expression of certain growth factors, could provide a safer method of delivery. In addition, given that several growth factors may need to be delivered simultaneously and locally for a combined effect, this might be best accomplished by using cellular transplants. However, the specific appeal of cellular transplantation for motor neuron disease is the possibility to restore already lost functions, by replacing degenerated host motor neurons, something which is unlikely to be accomplished by growth factor delivery alone. Furthermore, cellular transplantation could also replace reactive host cells such as microglia and astrocytes that appear to be part of the degenerative process in ALS (Kawamata *et al*., 1992; Hall *et al*., 1998; Beers *et al*., 2006; Boillee *et al*., 2006b; Kim *et al*., 2006; Di Giorgio *et al*., 2007; Nagai *et al*., 2007) and perhaps also in the other motor neuron diseases (Rathke-Hartlieb *et al*., 1999). For these purposes, ES cells, neural stem cells (NSCs), umbilical cord blood cells (UCBCs) and bone marrow cells (BMCs) are potential cellular sources.

Derivation and differentiation of stem cells

Embryonic stem cells

Embryonic stem cells are pluripotent cells that are conventionally isolated from the inner cell mass of blastocysts (Evans & Kaufman, 1981; Martin, 1981; Thomson *et al*., 1998), but can also be derived from a single blastomere (Chung *et al*., 2006; Klimanskaya *et al*., 2006) and from the epiblast (Tesar *et al*., 2007). Blastocyst-derived mouse ES cells can be maintained and expanded in an undifferentiated state by growth on primary murine embryonic fibroblast (PMEF) feeder layers (Martin, 1981) and/or in the presence of leukemia inhibitory factor (LIF) (Smith *et al*., 1988; Williams *et al*., 1988; Nichols *et al*., 1990). However, while expansion on PMEF can maintain primate ES cells, including human, in an undifferentiated state, LIF alone cannot (Thomson *et al*., 1998, 1995). A multitude of *in vitro* differentiation protocols aimed at deriving specific neuronal and glial subtypes from ES cells have been developed. Exposure to retinoic acid (RA) and Sonic hedgehog (Shh) differentiates ES cells into motor neurons and interneurons (Wichterle *et al*., 2002; Li *et al*., 2005), while other signaling molecules can direct differentiation into dopamine neurons (Lee *et al*., 2000; Kawasaki *et al*., 2000; Kawasaki *et al*., 2002; Barberi *et al*., 2003; Perrier *et al*., 2004; Roy *et al*., 2006), telencephalic neurons (Watanabe *et al*., 2005), astrocytes (Gossrau *et al*., 2007), oligodendrocytes (Glaser *et al*., 2005; Nistor *et al*., 2005) and microglia (Tsuchiya *et al*., 2005).

Neural stem cells

Cells with stem cell potential are widely distributed in the embryonic CNS, while in the adult they are restricted to two main regions: the hippocampal dentate gyrus and the subventricular zones of the lateral ventricles (Gage, 2000; Alvarez-Buylla & Garcia-Verdugo, 2002). Depending on the region where the cells are isolated from and the age of the animal, the neural stem cells will either be multipotent with a broad self-renewing potential and with the capacity to generate neurons, astrocytes and oligodendrocytes or lineage restricted neural progenitors with limited self-renewal and commitment to either neuronal or glial fates (Gage, 2000). For isolation of neural stem cells, the tissue is usually dissociated and cells are subsequently exposed to high concentrations of mitogens such as fibroblast growth factor 2 (FGF-2) (Gensburger *et al*., 1987; Richards *et al*., 1992) and/or epidermal growth factor (Reynolds & Weiss, 1992; Reynolds *et al*., 1992). Cells can be conditionally immortalized using oncogens such as simian virus 40 large T antigen (Noble, 1999) or v-myc (Hoshimaru *et al*., 1996), for facilitated proliferation. After a time of proliferation, the cells can subsequently be differentiated *in vitro* either by withdrawal of the mitogen and/or by exposing the cells to different factors. RA and Shh can induce a motor neuron phenotype also from NSCs (Gao *et al*., 2005).

Bone marrow cells and umbilical cord blood cells

Bone marrow contains hematopoietic and mesenchymal stem cells and is usually harvested from a large bone of the donor, such as the pelvis. Hematopoietic stem cells give rise to leucocytes, erythrocytes and thrombocytes, whereas mesenchymal cells have the ability to differentiate into osteoblasts, chondrocytes, myocytes and many other cell types (Kolf *et al*., 2007). BMCs have been shown to generate cardiac fibers *in vivo* in infarcted or dystrophic heart (Jackson *et al*., 2001; Orlic *et al*., 2001). BMCs have also been described to contribute to Purkinje neuron population after transplantation (Priller *et al*., 2001; Weimann *et al*., 2003a), although this might be solely due to reprogramming after cell fusion (Weimann *et al*., 2003b).

UCBC samples are collected from placentas and umbilical cord and are rich in hematopoietic stem cells, and therefore utilized as an alternative to bone marrow transplantation when no sibling donors are available (Schoemans *et al*., 2006). UCB also contains small amounts of mesenchymal stem cells (Lee *et al*., 2004) and endothelial progenitor cells (Zhang *et al*., 2006), which could give rise to a multitude of cell types and perhaps even participate in regenerative processes after transplantation (Ott *et al*., 2005).

Genetic animal models of progressive motor neuron disorders

Mutant SOD1-overexpressing mice and rats as models of ALS

Since the establishment of the mutant SOD1-overexpressing transgenic mice and rats, they have become the most commonly used motor neuron disease models in cell transplantation and growth factor delivery studies (Tables 1 and 2). Over-expression of mutant forms of human SOD1 in mice and rats result in ALS-like motor neuron disease (Tu *et al*., 1996; Wong *et al*., 1998; Howland *et al*., 2002). Paralysis is initiated at 3 months of age in the $SOD1^{G93A}$ mouse and animals die within 2 months after the appearance of clinical symptoms (Gurney *et al*., 1994; Tu *et al*., 1996). Electric properties of lumbar motor neurons and axonopathy are initiated already during the first and second month of age, long before motor neuron cell bodies are lost (Durand *et al*., 2006; Pun *et al*., 2006). In addition to motor neuron death, as in the human disease, the SOD1 transgenic models also display loss of spinal interneurons (Morrison *et al*., 1996, 1998). Transgenic $SOD1^{G93A}$ rats show a similar, but quicker progression of disease (Nagai *et al*., 2001; Howland *et al*., 2002; Storkebaum *et al*., 2005; Matsumoto *et al*., 2006; E. Hedlund *et al*., unpublished observations). $SOD1^{G93A}$ rats and mice show variability in disease course. The rats either display hindlimb onset (Nagai *et al*., 2001; Storkebaum *et al*., 2005; Howland *et al*., 2002; Matsumoto *et al*.,

2006) or forelimb onset (Matsumoto *et al*., 2006; Storkebaum *et al*., 2005; E. Hedlund *et al*., unpublished observations). Already during early stages of disease, even prior to loss of motor neurons, there is an increase in the numbers of reactive astrocytes and activated microglia (Hall *et al*., 1998; Henkel *et al*., 2006) and a subsequent up-regulation of cytokines (Alexianu *et al*., 2001; Elliott, 2001; Nguyen *et al*., 2001; Hensley *et al*., 2002).

The fast progression of disease in these models makes them suboptimal for use in cell replacement transplantation strategies initiated after onset, as regenerative processes have insufficient time to take place. Consequently, most cellular transplantations have been performed on presymptomatic SOD1 transgenic animals and studies aimed at regeneration have used acute motor neuron injury models where the life span of the animals is not affected by the motor neuron death (Tables 1 and 2).

Neuromuscular degeneration (nmd) mouse as a model of SMARD1

The *nmd* mouse, which carry a spontaneous autosomal recessive *Ighmbp2* mutation, display symptoms similar to SMARD1 (Cox *et al*., 1998). Degeneration in the *nmd* mouse is visible as a dorsal contraction and paralysis of the hindlimbs, with forelimbs subsequently affected to variable degrees and animals eventually dying due to respiratory failure (Cook *et al*., 1995; Cox *et al*., 1998). The life span for homozygous mice ranges from 12 to 138 days (Cook *et al*., 1995, 1998; Grohmann *et al*., 2004; Maddatu *et al*., 2004; Corti *et al*., 2006). Rescue of motor neuron atrophy in the *nmd* mouse by transgenic expression of Ighmbp2 in neurons only revealed that Ighmbp2 is important also for skeletal and cardiac myocyte survival, with animals dying from cardiomyopathy with secondary respiratory failure (Maddatu *et al*., 2004). The *nmd* mouse has been previously used in cell transplantation experiments (Table 1).

Cell transplantation aimed at providing a trophic environment for endogenous motor neurons and/or replacement of non-motor neurons

Transplantation of human and mouse neural stem cells and delivery of trophic factors

In an attempt to preserve host motor neurons, human NSCs (hNSCs) of two different origins have been used for intraspinal grafting of presymptomatic $SOD1^{G93A}$ rats. In the first study, cortex-derived hNSCs were infected with a viral construct for over-expression of GDNF (Klein *et al*., 2005) and predifferentiated with ciliary neurotrophic factor (CNTF) to induce a higher content of astrocytes (Caldwell *et al*., 2001), the premise being that healthy astrocytes overexpressing GDNF could exhibit a protective role on mutant motor neurons (Henderson *et al*., 1994; Clement *et al*., 2003), and thereafter transplanted (Table 1). The transplanted cells showed limited migration and GDNF secretion within the grafted region only. Host fibers in close proximity to the graft up-regulated cholinergic markers, but there was no protection of host motor neurons and no effect on disease onset or life span. The grafts contained no mature neurons and only a small fraction of astocytes, and were instead mainly composed of $nestin^{+}$ neural precursors (Klein *et al*., 2005). The lack of a benefit of the GDNF release within the spinal cord in this study could be due to ineffective anatomical targeting, rather than the identity of the cells secreting the growth factor, although this remains to be investigated. Thus, additional injection sites along the rostrocaudal axis of the spinal cord may improve motor neuron survival in the transgenic SOD rats. Specific transgene expression of GDNF in astrocytes of the $SOD1^{G93A}$ mouse had no effect on disease onset or progression (Li *et al*., 2006). However, it is likely that GDNF secretion from a normal astrocyte will give a more favorable result on motor neuron survival, based on previous findings of toxicity of mutant SOD-expressing glial cells (Beers *et al*., 2006; Boillee *et al*., 2006b; Di Giorgio *et al*., 2007; Nagai *et al*., 2007). Overexpression of GDNF by endogenous mutant SOD1-expressing or transplanted wild-type myoblasts delay onset and disease progression with concomitant decrease in motor neuron loss (Mohajeri *et al*., 1999; Li *et al*., 2006), indicating that muscle should be considered as a target for cellular and growth delivery therapies.

In a second study, transplantation of hNSCs derived from the cervical spinal cord resulted in a modest delay in disease onset and an increase in survival (Table 1), which correlated with a reduction in motor neuron loss (Xu *et al*., 2006). Grafted cells formed synapses on host rat motor neurons and the parenchyma and cerebrospinal fluid (CSF) of cell-grafted animals contained significantly higher levels of GDNF and brain-derived neurotrophic factor (BDNF) (Xu *et al*., 2006). Thus, it appears that the effect of hNSCs on degenerating motor neurons was mediated mainly by delivery of these growth factors, which exhibit known motor neuron protective effects (Henderson *et al*., 1993, 1994). The more positive outcome of this study may be related to improved graft placement, implantation into younger animals, difference in phenotype of implanted cells and growth factor secretion (Table 1) or even the use of different immunosuppressants, cyclosporin (Klein *et al*., 2005) vs. FK506 (Xu *et al*., 2006), given that FK506 has been shown to increase neurite outgrowth (Steiner *et al*., 1997).

Mouse NSCs (mNSCs) have been utilized for transplantation into presymptomatic nmd (Corti *et al*., 2006) and $SOD1^{G93A}$ mice (Corti *et al*., 2007). Stem cell populations were either isolated from embryonic spinal cords and subjected to fluorescent activated cell sorting (FACS) based on high aldehyde dehydrogenase (ALDH) expression and a low side scatter profile (Corti *et al*., 2006) or from adult brain and purified based on Lewis X (stage-specific embryonic antigen, SSEA-1) and the chemokine receptor CXCR4-expression (Corti *et al*., 2007). Cells were primed into a motor neuron phenotype and subsequently transplanted (Corti *et al*., 2006, 2007). Animals in both studies showed a 3-week delay in onset, correlated with a partial preservation of host spinal motor neurons, and also a 3-week increase in mean survival time (Corti *et al*., 2006; 2007) (Table 1). The mean disease progression time was not altered (Corti *et al*., 2007), demonstrating that the grafted cells had no beneficial effect once clinical disease had been initiated. The grafts were mainly composed of neurons, 20% of which were of a motor neuron phenotype, and also contained neural precursors, astrocytes and a small amount of oligodendrocytes (Corti *et al*., 2006, 2007). Morphological processes from grafted cells were present within the ventral roots, but no analysis of possible contributions to NMJs was performed (Corti *et al*., 2006). The beneficial effect of the grafted cells on host motor neuron survival and disease onset appeared to be through trophic support (Corti *et al*., 2006, 2007), based on graft release of IGF-1 and VEGF (Corti *et al*., 2007). The levels of these growth factors were measured within the lumbar spinal cord; however, no such data were obtained in other parts of the spinal cord, or in the CSF, precluding a more specific analysis of general trophic effects that may have contributed to the outcome. Histological end-stage analysis showed limited migration of the transplanted cells. If the growth factor release from these cells were only local, a delayed display of hindlimb paralysis would have been expected, given the lumbar spinal cord graft placement, and not a general delay in onset. However, if the growth factors diffused

throughout the spinal cord, which is the likely event in this case, the entire spinal cord would benefit from such trophic support.

It is still not known if an NSC graft approach, in which the main protective effect may be mediated by growth factor release, can be beneficial when initiated after the onset of symptoms, which is the most needed treatment period from the perspective of patients. Current NSC grafting studies on SOD1 transgenic animals have all utilized presymptomatic animals and relied on a small number of implantations sites, producing no or a small effects on disease progression time span (Klein *et al*., 2005; Corti *et al*., 2006, 2007; Xu *et al*., 2006). Nevertheless, it is possible that multiple grafting sites along the spinal cord would provide a more effective trophic support for a beneficial effect of NSCs on disease progression time span, thereby indicating their usefulness even after onset of disease.

Transplantation of bone marrow cells and umbilical cord cells

Human umbilical cord blood cells (hUCBCs) (Ende *et al*., 2000) and mouse bone marrow cells (mBMCs) (Ende *et al*., 2000; Corti *et al*., 2004) delay onset and increase survival with a few weeks following delivery into the presymptomatic $SOD1^{G93A}$ mouse (Table 1). Disease duration appeared only to have been affected in the mBMC group, indicating that this cell population could be effective also when delivered after onset of disease. Graft survival appeared limited in the hUCBCs transplanted animals, and the positive outcome could have been due to transient graft survival or the presence of cells in tissues not analysed, such as muscle and/or spinal cord. mBMCs were identified in brain, but to a higher extent in skeletal and heart muscle (Corti *et al*., 2004). Most striking was the contribution of transplanted cells to the microglial population, with almost one-third of the microglia being graft derived (Corti *et al*., 2004) (Table 1).

A recent study showed that expression of mutant SOD1 in muscle does not appear to affect either onset or survival of the disease, and neither does an enhancement of muscle mass (Miller *et al*., 2006). It therefore seems likely that the main effect of the mBMC transplant in the two studies mentioned above (Ende *et al*., 2000; Corti *et al*., 2004) may have been caused by a substitution of mutant microglia with wild-type cells. Indeed, CD11b-Cre-mediated removal of mutant SOD1 from the microglial population only, in $SOD1^{G37R}$ mice, was shown to prolong the mean survival of these animals by around 100 days (Boillee *et al*., 2006b). Additionally, when $PU1^{-/-}$ mice, which are unable to develop myeloid and lymphoid cells, were bred with $SOD1^{G93A}$ mice, wild-type donor-derived microglia substantially slowed disease progression in the resulting $SOD1^{G93A}/PU1^{-/-}$ mice (Beers *et al*., 2006). Furthermore, mES cell-derived motor neurons were selectively killed when transplanted onto spinal cord slices from $SOD1^{G93A}$ mice, which showed large microglial activation, but not when grafted onto mutant hippocampal or wild-type spinal cord slices containing less activated microglia (Kim *et al*., 2006). However, LPS stimulation of microglia rendered also hippocampal $SOD1^{G93A}$ slices toxic to motor neurons, showing that there was not a tissue-specific toxicity of microglia, but rather a regional-specific activation pattern (Kim *et al*., 2006). Indeed, both primary spinal cord and cortical glial cells over-expressing mutant SOD1 have been shown to be selectively toxic to motor neurons *in vitro* (Kim *et al*., 2006; Di Giorgio *et al*., 2007; Nagai *et al*., 2007), with secreted factors from astrocytes being more toxic to ES cell-derived motor neurons *in vitro* than those secreted from microglia (Nagai *et al*., 2007). Furthermore, lowering microglial activation in the $SOD1^{G37R}$ animals by minocycline administration in the late presymptomatic stage slowed disease progression, delayed motor neuron degeneration and increased the life span of the animals by approximately 5 weeks in the majority of the animals (Kriz *et al*., 2002). $SOD1^{G93A}$-overexpressing spinal cord tissue secretes higher levels of nitric oxide, interleukin (IL)-1β, IL-6 and IL-12p70 than wild-type spinal cords and lower levels of VEGF (Kim *et al*., 2006). Furthermore, neutralizing these factors could decrease the toxicity of the mutant spinal cords (Kim *et al*., 2006). IL-1β and IL-6 were found to be similarly produced by mutant and wild-type spinal cord astrocytes (Nagai *et al*., 2007), indicating that these factors might instead have been secreted from activated microglial cells.

Interestingly, as shown in both the transgenic approaches where mutant SOD1 was removed from microglia and in the minocycline study, expression of mutant SOD1 in microglia appears to be important for the progression of the disease (Beers *et al*., 2006; Boillee *et al*., 2006b). Expression of mutant SOD1 in motor neurons on the other hand appears to be important for disease onset and early disease progression (Boillee *et al*., 2006b). In the BMC transplantation studies there was a slight, but similar effect on both onset and duration of the disease (Ende *et al*., 2000; Corti *et al*., 2004) (Table 1). This discrepancy could perhaps be attributed to the much smaller proportion of mutant microglia being replaced with wild-type microglia in the case of the transplantation studies compared with the transgenic approaches (Corti *et al*., 2004; Beers *et al*., 2006; Boillee *et al*., 2006b). This could also account for the smaller effect in general on survival time. There is a relatively high mortality rate associated with allogenic BMC transplantation (for a review, see Grewal *et al*., 2003). The somewhat low benefit from these BMC transplants makes it an unlikely treatment strategy for motor neuron diseases. However, the insights gathered from these studies, including the demonstration that decreasing microglial activation (Kriz *et al*., 2002) or replacing mutant SOD1-overexpressing microglia with wild-type microglia (Ende *et al*., 2000; Corti *et al*., 2004) could delay the onset of disease, provide valuable ideas for the design of future therapies.

Cell transplantation aimed at replacing motor neurons

Transplantation of embryonic stem cell- or neural stem cell-derived motor neuron precursors and motor neurons

The selective degeneration of motor neurons, located in discrete regions of the brain and spinal cord, in ALS, SMA and SBMA, are encouraging for cell replacement strategies using stem cell-derived motor neurons (Isacson, 2003; Wichterle *et al*., 2002). Towards this goal, mouse ES cell-derived motor neurons were recently used successfully in a rat model of virus-mediated acute motor neuron death (Harper *et al*., 2004; Deshpande *et al*., 2006). Here, spinally implanted ES cell-derived motor neurons could extend axons into the ventral roots when animals were co-infused with dibuturyl cAMP (dbcAMP) (Harper *et al*., 2004), a molecule known to increase axon outgrowth (Cai *et al*., 2001; Qiu *et al*., 2002). Furthermore, if target muscles were transplanted with NSCs over-expressing GDNF and animals treated with dbcAMP and the phophodiesterase 4 inhibitor rolipram, which can also overcome myelin repulsion (Nikulina *et al*., 2003), significantly more axons extended into the ventral roots and the muscles, neuromuscular junctions were formed and the animals recovered partially from the paralysis (Deshpande *et al*., 2006) (Table 1).

Using another approach, hNSCs, primed into a motor neuron fate, were implanted into animals with chronic loss of motor neurons from a neonatal sciatic axotomy (Gao *et al*., 2005). Such transplantation appears to have given an initial behavioral improvement due to trophic effects from the grafted cells. Later, behavioral outcome appeared to correlate with transplanted motor axons reaching target muscles and

forming NMJs (Gao *et al*., 2005). However, a more rigorous analysis to clearly identify the graft-derived component of the NMJs is warranted to make such interpretations. In this study, no treatment was used to stimulate neurite outgrowth of grafted cells. Analysis of fetal ventral mesencephalic cell transplants from xenogeneic tissue (Brundin *et al*., 1985, 1988; Galpern *et al*., 1996) into lesion models such as the parkinsonian rat has revealed differences in the time needed to reverse disease symptoms, which primarily correlate with the rate of neuronal maturation of the donor tissue species (Isacson & Deacon, 1997). Therefore, the apparent ability of the hNSC-derived motor neurons to reach muscle targets without co-treatment with factors that would promote axon outgrowth, in the study by Gao *et al*., could be due to human cells being used instead of mouse, with the human cells reaching maturation more slowly than mouse cells, thereby enabling more extensive axon outgrowth, without myelin repulsion, for an extended period of time. Furthermore, the difference in predifferentiation strategies might in part cause this difference. In the mES cell studies, RA was used in combination with Shh, whereas in the hNSC study Shh, but no RA, was used. RA terminally differentiates cells into neurons, thereby decreasing the chance of teratoma formation but also the outgrowth properties of the neurites. It is likely that the cells transplanted in the hNSCs study were more immature than those transplanted in the mES cell study and thereby had a better chance to grow out and reach the target muscle without any additional manipulation. It is not yet known if similar cellular treatment approaches will be successful in a model of chronic motor neuron degeneration, such as ALS, and replacement of motor neurons in humans still faces vast challenges.

Additional transgenic animal models of motor neuron disease for further exploration of cellular therapies

Wobbler mouse as a model for motor neuron disease

The autosomal recessive wobbler (*wr*) mutation of the mouse causes spinal muscular atrophy and defective spermatogenesis (Falconer, 1956; Duchen & Strich, 1968; Mitsumoto & Bradley, 1982; Heimann *et al*., 1991). The mutation is associated with the gene that encodes for the vacuolar-vesicular protein sorting 54 (Vps54) and which plays a role in vesicular trafficking (Schmitt-John *et al*., 2005). The wobbler mouse displays only lower motor neuron disease and muscle weakness is largely restricted to the neck and forelimbs, with sparing of the hindlimbs (Falconer, 1956; Mitsumoto & Bradley, 1982; Mitsumoto & Gambetti, 1986). Neurodegeneration of the brain and spinal cord starts at two weeks of age, with motor neuron symptoms appearing from 3 to 4 weeks of age (Rathke-Hartlieb *et al*., 1999). The progression of the disease is slow, with a life expectancy of 6 months (Mitsumoto & Bradley, 1982). In contrast to the SOD1 transgenic models of ALS and the pmn mouse, motor neuron degeneration in the wobbler mouse appears to be a primary neuronopathy, initially affecting the motor neuron cell body, and subsequently causing an axonopathy (Mitsumoto & Bradley, 1982; Mitsumoto & Gambetti, 1986). In contrast to the SOD1 transgenic models of ALS, astrocyte and microglial activation has been reported to be initiated after motor neuron loss has begun (Rathke-Hartlieb *et al*., 1999). Consequently, a study using chimeras indicated that the effect of the *wr* gene (Vps54) is cell-autonomous (Augustin *et al*., 1997), although wobbler astrocytes have been shown to influence the survival of motor neurons (Ait-Ikhlef *et al*., 2000) and are probably contributing to motor neuron death in later stages of disease. The wobbler mouse has been widely used in growth factor delivery studies (Table 2). However, the early onset and slow progression of disease in this model in fact make it very appealing for future ES cell-derived motor neuron graft studies, where ample time exists to study the outgrowth of transplanted neurons and associated effects after possible reconnection with host muscle after onset of disease. In addition, the presence of activated microglia after onset in this model gives an opportunity to evaluate survival of grafted cells in a hostile environment.

Mice expressing androgen receptor with ≥100 CAG repeats as a model for SBMA

Animal models utilizing AR with 100 CAG repeats or more recapitulate SBMA well (Abel *et al*., 2001; McManamny *et al*., 2002; Sopher *et al*., 2004). Gait impairment is visible at 13–18 months of age and animals die at age 15–24 months depending upon AR100 expression level (Sopher *et al*., 2004). Mutant AR-induced death of motor neuron-like cells *in vitro* can be rescued by VEGF (Sopher *et al*., 2004) and inhibition of Hsp90 clearly slows down motor impairment in the AR100 transgenic mouse by reducing AR100 levels in the cells (Waza *et al*., 2005). Future experiments will determine if growth factor delivery and/or cell transplantation into this SBMA model can be successful.

Peripherin over-expression as a model of late-onset motor neuron disease

Transgenic mice over-expressing peripherin, a type III neuronal intermediate filament (IF) protein, develop a late-onset, selective motor neuron disease, characterized by a deficiency of neurofilament light (NF-L) protein, impaired axonal transport and IF inclusions (Beaulieu *et al*., 1999; Millecamps *et al*., 2006). Symptoms appear at 2 years of age (Beaulieu *et al*., 1999). Over-expression of peripherin in NF-L null mice results in an earlier onset disease with symptoms appearing around 8 months of age and paralysis at 14 months (Beaulieu *et al*., 1999). Interestingly, peripherin is also a component of IF inclusions in ALS patients (Corbo & Hays, 1992; Migheli *et al*., 1993) and in the SOD1 transgenic mouse models of ALS (Wong *et al*., 1995; Tu *et al*., 1996). However, neither up-regulation nor suppression of peripherin in $SOD1^{G37R}$ mice has any effect on disease onset, duration or loss of motor neurons, indicating that peripherin is not a contributing factor to motor neuron disease in this model (Lariviere *et al*., 2003). The peripherin over-expressing mice have not been used in cell- or trophic factor-delivery experiments, but could provide a useful setting due to the slow progression of disease.

Dynein-dynactin dysregulation as a model of motor neuron disease

Recently, manipulations of dynein-dynactin-mediated axonal transport have been shown to cause late-onset motor degeneration in mice, without a significant change in life span (LaMonte *et al*., 2002; Hafezparast *et al*., 2003). The resulting animal models have so far not been used in cell transplantation or growth factor delivery experiments, but they may have potential for studies of cellular integration.

Motor neuron degeneration (mnd) mouse as a model of motor neuron disease

The mnd or *m*/m mouse is an autosomal dominant mouse mutant characterized by late-onset (5–11 months) hindlimb weakness

progressing to spastic paralysis of all limbs and premature death, resulting from degeneration of upper and lower motor neurons (Messer & Flaherty, 1986; Messer *et al*., 1987). Similar to ALS, spinal motor neurons show deposits of ubiquitin and changes in neurofilament distribution (Callahan *et al*., 1991; Mazurkiewicz, 1991; Mazurkiewicz *et al*., 1993). However, cells in various brain regions and in the retina, which are not affected in ALS, degenerate in this model (Messer *et al*., 1993, 1987). The mnd mouse has been previously used in growth factor delivery studies aimed at neuroprotection prior to onset (Table 2). The relatively slow progression of disease could provide a useful platform also for studies on cellular integration.

Progressive motor neuropathy (pmn) mouse as a model of motor neuron disease

The pmn mouse is an autosomal-recessive mutant displaying retrograde degeneration of motor axons followed by death of motor neuron cell bodies. Hindlimb muscle weakness is evident at the end of the third postnatal week and all the mice die at age 6–7 weeks (Schmalbruch *et al*., 1991). Mutations in the tubulin-specific chaperone E (Tbce), which plays a critical role in microtubule stability, is responsible for this progressive motor neuronopathy (Bommel *et al*., 2002; Martin *et al*., 2002). This animal model has been extensively used in earlier growth factor delivery studies (Table 2). The fast progression of disease in this model makes it unsuitable for analysis of motor neurons replacement after initiation of onset, as the axons of transplanted cells will not have sufficient time to reach their targets. However, this model could be used to analyse the effect of cellular transplants aiming to replace host microglia or delivering growth factors.

Smn null/SMN2 mice as model for SMA

SMA is caused by mutations in the telomeric survival motor neuron gene (SMN1) (Bussaglia *et al*., 1995; Lefebvre *et al*., 1995; Parsons *et al*., 1996; Hahnen *et al*., 1997; Talbot *et al*., 1997). Species other than humans have only one SMN gene (DiDonato *et al*., 1997; Viollet *et al*., 1997) and deletion of this is embryonically lethal (Schrank *et al*., 1997). The human SMN2 gene can, however, rescue the embryonic lethality of the Smn null mice, creating a mouse with a severe form of spinal muscular atrophy that dies at the end of the first postnatal week (Monani *et al*., 2000). This model has so far not been used in any cell transplantation or growth factor delivery experiments. Perhaps a mutant line utilizing a higher copy number of the human SMN2 gene might slow disease progression and thus offer more opportunities for studying neuroregeneration and neuroprotection.

Prospects and considerations for future use of stem cells transplantation in motor neuron diseases

From the reviewed studies it appears that the use of stem cell transplantation for release of trophic factors and/or replacement of motor neuron, interneurons and/or microglia are valuable approaches. Although it is clear that ES cells can differentiate into functional motor neurons *in vitro* (Wichterle *et al*., 2002; Harper *et al*., 2004; Miles *et al*., 2004; Li *et al*., 2005), it still remains to be elucidated how the differentiation can be directed to generate specific subtypes of motor neurons. Using currently available protocols, >95% of the motor neurons formed from mES cells appear to be of a medial motor column neuron identity with the preferential projection to axial musculature after transplantation (Soundararajan *et al*., 2006). Given that in motor neuron diseases there is a need to replace also lateral motor column neurons, which innervate limb musculature, it is pivotal to be able to apply the cues that determine the difference in developmental lineages between medial and lateral motor column motor neurons to cultures *in vitro*. Furthermore, motor neurons of mainly a cervical identity can be generated from mES cells (Wichterle *et al*., 2002), whereas motor neurons of a thoracic character can be induced from hES cells (Li *et al*., 2005). The generation of motor neurons of a more posterior phenotype from hES than mES cells has been explained by the use of FGF2, which has caudalizing activity (Kudoh *et al*., 2002), to neuralize the hES cells, followed by an early RA treatment and subsequent addition of Shh (Li *et al*., 2005). For mES cells, RA alone was used to neuralize and caudalize the cells (Wichterle *et al*., 2002). For transplantation to motor neuron diseases, it is necessary to be able to pattern cells *in vitro* properly to generate spinal motor neurons of a lumbar, thoracic and cervical identity.

In the developing embryo, it has been carefully delineated that both the identity of motor neurons along the rostrocaudal axis of the spinal cord as well as the motor neuron columnar and pool identity and thereby that target–muscle connectivity are established through Hox regulatory networks (Dasen *et al*., 2003; Dasen *et al*., 2005). The sequential phases of Hox genes are in turn activated in response to graded FGF, growth and differentiation factor 11 (Gdf11) and RA signals (Liu *et al*., 2001; Dasen *et al*., 2003; Liu, 2006). The careful sequential use of these signals together with Shh in ES cell cultures could perhaps enable the differentiation of ES cells into motor neurons of all spinal rostrocaudal identities as well as of correct target-muscle connectivity type. In addition to the loss of spinal motor neurons, cortico-spinal, bulbo-spinal and rubro-spinal motor neurons are also lost in ALS. Quantification using the $SOD1^{G93A\ G1H}$ mouse showed that close to half of these neurons were lost in animals at the age when symptoms occur (Zang & Cheema, 2002). Furthermore, evidence from clinical studies (Eisen & Weber, 2001) and analysis of the $SOD1^{G93A}$ mouse (Browne *et al*., 2006) indicate that abnormalities in cerebral motor pathways precede spinal cord pathology, perhaps leading to anterograde trans-neuronal degeneration of motor neurons of the spinal cord in ALS. It therefore appears that motor neurons of the cortex and the brainstem might need to be replaced in ALS, and that it is of importance to know how to also generate these *in vitro and* how to promote their outgrowth in vivo after transplantation. Recently, several factors that are involved in the specification of cortico-spinal motor neurons (CSMNs), such as Fezl (Molyneaux *et al*., 2005), and axonal projection of CSMNs to the spinal cord, for instance Ctip2 (Arlotta *et al*., 2005), were defined. Furthermore, IGF-I was shown to increase axon outgrowth of CSMNs whereas BDNF induced branching and arborization (Ozdinler & Macklis, 2006). An ES cell transplantation approach could potentially utilize these different factors initially to genetically engineer cells to develop into an appropriate CSMN *in vitro* and thereafter drive the cell to reach and synapse onto its targets after transplantation by the use of growth factor delivery.

For appropriate exposure of ES cells to different signaling molecules it might be necessary to grow the cells in structures more resembling neural tubes, and using a point source from where growth factors are secreted. Furthermore, it will be exceptionally difficult to generate motor neurons of one identity only, e.g. lumbar motor neurons, which can innervate axial muscles, in the cultures. Therefore, to specifically select appropriate motor neurons to be transplanted into a specific site in the spinal cord or the brain will probably necessitate the isolation of specific motor neuron types from the cultures by the use of surface markers and FACS.

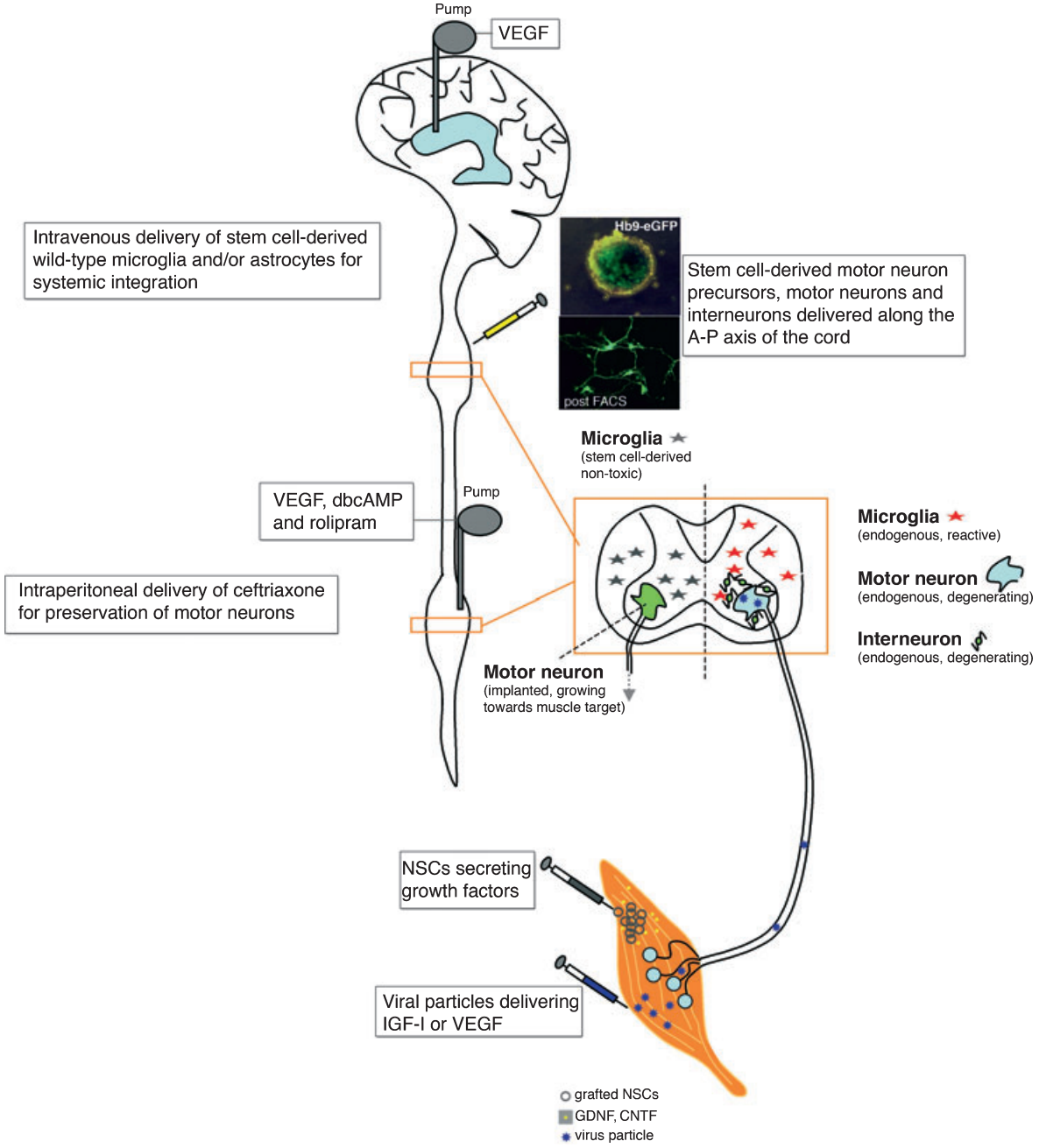

FIG. 1. Possible combination treatment strategies for motor neuron diseases using cellular transplants and growth factors. A treatment strategy to replace degenerating motor neurons and reactive immunological cells such as microglia while preserving remaining endogenous motor neurons could include several of the following procedures: (i) intraspinal implantation of ES cell-derived motor neuron precursors, motor neurons as well as interneurons along the A–P axis of the spinal cord, (ii) intramuscular placement of cells secreting growth factors to attract axons of grafted motor neurons, e.g. GDNF, (iii) intrathecal delivery of dbcAMP for increased axonal outgrowth of transplanted motor neurons, (iv) intravenous delivery of ES cell- or NCS-derived microglia and astrocytes for systemic replacement of host reactive glial cells, (v) intramuscular placement of cells secreting growth factors to preserve endogenous motor neuron terminals, e.g. CNTF, (vi) intracerebroventricular and intrathecal delivery of VEGF through infusion pumps to preserve endogenous upper, bulbar and lower motor neurons, (vii) intramuscular delivery of viral vector-expressing growth factors such as IGF-I or VEGF for retrograde transport and neuroprotection of remaining endogenous motor neurons and for attraction of grafted CSMNs axons, and (viii) intraperitoneal delivery of ceftriaxone to regulate glutamate levels (Rothstein *et al*., 2005).

Furthermore, the axons of transplanted motor neurons will encounter an environment that is hostile to axon outgrowth (Kolf *et al*., 2007). In addition, studies on retinal ganglion cells and brain stem neurons have shown that CNS neurons fail to regenerate not only due to CNS glial inhibition, but also due to loss of intrinsic axonal growth capacity during maturation (Goldberg *et al*., 2002; Blackmore & Letourneau, 2006). Even if these extrinsic and intrinsic contributions to growth inhibition can be overcome, as shown in the studies discussed above, the axons of transplanted motor neurons would need an extensive amount of time to reach target muscles of an adult. Assuming that a transplanted motor neuron axon could grow at the fastest rate of a regenerating motor axon (approaching 4 mm/day; Fugleholm *et al*., 1994), it would take 250–300 days to reach the muscle targets in an adult. Should the growth rate be much less, which is most likely, it would take more than a year before NMJs could begin to form. In some of the more aggressive forms of ALS, the patient has

less than a year to live from the time of diagnosis and would therefore not benefit from such a treatment strategy. Infants with types II and III SMA and patients with Kennedy's disease or a slow progressing form of ALS are more likely to benefit from such a treatment strategy. Furthermore, the cell bodies of the motor neurons, which are lost in ALS, are dispersed throughout the spinal cord, brain stem and the motor cortex of the brain, requiring extensive implantation surgery with multiple injections to accomplish significant medical recovery.

In addition to replacing lost host motor neurons it is appealing to consider the replacement of reactive host microglia and/or astrocytes with normal glial cells, which might cause less damage to endogenous as well as transplanted motor neurons. From the studies by Corti *et al*. it is evident that microglia can be generated from BMCs (Corti *et al*., 2004). Due to the risks associated with non-autologous bone marrow transplantation ES or NSC cells would perhaps be a better source of these cells. In fact, astrocytes (Brustle *et al*., 1999) as well as microglia can also be derived from mES cells (Tsuchiya *et al*., 2005). mES cell-derived microglia have shown limited migration capacity (Tsuchiya *et al*., 2005), but adaptations in the differentiation protocol might result in cells with a more widespread distribution and with potential for further studies in animal models of motor neuron loss. Furthermore, NSCs, which have an extensive migration capacity, directed towards sites of injury (Snyder *et al*., 1997; Flax *et al*., 1998; Aboody *et al*., 2000; Imitola *et al*., 2004) can reduce host microglial activation significantly after intraventricular transplantation into an animal model of Sandhoff's neurodegenerative metabolic disease (Lee *et al*., 2007). The effect of combined intrathecal and intraventricular transplantation of unprimed NSCs on models of ALS for suppression of host glial cells would be interesting to explore.

Finally, we believe that combining cellular replacement strategies with growth factor delivery could give the most beneficial effects in motor neuron diseases and that such treatment would include several of the following steps (Fig. 1): (i) multiple intraspinal injections of ES cell-derived motor neuron precursors, motor neurons and interneurons transplanted to replace dying motor neurons and interneurons, (ii) intramuscular injection of NSCs secreting GDNF to attract grafted motor neurons to their target muscles, (iii) intrathecal delivery of dbcAMP and rolipram for increased axonal outgrowth of transplanted motor neurons, (iv) intravenous injection of BMCs or ES cell- or NCS-derived microglia and astrocytes for systemic replacement or suppression of reactive host glial cells, (v) intramuscular placement of cells secreting growth factors, e.g. CNTF, to preserve endogenous motor neuron terminals, (vi) use of intracerebroventricular and intrathecal pumps to infuse VEGF for preservation of the still-remaining endogenous upper and lower motor neurons, (vii) viral delivery of IGF to endogenous motor neurons through intramuscular injection and retrograde transport, and (viii) intraperitoneal delivery of ceftriaxone to regulate glutamate levels and preserve endogenous motor neurons (Rothstein *et al*., 2005).

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Abbreviations

ALS, amyotrophic lateral sclerosis; BMCs, bone marrow cells; BNDF, brain-derived neurotrophic factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; ES, embryonic stem; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; IGF-1, insulin-like growth factor-1; NMJs, normal neuromuscular junctions; NSCs, neural stem cells; RA, retinoic acid; SBMA, spinal bulbar muscular atrophy; Shh, sonic hedgehog; SMA, spinal muscular atrophy; SMARD1, spinal muscular atrophy with respiratory distress 1; SOD1, Cu/Zn superoxide dismutase 1; UCBCs, umbilical cord blood cells; VEGF, vascular endothelial growth factor.

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