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Genetic strategies to study TDP-43 in rodents and to develop preclinical therapeutics for amyotrophic lateral sclerosis

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Abstract

The neuropathological hallmark of the majority of amyotrophic lateral sclerosis (ALS) and a class of frontotemporal lobar degeneration is ubiquitinated cytoplasmic aggregates composed of transactive response DNA binding protein 43 kDa (TDP-43). Genetic manipulation of TDP-43 in animal models has been used to study the protein's role in pathogenesis. Transgenic rodents for TDP-43 have recapitulated key aspects of ALS such as paralysis, loss of spinal motor neurons and muscle atrophy. Viral vectors are an alternate approach to express pathological proteins in animals. Use of the recombinant adeno-associated virus vector serotype 9 has permitted widespread transgene expression throughout the central nervous system after intravenous administration. Expressing TDP-43 in rats with this method produced a phenotype that was consistent with and similar to TDP-43 transgenic lines. Increased levels of TDP-43 in the nucleus are toxic to neurons and sufficient to produce ALS-like symptoms. Animal models based on TDP-43 will address the relationships between TDP-43 expression levels, pathology, neuronal loss, muscle atrophy, motor function and causative mechanisms of disease. New targets that modify TDP-43 function, or targets from previous ALS models and other models of spinal cord diseases, could be tested for efficacy in the recent rodent models of ALS based on TDP-43. The vector approach could be an important therapeutic channel because the entire spinal cord can be affected from a one-time peripheral administration.

Keywords

adeno-associated virus; amyotrophic lateral sclerosis; frontotemporal lobar degeneration; gene therapy; gene transfer

Introduction

Advances in the development of animal models expressing neuropathological proteins have led to increased understanding of neurodegenerative disease mechanisms. Rodents are a gateway for drug development to man because of their similar anatomy and well-

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characterized models. Rodent models are also faster than larger animals such as cats, dogs, pigs and monkeys. Transgenic and knockout manipulation of the germ-line is a gold-standard approach to study disease hallmark pathologies because of the permanent and consistent effect on gene expression levels. Complementary expression systems can offer higher throughput and more rapid and thus cost-effective means to address key questions about pathogenesis. Research on amyotrophic lateral sclerosis (ALS) has historically been focused on familial forms of the disease involving mutations in superoxide dismutase 1 (SOD1). Following the discovery of the role of transactive response DNA binding protein 43 kDa (TDP-43) in ALS and other neurodegenerative diseases, a number of model systems have been used, which were developed during the course of studying other hallmark neuropathological proteins such as amyloid precursor protein, microtubule-associated protein tau, alpha-synuclein, huntingtin and SOD1. Based on the knowledge gained from the earlier research on these proteins, advances on animal models of TDP-43 and the underlying disease mechanisms have evolved quickly. As most neurodegenerative diseases have limited therapeutic options, it is naturally hoped that the rapid advancements in model development will drive towards clinical translation.

TDP-43 is a predominantly nuclear protein with functions related to transcription and RNA processing and transport (Buratti & Baralle, 2008). In 2006, it was discovered that TDP-43 is a major component of the cytoplasmic ubiquitinated inclusions in the neurodegenerative diseases frontotemporal lobar degeneration with ubiquitin-positive inclusions and ALS (Arai *et al.*, 2006; Neumann *et al.*, 2006). The branch of frontotemporal lobar degeneration with TDP-43 pathology has since been renamed FTLD–TDP (Mackenzie *et al.*, 2010). The neuropathological pattern in these diseases involves TDP-43 mislocalization to the cytoplasm, a clearing of nuclear TDP-43, and ubiquitinated and hyperphosphorylated inclusions found throughout the central nervous system (CNS) (Neumann *et al.*, 2006; Geser *et al.*, 2008), although pathological markers may vary depending on the CNS region affected (Igaz *et al.*, 2008; Neumann, 2009; Mackenzie *et al.*, 2010). A recent study implicates deficient nuclear import of TDP-43 leading to its cytoplasmic deposition (Nishimura *et al.*, 2010). TDP-43 can form inclusions within neuronal nuclei, dystrophic neurites and glia in FTLD–TDP subtypes (Neumann *et al.*, 2007; Mackenzie *et al.*, 2010). FTLD–TDP and ALS may share similar mechanisms of pathogenesis involving TDP-43, and both diseases lack efficacious medicine to slow disease progression. ALS is a neuromuscular disease involving the loss of motor neurons and atrophy of the muscles that they innervate, with differential onset of symptoms that can be tied to early degeneration of either brainstem or spinal motor neurons (Eisen, 2009). Familial mutations account for 10% of the ALS population (Van Damme & Robberecht, 2009). The vast majority of ALS research is focused on SOD1 (Rosen *et al.*, 1993; Boillée *et al.*, 2006), although it has been estimated that only 2% of ALS involves mutations in SOD1 (Mackenzie *et al.*, 2007). However, TDP-43 neuropathology is found in both sporadic and familial ALS, estimated to be as much as 98% of ALS (Mackenzie *et al.*, 2007). An enormous wealth of resources and knowledge has accrued from studying familial SOD1 mutations (Boillée *et al.*, 2006), although there remains much frustration in terms of translational value. Because the vast majority of ALS is now known to involve some degree of TDP-43 pathology, research on this protein may be more broadly relevant to ALS.

Familial mutations in TDP-43 associated with ALS (Gitcho *et al.*, 2008; Sreedharan *et al.*, 2008) suggest either loss of function or toxic gain of function as possible pathogenic mechansims. Yeast (Johnson *et al.*, 2008), *Caenorhabditis elegans* (Ash *et al.*, 2010; Liachko *et al.*, 2010), *Drosophila* (Li *et al.*, 2010), zebrafish (Kabashi *et al.*, 2010) and chick embryo (Sreedharan *et al.*, 2008) models have recapitulated the molecular and cellular aspects of FTLD–TDP and ALS in order to address the key question of TDP-43 function/dysfunction in disease (Gendron *et al.*, 2010). These animal models, complemented by cell culture

models (e.g. Caccamo *et al.*, 2009; Nonaka *et al.*, 2009; Zhang *et al.*, 2009; Barmada *et al.*, 2010), offer rapid screening of drugs and gene targets to address the mechanisms. The logical next step from any of these high-throughput systems is translation to a mammalian system, usually in a rodent or a non-human primate model. Due to their relevant neuroanatomy and myoanatomy, and well-characterized behavioral and toxicological paradigms, mice and rats are essential for proof of concept discoveries on TDP-43 function as they relate to disease.

TDP-43 overexpression in rodents via germ-line manipulation

The breakthrough study by Neumann *et al.* (2006) established TDP-43 as a neuropathological substrate protein in FTLD–TDP and ALS, and sparked efforts to generate transgenic mice based on this protein (Wegorzewska *et al.*, 2009; Shan *et al.*, 2010; Stallings *et al.*, 2010; Tsai *et al.*, 2010; Wils *et al.*, 2010; Xu *et al.*, 2010; Zhou *et al.*, 2010; Igaz *et al.*, 2011). Different promoter strategies have been used to drive expression, e.g. ubiquitous expression with the prion promoter (Wegorzewska *et al.*, 2009; Stallings *et al.*, 2010; Xu *et al.*, 2010), specific neuronal expression with the thymus cell antigen 1 promoter (Shan *et al.*, 2010; Wils *et al.*, 2010) or conditional expression with the forebrain specific calcium–calmodulin-dependent kinase II (CaMKII) promoter (Tsai *et al.*, 2010; Igaz *et al.*, 2011). A consensus of motor effects and morbidity and mortality has generally resulted from TDP-43 overexpression despite the different promoter strategies, form of TDP-43 used and the degree of TDP-43 pathology, underscoring great sensitivity to changes in TDP-43 levels and functionality (Table 1). ALS and FTLD–TDP are separated in Table 1, which is an oversimplification because there are both spinal cord and brain effects in the animal models and in disease (Geser *et al.*, 2008).

The first transgenic TDP-43 mice reported in the literature utilized the A315T mutant form of human TDP-43 linked to familial ALS, whereas wild-type TDP-43 mice were embryonic lethal in that study (Wegorzewska *et al.*, 2009). The mutant TDP-43 mice displayed several salient features of ALS, such as limb paralysis and muscle wasting over 3–9 months, and electromyographic traces from the hindlimb consistent with denervation. There was selective ubiquitination in layer V of the cortex and in the spinal cord, which could have included upper and lower motor neurons, although disease-like cytoplasmic hyperphosphorylated aggregates of TDP-43 were not detected. However, there was evidence of disease-relevant TDP-43 protein fragments. The mice used by Wegorzewska *et al.* (2009) are a milestone in the progress on TDP-43′s role in ALS, and many of their features have been reproduced in other TDP-43 mice and rats. Wils *et al.* (2010) were able to generate wild-type TDP-43 mice, with several lines with different levels of overexpression. This yielded time- and gene-dosage-dependent effects of TDP-43 to induce functional motor disease states, with more rapid, similar or slower time courses relative to the A315T mice (Wegorzewska *et al.*, 2009) depending on the TDP-43 level. The two studies shared many similar outcomes of paralysis, morbidity, mortality, motor neuron loss, gliosis and, notably, similar ubiquitination in the cortex and spinal cord. The study of Wils *et al.* (2010) showed evidence of apparent proteolytic cleavage of TDP-43 that corresponded with the severity of the disease state, and disease-relevant cytoplasmic and nuclear inclusions of hyperphosphorylated TDP-43 (Arai *et al.*, 2006, 2010; Neumann *et al.*, 2006), which were not found in the mice used by Wegorzewska *et al.* (2009). TDP-43 phosphorylation was determined with phospho-specific antibody, and cytoplasmic aggregates have been detected in several studies by this method (Stallings *et al.*, 2010; Xu *et al.*, 2010; Zhou *et al.*, 2010; Igaz *et al.*, 2011). Regarding the relevant recapitulation of TDP-43 proteinopathy in glial cells (Nishihira *et al.*, 2008; Zhang *et al.*, 2008), several transgenic models have used neuron-specific promoters, such as thymus cell antigen 1 and CaMKII (Tsai *et al.*, 2010; Wils *et al.*, 2010; Igaz *et al.*, 2011), whereas one study using the ubiquitous prion protein

promoter reported an increase in nuclear, but not cytoplasmic, TDP-43 within glia (Wegorzewska *et al.*, 2009).

Two studies modeled FTLD–TDP by overexpressing TDP-43 with the CaMKII promoter, which is active in the hippocampus, striatum and cortex but not in other brain regions such as the cerebellum and spinal cord (Tsai *et al.*, 2010; Igaz *et al.*, 2011). The CaMKII promoter is also developmentally regulated, which is advantageous to avoid embryonic lethality. In the study of Tsai *et al.* (2010), TDP-43 mice were deficient in multiple memory tasks by 2 months, concomitant with electrophysiological changes in long-term potentiation and miniature excitatory postsynaptic currents, and neuronal loss in the hippocampus. Surprisingly, motor impairments developed in these mice by 6 months of age, similar to other TDP-43 transgenic mice using different promoters, suggesting that damage to upper motor neurons and the corticospinal tract contributes to the motor pheno-types, as no spinal motor neuron loss or spinal TDP-43 expression was observed. The study of cognitive dysfunction is relevant to frontotemporal lobar degeneration (Chen-Plotkin *et al.*, 2010; Geser *et al.*, 2010) as well as ALS (Abe *et al.*, 1997). However, in TDP-43 transgenic mice with spinal expression, damage to the motor system renders memory-related tasks unfeasible. Furthermore, the sensitivity of the assays of cognitive function may not be able to detect subtle changes, although memory-related performance was impaired when TDP-43 expression was limited to the forebrain (Tsai *et al.*, 2010). Another way of studying the effects of TDP-43 on memory-related behaviors would be to target expression to specific brain regions such as the hippocampus with vector gene transfer, which we have been attempting.

The study of Igaz *et al.* (2011) utilized a CaMKII/tetracycline operator promoter system, whereby forebrain expression of TDP-43 was induced when mice were withdrawn from the drug doxycycline. The authors used this to compare the effects of human TDP-43 with a defective nuclear localization signal (resulting in cytoplasmic expression) or wild-type TDP-43 (nuclear expression) without developmental effects. Both forms of TDP-43 induced relevant FTLD–TDP neuropathology and degeneration. However, the mutated TDP-43 that forced cytoplasmic expression was surprisingly unable to potentiate disease-relevant pathology in terms of increased cytoplasmic inclusions. Furthermore, the cytoplasmic TDP-43 expression exacerbated the depletion of endogenous mouse TDP-43, which correlated with neuron loss and motor dysfunction. Simultaneous and consistent with the loss of endogenous mouse TDP-43, there were gene expression changes in chromatin assembly pathways. The mice used by Igaz *et al.* (2011) are unique for the conditional TDP-43 expression pattern, and offer two distinct mechanisms of TDP-43 toxicity, in either the nucleus or cytoplasm, that can be studied.

Mitochondrial dysfunction has been implicated in the neurodegenerative mechanism of TDP-43 (Shan *et al.*, 2010; Xu *et al.*, 2010), based on research on ALS and transgenic SOD1 mice (Boillée *et al.*, 2006). The TDP-43 mice in Xu *et al.* (2010) showed abnormal clustering of degenerating mitochondria that formed aggregates in the spinal motor neurons, concomitant with changes in expression of specific mitochondrial gene products. These mice also had an overall motor phenotype similar to the TDP-43 mice used by Wegorzewska *et al.* (2009) and Wils *et al.* (2010). Utilizing a double transgenic strategy to visualize mitochondria, TDP-43 mice in Shan *et al.* (2010) also demonstrated mitochondrial clustering in neuron cell bodies coinciding with depletion of mitochondria in neuromuscular junction nerve terminals, which could have been related to their motor dysfunction. This study reported an increase in and altered localization of Gemini coiled bodies, which are nuclear structures involved in premessenger RNA splicing. There were also specific changes in gene expression, such as decreases in neurofilament proteins that could have been related to the axonopathy that was found in the ventral roots of the spinal cord. It is of note that

knockdown of TDP-43 expression within this study blocked the formation of Gemini bodies in the nucleus. The onset and severity of motor deficits were more rapid and pronounced in the males of this TDP-43 transgenic line, coinciding with increased TDP-43 accumulation in their spinal cords.

Another emerging theme is the loss of endogenous mouse TDP-43 expression when human TDP-43 is overexpressed. The TDP-43 mice from Xu *et al.* (2010) and those from Igaz *et al.* (2011) showed loss of endogenous mouse TDP-43 mRNA and protein, respectively, which could have relevance in terms of the loss of TDP-43 staining in the nucleus in disease samples (Neumann *et al.*, 2006, 2007) and transgenic models (Wegorzewska *et al.*, 2009; Wils *et al.*, 2010). The mice may be downregulating their own TDP-43 levels (Ayala *et al.*, 2011) in the face of overexpression, or cytoplasmic TDP-43 aggregates could be recruiting nuclear TDP-43 to the cytoplasm (Caccamo *et al.*, 2009; Nonaka *et al.*, 2009).

The functional impact of inherited familial mutations in TDP-43 has been studied using transgenic mice. Results from yeast (Johnson *et al.*, 2008), C. elegans (Liachko *et al.*, 2010), zebrafish (Kabashi *et al.*, 2010), chick embryos (Sreedharan *et al.*, 2008) and cell culture (Barmada *et al.*, 2010) have implicated the specific pathogenicity of familial mutations such as the M337V and A315T TDP-43 mutations. Similar conclusions have been reached in TDP-43 transgenic mice with either the A315T or M337V TDP-43 mutations (Stallings *et al.*, 2010) and in TDP-43 transgenic rats using the M337V TDP-43 mutation (Zhou *et al.*, 2010). However, both wild-type (Wils *et al.*, 2010; Xu *et al.*, 2010) and mutant TDP-43 (Wegorzewska *et al.*, 2009; Zhou *et al.*, 2010) have led to comparably robust ALS-relevant phenotypes.

Transgenic rats for mutant TDP-43 manifest an ALS-relevant motor phenotype with paralysis, motor neuron loss and muscle atrophy (Zhou *et al.*, 2010), so the consequence of overexpressing TDP-43 is shared in mice and rats. A `tet-off' promoter system was used to avoid overexpression during embryogenesis. This model yielded unique evidence for the direct ubiquitination of TDP-43 by co-immunoprecipitation, not reported in TDP-43 mice. Rat models are particularly advantageous for fine anatomical manipulations, and specific behavioral and toxicological assays designed for rats, such as food-reward paradigms.

A variety of germ-line strategies have been employed to express mutant and wild-type TDP-43 in rodents involving different regulatable or conditional promoters, achieving TDP-43 expression in select tissues and cell types and at certain ages (Table 1). Despite the different strategies, increasing TDP-43 levels in the nucleus results in consistent functional outcomes such as paresis/paralysis and morbidity (Wegorzewska *et al.*, 2009; Shan *et al.*, 2010; Stallings *et al.*, 2010; Tsai *et al.*, 2010; Wils *et al.*, 2010; Xu *et al.*, 2010; Zhou *et al.*, 2010; Igaz *et al.*, 2011), with several studies showing modest loss of spinal motor neurons (Wegorzewska *et al.*, 2009; Wils *et al.*, 2010; Zhou *et al.*, 2010) and muscle wasting (Wegorzewska *et al.*, 2009; Stallings *et al.*, 2010; Zhou *et al.*, 2010). Despite similar behavioral outcomes, some of the main differences among the lines involve TDP-43 inclusion formation and apparent proteolytic processing (Wegorzewska *et al.*, 2009; Shan *et al.*, 2010; Igaz *et al.*, 2011), which suggest that these features are non-essential for the phenotype of motor paralysis.

TDP-43 knockout mice

Loss of TDP-43 function may play a role in disease pathogenesis due to the clearing of nuclear TDP-43 that occurs concomitantly with cytoplasmic inclusions. TDP-43 knockout flies in the study of Feiguin *et al.* (2009) had motor impairment and defects in motor neuron synapses, which could be rescued by the expression of human TDP-43. An antisense oligonucleotide strategy in zebrafish reduced TDP-43 levels and induced a swimming

impairment, similar to that seen when TDP-43 was overexpressed (Kabashi *et al.*, 2010). That a similar motor phenotype could be caused in zebrafish by either TDP-43 knockdown or TDP-43 overexpression suggests that loss of TDP-43 function could underlie both disease and, paradoxically, the phenotype when TDP-43 is overexpressed in animals. Studying the loss of TDP-43 function in mice has been challenging due to embryonic lethality, and has underscored the great sensitivity to altering levels of TDP-43 in rodents. Homozygous knockouts are consistently embryonic lethal (Kraemer *et al.*, 2010; Sephton *et al.*, 2010; Wu *et al.*, 2010), which suggests a critical function during embryogenesis that lacks redundancy with other ribonucleoproteins. However, heterozygous mice do not show any level of TDP-43 knockdown, consistent with TDP-43 being able to regulate its own levels (Ayala *et al.*, 2011). The TDP-43 heterozygous knockouts from Kraemer *et al.* (2010) had muscle weakness after 1 year, although there was no evidence of neuronal loss or muscle atrophy. To permit the study of homozygous knockout in mice, Chiang *et al.* (2010) employed a loxP/Cre recombinase system to create conditional knockout mice when the drug tamoxifen was given. Homozygotes had a drastic decrease in TDP-43 expression that led to weight loss and death, and concomitant dysregulation of genes involved with obesity. Modeling the loss of TDP-43 function in rodents is limited by the embryonic lethality and tight regulation of TDP-43 levels, and has yet to produce ALS-related sequelae such as loss of motor neurons or muscle atrophy.

TDP-43 overexpression in rats via somatic cell gene transfer strategy

The viral vector overexpression of neuropathological proteins is also used to mimic salient features of neurodegenerative diseases in rodents or non-human primates (e.g. Senut *et al.*, 2000; Kirik *et al.*, 2002, 2003; Klein *et al.*, 2002, 2004). The injection of a gene vector can target disease-relevant areas, which was advantageous for overexpression of the Parkinson's disease-related protein alpha-synuclein (Kirik *et al.*, 2002; Klein *et al.*, 2002; Lo Bianco *et al.*, 2002) because, overall, the transgenic mice do not exhibit disease-relevant loss of dopamine neurons in the substantia nigra (Chesselet, 2008). Adeno-associated virus (AAV) is a single-stranded DNA virus that is not attributed to a specific known disease or pathology (Daya & Berns, 2008). The recombinant vectors based on AAV express no viral genes and are therefore considered to be relatively safe. AAV vectors have been administered to humans in clinical research for Parkinson's disease using several different gene targets (Kaplitt *et al.*, 2007; Christine *et al.*, 2009; Marks *et al.*, 2010). Because AAV vectors are efficient for expressing specific genes in neurons without causing nonspecific side effects due to the vector, they have become a key tool in basic neuroscience research.

Compared with germ-line mice, vector-based models do not produce the same level of expression and are more subject to variability due to gene transfer efficiency in each individual subject. However, stable long-term expression levels and reproducible results have demonstrated sufficient consistency to address hypotheses that would take longer and cost more in germ-line transgenics to answer similar questions. Gene variants could be quickly screened and compared in a rodent without having to generate, maintain and genotype transgenic lines. Improved targeting, facility for gene combinations and the presence of within-subject non-transduced internal controls are some of the unique advantages of the vector approach. The ability to turn on expression in specific CNS regions gives the experimenter control of optimizing the expression and disease relevance, as well as avoiding embryonic lethality. If the vector technique can be sufficiently consistent in producing ALS-like symptoms, it could be more cost-effective than transgenic lines, given the advent of intravenous gene delivery with the AAV type 9 vector, which widely transduces the spinal cord and brain (Foust *et al.*, 2009).

Tatom *et al.* (2009) studied focal injections of an AAV9 human TDP-43 vector to the substantia nigra of rats, a brain region with TDP-43 pathology and/or neuron loss in several neurodegenerative diseases with parkinsonian symptomatology (Leverenz *et al.*, 2007; Geser *et al.*, 2008; Zhang *et al.*, 2008; Wider *et al.*, 2009). The AAV9 TDP-43 produced potent toxicity to the dopamine neurons that was vector dose-dependent, along with a progressive effect on rotational behavior over time. The focal vector injections thus produced a highly potent and consistent assay of TDP-43-induced neurodegeneration and behavioral deficit with disease relevance in the rat. The human TDP-43 expression was mainly nuclear, and estimated to be threefold higher than endogenous rat TDP-43 levels, so as in other TDP-43 models, small alterations in TDP-43 levels in the nucleus were toxic to neurons. There were examples of cytoplasmic TDP-43 deposition and cytoplasmic ubiquitination in the TDP-43 vector group, but they were infrequent and marginal compared with the massive gliosis and neuronal loss that occurred in the TDP-43 transduced substantia nigra. Although TDP-43 is relevant in regard to the substantia nigra, the focal vector approach seemed limited for the transduction of upper and lower motor neurons in relation to ALS. The widespread TDP-43 pathology (Geser *et al.*, 2008) in ALS could be mimicked with the widespread expression in germ-line transgenics (e.g. Wegorzewska *et al.*, 2009), but results with vectors until now could not generate similar widespread expression (e.g. Foust *et al.*, 2008b).

That changed when Foust *et al.* (2009) demonstrated green fluorescent protein expression throughout the mouse CNS with intravenous AAV9 vector administration. By injecting neonatal mice, a still-developing blood–brain barrier allows vector entry to the CNS that is more difficult to achieve in older subjects. Wang *et al.* (2010) translated this technique to rats to overexpress TDP-43, which produced paresis and paralysis, an overall functional phenotype common to the TDP-43 transgenic mice and rats. The spread of green fluorescent protein expression in the CNS of rats was unprecedented, visualized by biophotonic imaging in Fig. 1A. The TDP-43 rats had progressive weight loss, morbidity and mortality relative to green fluorescent protein rats, with modest loss of spinal motor neurons, but remarkably severe muscle atrophy (Fig. 1B) and hindlimb paralysis. As cytoplasmic TDP-43 neuropathology was not found in these rats, the data again implicate the toxicity of nuclear TDP-43 overexpression. The hindlimb paralysis was quantified by rotarod and analysis of rearing behavior, and both readouts were highly consistent, demonstrating that the behavioral phenotype for TDP-43-induced paralysis is reproducible and comparable to data from transgenic mice. Although consistent, the onset of the disease state was rapid within a few weeks, so controlling expression to the adult onset of symptoms will be more relevant to ALS. Other refinements will be more selective expression in the CNS and motor neurons, as the intravenous AAV9 injections also caused peripheral expression, which also occurs in TDP-43 transgenic mice using the prion promoter (e.g. Xu *et al.*, 2010). The corticospinal tract of rodents and primates varies because, in rodents, it is located in the dorsomedial spinal cord, whereas in primates it is in the lateral spinal cord (Watson & Harvey, 2008). Functionally, it is also more of a pure motor pathway in primates compared with rodents (Watson & Harvey, 2008). A non-human primate model might be essential for greater relevance, which can be achieved via vector gene transfer. Apart from the utility in modeling neurodegenerative diseases through the overexpression of neurotoxic proteins, viral vectors also have therapeutic potential. Vector gene transfer has been the best method to deliver therapeutic proteins in a stable manner to the CNS, and recent advancements of widespread CNS transduction using a one-time peripheral injection have broadened the scope for affecting large portions of the spinal cord, and thus spinal cord diseases.

Preclinical gene therapy in the spinal cord

Amyotrophic lateral sclerosis is a candidate for gene therapy because it is life threatening and without cure, and because there are forms of the disease with genetic etiology. To

counteract the neurodegeneration in ALS, the whole spinal cord may need to be treated on a long-term basis. Several strategies have been used for AAV gene delivery to the spinal cord (Fig. 2), with recent efforts succeeding for widespread and long-term spinal transduction. The discussion is centered on data with AAV, although similar strategies have been used with lentivirus vectors (Azzouz *et al.*, 2004; Ralph *et al.*, 2005; Raoul *et al.*, 2005) or adenovirus vectors (Bordet *et al.*, 2001; Acsadi *et al.*, 2002; Manabe *et al.*, 2002; Yamashita *et al.*, 2002) for gene transfer in mutant SOD1 mice. Peel *et al.* (1997) used an AAV2 vector to achieve green fluorescent protein expression in the spinal cord via direct injections into the cervical spinal cord. Azzouz *et al.* (2000) used this route of administration and demonstrated neuroprotection in mutant SOD1 mice using an AAV vector for the antiapoptotic gene B-cell lymphoma 2. Other studies have shown neuroprotection using the growth factors insulin-like growth factor 1 (Lepore *et al.*, 2007; Franz *et al.*, 2009) and granulocyte-colony-stimulating factor (Henriques *et al.*, 2011) via spinal injections. Dodge *et al.* (2008) injected an insulin-like growth factor 1 vector into the deep cerebellar nuclei, a brain region with extensive spinal projections, resulting in insulin-like growth factor 1 expression throughout the spinal cord. Secretable peptidergic factors can spread beyond the area of the transduced cells to exert more widespread effects.

Different routes of administration that avoid direct injections to the spinal cord parenchyma have been developed (Fig. 2), because it would be beneficial to affect the entire spinal cord without having to inject it. Kaspar *et al.* (2003) successfully delivered a vector for insulin-like growth factor 1 to muscle, relying on vector uptake in the axons of innervating spinal neurons, to treat SOD1 mice. The spread of spinal cord neuronal transduction was limited, but the secretable growth factors could have spread further. The efficiency of transduction of motor neurons after intramuscular administration has improved with newer types of the AAV vector (Towne *et al.*, 2010). Intracerebroventricular vector administration leads to more widespread spinal transduction (Dodge *et al.*, 2010; Passini *et al.*, 2010), and this route of gene delivery was successful in extending lifespan in a mouse model of spinal muscular atrophy by expressing and thereby rescuing the deficiency in the survival motor neuron protein (Passini *et al.*, 2010). An intravenous route of administration could be preferable because it allows for widespread gene transfer derived from a peripheral administration. An early study administering AAV intravenously to neonatal mice was effective in correcting a model of lysosomal storage disease in the brain (Daly *et al.*, 1999), although transgene expression in the spinal cord was not discussed. Intravenous AAV8 vector delivery to mice led to limited spinal cord and brain expression previously (Foust *et al.*, 2008b). However, the AAV9 vector resulted in more widespread spinal cord expression after intravenous delivery to neonatal mice (Foust *et al.*, 2009), rats (Wang *et al.*, 2010) and macaques (Foust *et al.*, 2010), and adult cats and mice (Duque *et al.*, 2009; Foust *et al.*, 2009). The recent consistent success of intravenous gene delivery of survival motor neuron protein to spinal muscular atrophy mice (Bevan *et al.*, 2010; Foust *et al.*, 2010; Valori *et al.*, 2010; Dominguez *et al.*, 2011) has provided key preclinical data necessary for human translation. A neonatal approach would be limited in order to treat a patient with symptoms. However, some of the data suggest that this approach can efficiently affect the adult spinal cord (Duque *et al.*, 2009), and transduction in adults can be bolstered by temporary disruption of the blood–brain barrier with mannitol (McCarty *et al.*, 2009). Further research will determine if AAV9 is unique in its ability to widely transduce the CNS compared with other natural vector serotypes. However, engineered versions of the vector could improve the gene transfer to adults (Gray *et al.*, 2010).

Several transgene targets have been efficacious in the SOD1 model of ALS using a variety of AAV administration routes in terms of extension of lifespan, phenotypic delay, preservation of spinal motor neurons and reduction of neuropathology (Table 2). AAV strategies have also been used for other spinal cord diseases, such as spinal muscular

atrophy, spinal cord injury and neuropathic pain (Table 2). For example, gene delivery of survival motor neuron protein increased lifespan and motor function, and decreased muscle atrophy in an spinal muscular atrophy model (Passini *et al.*, 2010), and gene delivery of neurotrophic factors increased motor function and motor neuron survival in spinal cord injury models (Blits *et al.*, 2003, 2004). It is possible that a secreted growth factor that was efficacious in a mutant SOD1 model could also work in a TDP-43 model if there are shared mechanisms of spinal neurodegeneration. There could also be a mechanistic overlap of deficiency of survival motor neuron protein with ALS (Piao *et al.*, 2011), mutant SOD1 models of ALS (Turner *et al.*, 2009) and TDP-43 models of ALS. As the field of research on TDP-43 develops, the emergence of proteins that can degrade or detoxify pathological TDP-43 will provide new therapeutic targets. The ability to affect the entire spinal cord from a peripheral route of administration will enhance the development of therapeutic strategies in ALS rodent models based on TDP-43, as well as in a variety of other spinal cord disease paradigms.

Rodent model caveats and outlook

Rodent models for TDP-43 have definitively demonstrated the specific toxicity of this protein to cause motor neuron symptoms. It will be important to determine how well the disease states accurately mimic specific human disease subtypes, and to improve upon the clinical relevance. The diseases now known as TDP-43 proteinopathies include clinicopathological syndromes ranging from dementia to motor neuron disease, subclassified as a spectrum of subtypes based on specific types of TDP-43 pathology and genetic etiologies (recently reviewed in Chen-Plotkin *et al.*, 2010; Geser *et al.*, 2010). The majority of the TDP-43 transgenic mice showed expression and pathology in both the brain and spinal cord, which could represent disease forms with overlapping brain and spinal cord pathology (Chen-Plotkin *et al.*, 2010; Geser *et al.*, 2010). The two studies using the CaMKII promoter successfully limited TDP-43 expression to the forebrain, which is thus more relevant to frontotemporal lobar degeneration subtypes and not ALS (Tsai *et al.*, 2010; Igaz *et al.*, 2011). However, the probability of cytoplasmic TDP-43 inclusions forming in rodent models may be different to that in human disease as most of the models showed predominantly nuclear expression, which underscores a potential nuclear mechanism in disease, at least in these models. The TDP-43 mice used by Wils *et al.* (2010) may have demonstrated the most comprehensive relevance, with specific loss of motor neurons, specific ubiquitination of motor cortex and spinal cord neurons, nuclear and cytoplasmic TDP-43 aggregates, and other relevant markers such as TDP-43 hyperphosphorylation and proteolytic cleavage (Arai *et al.*, 2006, 2010). Although relevant, the disease states generated in rodents based on overexpression cannot mirror human diseases with normal levels of TDP-43, and thus the disease mechanism may be different. More ideal models would more closely mimic specific subtypes in terms of targeting expression to the neuronal populations that are vulnerable in human disease, with more frequent TDP-43 cytoplasmic inclusions, and with disease state pathogenesis and progression similar to the human time course, e.g. adult onset of symptoms.

Overexpression is an indispensable way of addressing disease mechanisms, but there is overall frustration about the extent of clinical translation that has directly developed from transgenic mouse models. Clinical research derived from beta-amyloid mice regarding immunotherapy or secretase inhibition has so far not translated successfully (Kokjohn & Roher, 2009; Extance, 2010). There have been a number of preclinical therapeutic strategies tested in mutant SOD1 mice for ALS that have also been attempted in clinical trials, such as lithium (Fornai *et al.*, 2008) and antisense oligonucleotides against mutant SOD1 (Smith *et al.*, 2006), although they have not yet yielded new approved drugs for ALS (Siciliano *et al.*, 2010). Although disease models should platform for testing new drugs, some of the biggest

gains from rodent models are their contributions to understanding disease mechanisms and determining the most appropriate proteins that could be targeted with a drug (e.g. Ittner *et al.*, 2010; Oddo *et al.*, 2003; Roberson *et al.*, 2007).

Although AAV vector strategies are promising for gene therapy as well as studying disease mechanisms, gene therapy clinical trials for hemophilia, for example, have had difficulty in translation, despite clear-cut efficacy in animal models (Mingozzi & High, 2011). However, current gene therapy clinical trials with AAV for a type of blindness are more promising (Simonelli *et al.*, 2010).

Conclusions

Diverse strategies have been used to generate rodents that overexpress TDP-43 and produce a consensus ALS-like syndrome (Wegorzewska *et al.*, 2009; Wils *et al.*, 2010; Xu *et al.*, 2010). Improved vector gene transfer efficiency of the spinal cord has permitted a consistent disease state with paralysis induced by TDP-43 (Wang *et al.*, 2010). The peripheral to central administration is also an advance that could be used for new gene therapy strategies in ALS animal models based on TDP-43, and for other spinal cord diseases, like ALS, that are orphan diseases with respect to efficacious medication.

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Abbreviations

AAV	adeno-associated virus
ALS	amyotrophic lateral sclerosis
CaMKII	calcium–calmodulin-dependent kinase II
CNS	central nervous system
FTLD–TDP	frontotemporal lobar degeneration with TDP-43
SOD1	superoxide dismutase 1
TDP-43	transactive response DNA binding protein 43 kDa

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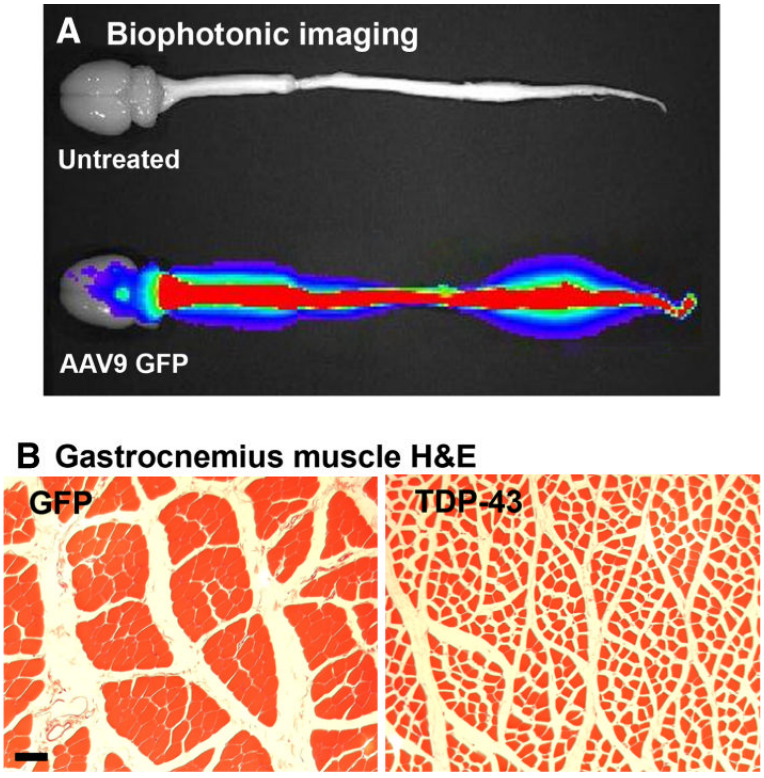
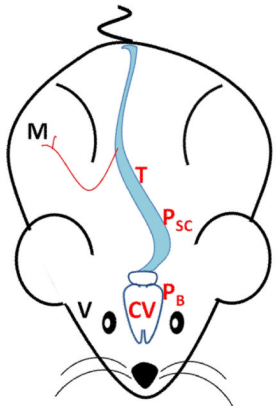


Fig. 1.

Peripheral intravenous injections of an AAV9 vector to neonatal rats led to widespread CNS expression of green fluorescent protein (GFP) or TDP-43. (A) GFP expression viewed by biophotonic imaging at 12 weeks of age. There is robust expression in the spinal cord of the GFP rat, but not in an age-matched uninjected control. The level sets of photonic emission are reflected by the color scheme, i.e. red denotes greater levels of GFP epifluorescence, and blue denotes lower levels. (B) Hematoxylin and eosin stain from the gastrocnemius muscle of a GFP rat at 4 weeks (left panel). AAV9 TDP-43 caused uniform shrinkage of myofibers indicative of widespread denervation (right panel). Bar: 67 μm for both panels in B. Reprinted from Wang *et al.* (2010).



Vector gene transfer to the spinal cord		
Route	CNS Peripheral	Example Reference
Intraparenchymal	$P_{Spinal\ Cord}$	Peel et al., 1997
Intraparenchymal	P_{Brain}	Foust et al., 2008a
Intrathecal	T	Storek et al., 2008
Intracerebroventricular	CV	Passini et al., 2010
Intramuscular	M	Kaspar et al., 2003
Intravenous	V	Foust et al., 2009

Fig. 2.

Routes of AAV vector administration for spinal cord transduction. CNS administrations target injections directly to CNS tissues or the cerebrospinal fluid (red). Intraparenchymal injections can be into spinal cord (P_{SC}) tissue, or in brain tissues that have axonal projections into the spinal cord (P_B). Injections can be into the intrathecal cavity (T) or the cerebral ventricles (CV), which can enhance vector spread in cerebrospinal fluid. Peripheral routes of administration lead to transduction of motor neurons: intramuscular (M) or intravenous (V) injections (black). Intravenous injections efficiently transduce neurons throughout the brain and spinal cord, although the approach is limited by having to use very young subjects. Modified vectors that can cross the mature blood–brain barrier (Gray *et al.*, 2010) or the use of mannitol, which relaxes the blood–brain barrier (McCarty *et al.*, 2009), could make this route more applicable in adults. Certainly some of these approaches transduce brain neurons in addition to spinal cord neurons (e.g. Foust *et al.*, 2008a, 2009; Passini *et al.*, 2010), although the figure highlights strategies to affect spinal cord diseases.

Table 1

Rodent models of ALS and FTLD–TDP based on TDP-43

ALS	**Wegorzewska *et al.* (2009)**	**Wils *et al.* (2010)**	**Shan *et al.* (2010)**	**Xu *et al.* (2010)**	**Zhou *et al.* (2010)**	**Stallings *et al.* (2010)**	**Wang *et al.* (2010)**	**FTLD–TDP**	**Tsai *et al.* (2010)**	**Igaz *et al.* (2011)**
Promoter	PrP	Thy-1	Thy-1.2	PrP	miniCMV/tetO	PrP	CBA	Promoter	CaMKII	CaMKII/tetO
TDP-43 form	A315T	WT	WT	WT	M337V	A315T	WT	TDP-43 form	WT	WT, ΔNLS
Onset of symptoms	3 months	0.5–14 months	0.5–3 months	3 weeks	3 weeks	1 month	2 weeks	Onset of symptoms	2–6 months	1–4 weeks
TDP-43 pathological modifications	No	Yes	No	Yes	Yes	Yes	No	TDP-43 pathological modifications	Yes	Yes
TDP-43-positive inclusions	no	yes	no	yes	Rare	Rare	no	TDP-43 positive inclusions	yes	Rare
TDP-43 fragments	Yes	Yes	no	Yes	Yes	Yes	ND	TDP-43 fragments	Yes	no
Motor neurodegeneration	Yes	Yes	no	Yes	ND	Yes	Yes	Forebrain neurodegeneration	Yes	Yes
Muscle atrophy	Yes	ND	Yes	No	Yes	Yes	Yes	Cognitive impairment	Yes	ND
Motor impairment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Motor impairment	Yes	Yes
Mortality	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Mortality	Yes	No

Models of ALS have transgene expression in the spinal cord, which results in paralysis, motor neurodegeneration (either spinal motor neuron loss or corticospinal tract degeneration) or muscle atrophy. FTLD–TDP models have TDP-43 expression predominantly in the forebrain, resulting in cognitive dysfunction and neurodegeneration in the cortex or hippocampus. TDP-43 pathological modifications refer to either ubiquitination or phosphorylation, both of which were found in Zhou *et al.* (2010). PrP, prion protein; Thy, thymus cell antigen; CMV, cytomegalovirus; tetO, tetracycline operator; CBA, cytomegalovirus/chicken beta-actin; ND, not determined; NLS, nuclear localization signal; WT, wild-type.

Table 2

Preclinical strategies in rodent models of ALS and other spinal cord diseases using AAV vector gene transfer

Disease	Transgene	Route of administration	Vector	References
ALS (SOD1)	Bcl2	Spinal parenchyma	AAV	Azzouz *et al.* (2000)
ALS (SOD1)	GDNF	Forelimb and hindlimb muscles	AAV2	Wang *et al.* (2002)
ALS (SOD1)	IGF-1	Hindlimb and intercostal muscles	AAV2	Kaspar *et al.* (2003, 2005)
ALS (SOD1)	IGF-1	Spinal parenchyma	AAV2	Franz *et al.* (2009), Lepore *et al.* (2007)
ALS (SOD1)	IGF-1	Deep cerebellar nuclei	AAV1,2	Dodge *et al.* (2008)
ALS (SOD1)	IGF-1, VEGF	Ventricles	AAV4	Dodge *et al.* (2010)
ALS (SOD1)	G-CSF	Spinal parenchyma	AAV1/2	Henriques *et al.* (2011)
SMA	SMN1	Intravenous	AAV9	Bevan *et al.* (2010), Dominguez *et al.* (2011), Foust *et al.* (2010), Valori *et al.* (2010)
SMA	SMN1	Ventricles and spinal parenchyma	AAV8	Passini *et al.* (2010)
SCI	BDNF, NT-3, GDNF	Spinal parenchyma	AAV2	Blits *et al.* (2003, 2004)
SCI	L1	Spinal parenchyma	AAV5	Chen *et al.* (2007)
SCI	VEGF, Ang-1	Spinal parenchyma	AAV8	Herrera *et al.* (2010)
SCI	NT-3	Forelimb muscles	AAV5	Fortun *et al.* (2009)
Neuropathic pain	BDNF	Spinal parenchyma	AAV	Eaton *et al.* (2002)
Neuropathic pain	IL-10	Intrathecal	AAV2	Milligan *et al.* (2005)
Neuropathic pain	ppbetaEP, IL-10	Intrathecal	AAV8	Storek *et al.* (2008)

Various transgenes, delivery methods and AAV serotypes have been used. There are rational targets that are worth testing in the new TDP-43 mouse and rat models of ALS, using either a secretable growth factor or an intraneuronal protein that could directly interact with TDP-43. SMA, spinal muscular atrophy; SCI, spinal cord injury; Bcl2, B-cell lymphoma 2; GDNF, glial cell-line-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; G-CSF, granulocyte-colony-stimulating factor; SMN1, survival motor neuron 1; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; L1, cell adhesion molecule L1; Ang-1, angiopoeitin-1; IL-10, interleukin 10; ppbetaEP, prepro-beta-endorphin. The AAVs used in Azzouz *et al.* (2000) and Eaton *et al.* (2002) were probably AAV2, but could not be determined.