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CHAPTER 9

Animal Models for ALS

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9.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease which results in paralysis and death within two to five years after disease onset.1 It typically strikes adults in their mid-life and leads to progressive muscle weakness and atrophy in limbs, and eventually to death due to respiratory failure. These events are caused by a progressive and selective loss of upper motor neurons in the cerebral cortex and the lower motor neurons in the brainstem and spinal cord. The major histopathology of ALS-affected motor neurons is formation of abnormal protein aggregates/inclusions in the cytoplasm, which correlates well with the progression and severity of the clinical symptoms of the disease.1,2

A worldwide incidence of ALS is estimated at 2–8 new cases per 100 000 population each year, which makes the disease the most common adult-onset motor neuron disease.1 Approximately 90% of ALS cases are sporadic ALS (SALS), while inherited forms of ALS or familial ALS (FALS) constitutes approximately 5–10% of all cases.3 To date, more than 10 genetic loci have been linked to ALS.3,4 Most of them were shown to contain genes linked to FALS, including SOD1 (ALS1), Als1/ALS2 (ALS2), senataxin/SETX (ALS4), spastic paraplegia 11/SPG11(ALS5), FUS/TLS (ALS6), VAPB
(ALS8), Angigenin/ANG (ALS9), TARDBP/TDP-43 (ALS10), FIG4 (ALS11), Optineurin/OPTN (ALS12).\textsuperscript{3,4}

In 1993, Rosen et al. discovered that 20\% of all FALS cases carry specific point mutations in a gene encoding for Cu/Zn superoxide dismutase 1 (SOD1).\textsuperscript{5} This epoch-making discovery led to generation of transgenic rodents that express various types of ALS-related mutant SOD1s. The mutant SOD1 transgenic rodents display major ALS symptoms that lead to motor neuron degeneration and defects in neuromuscular junction.\textsuperscript{6,7} Importantly, over-expression of wild-type SOD1 does not lead to overt motor neuron degeneration, but slight toxicity to motor neurons.\textsuperscript{8} Ablation of the endogenous SOD1 gene has also very mild toxicity.\textsuperscript{9} These results support the 'gain of function' hypothesis proposing that SOD1 carrying an ALS-associated mutation can acquire a toxic function responsible for the selective motor neuron death. Although numbers of transgenic mice with known ALS-associated mutant genes have been generated, most of them could not provide evidence to show their pathological relevance to ALS.

The cause of selective motor neuron degeneration in ALS remains elusive. It is widely accepted that multiple mechanisms may contribute to the disease pathogenesis.\textsuperscript{1,3,4} The possible molecular mechanisms include glutamate excitotoxicity, endoplasmic reticulum stress, mitochondrial dysfunction, cytoskeletal abnormalities, deficiency of neurotrophic growth factors, abnormal accumulation of protein aggregates (i.e. SOD1, TDP-43, neurofilament), defects in

![Diagram of selective degeneration of motor neuron](image)

**Figure 9.1** A model for selective motor neuron degeneration in ALS. A current working model proposes that ALS may be caused by a combination of ALS-causal genes (i.e. mutations in SOD1, defects in cytoskeletal proteins or motor proteins implicated in axonal transport), and environmental cues to trigger the disease (i.e. excessive glutamate, oxidative mitochondria damage, endoplasmic reticulum stress). Their complicated interplay still remains speculative and unsolved.
axonal transport, activation of apoptotic pathways, and so on (Figure 9.1). Recent studies also suggest that motor neuron death in ALS depends not only on abnormal intracellular events in motor neurons but also malfunctions of surrounding non-neuronal cells.

In general, animal disease models have been used in developing and testing new drugs to evaluate their effectiveness to the disease. Established first in 1994, the SOD1 ALS mouse model remains a robust ALS model for preclinical research. Some of the recent breakthrough in ALS research are discoveries of ALS-associated novel mutations in TARDBP/TDP-43, FUS/TLS and optineurin which encode proteins implicated in RNA processing or intracellular trafficking. Future studies on these genes should hopefully expand our understanding of the disease pathogenesis and lead to generation of new animal models alternative for the mutant SOD1 mice.

In this chapter, we describe important studies of ALS animal models, most of which have been demonstrated in mutant SOD1 transgenic mice, and discuss the applicability of ALS rodent models for better understanding and preclinical studies of the human disease.

9.2 Genetics of ALS

9.2.1 Genes Related to Familial ALS

To date, 16 familial ALS (FALS) including classic FALS (ALS1–ALS12), FALS with frontotemporal lobar dementia (FTD) (ALS-FTD1 and ALS-FTD2), X chromosome-linked ALS (X-linked ALS) and dynactin-related FALS have been identified. Most of the classical FALS are inherited as an autosomal dominant disorder except alsin-linked ALS2 and spastic paraplegia 11 (SPG11)-linked ALS5. However, ALS1 (SOD1), ALS6 (FUS/TLS), and ALS12 (optineurin) are also found to be an autosomal recessive disorder in some cases. The aetiology of ALS could be even more complicated because mutations of some FALS-related genes such as SOD1, angiogenin, TARDBP/TDP-43 and FIG4 are also found in non-inherited SALS.

9.2.2 Genes Related to Sporadic ALS

Sporadic ALS (SALS) that are not associated with a family history account for 90–95% of ALS cases. SALS is clinically distinguishable from FALS, although some FALS-related genes are shared with SALS.

While the genetic basis of FALS has been well explored, the molecular basis of SALS is less well understood. To date, SALS-related mutations are exclusively found in charged multivesicular body protein 2B (CHMP2B), neurofilament heavy chain (NF-H) and peripherin genes.

9.2.2.1 Charged Multivesicular Body Protein 2B (CHMP2B) Gene

The mutation in charged multivesicular body protein 2B (CHMP2B) gene on chromosome 3 was first found in a large Danish pedigree with frontotemporal
dementia (FTD), which is characterized by frontotemporal lobar degeneration (FTLD) with ubiquitin-immunoreactive inclusions (FTLD-U). Subsequently CHMP2B mutants were found in patients with a non-inherited form of ALS with FTD symptoms designated as ALS-FTD3.

9.2.2.2 Neurofilament Heavy Chain Subunit Gene

Aberrant neurofilament accumulations are one of the major pathological features for both SALS and FALS. However, no conclusive linkage between mutations in three neurofilament genes and either SALS or FALS patients have been reported, although in-frame insertions or deletions within the normal array of 44-45 lysine-serine-proline (KSP) repeats region in the tail domain of NF-H have been reported in ~1% of SALS cases. In other investigations, dominant point mutations in the NF-L gene have been linked to a mild motor neuron generative disease, Charcot-Marie-Tooth (CMT) disease.

9.2.2.3 Peripherin Gene

A frame-shift mutation in peripherin on chromosome 12q12 has been found in SALS patients which produces a truncated peripherin that disrupts neurofilament assembly. Peripherin was found in intermediate filament (IF) inclusions in ALS patients.

9.3 SOD1 Mouse Model of ALS

9.3.1 SOD1 Mutations

Cu/Zn superoxide dismutase 1 (SOD1) is a ubiquitously distributed cytoplasmic enzyme that catalyses conversion of superoxide into peroxide and oxygen. Ever since mutations in SOD1 were linked to FALS, SOD1 have been a hallmark of ALS research. To date, more than 146 different mutations scattered throughout the SOD1 protein have been identified (www.alsod.org) in FALS (ALS1) cases, independent of exon, domains or motif. ALS-related SOD1 mutants (dismutase active G37R and G93A; dismutase inactive G85R, G86R, G127X, SOD1Q45, H46R, D90A) have been overexpressed in mice and examined if they develop disease pathologies similar to that observed in human ALS. These mutant SOD1 transgenic rodents develop comparable disease phenotypes such as mitochondria abnormalities and microglia activation in spinal cords, hindlimb weakness, progressive paralysis and muscular dystrophy but to different degrees between the transgenic strains. It is interesting that SOD1 gene knock-out mice do not lead to motor neuron disease and expression of G85R mutant in SOD1-deficient mice does not affect the disease course. In addition, overexpression of WT hSOD1 in WT mice has no effect on SOD1 activity which is regulated by catalytic copper-binding catalysed by CCS (copper chaperone for SOD1). Motor neurons of CCS-deleted mice have an increased sensitivity to axonmy-induced death.
Multiple molecular mechanisms have been proposed for SOD1-related ALS pathogenesis such as glutamate excitotoxicity, mitochondrial dysfunction, microglial dysfunction, axonal transport blockade, and inflammatory and apoptotic signals triggered by aberrant redox reaction or protein aggregation (Figure 9.1).

9.3.2 SOD1 Mutant Transgenic Mice

Studies of the mutant SOD1 transgenic rodents revealed that the levels and physical locations of mutant SOD1 alleles are critical for promotion of the disease. For example, abnormally shaped mitochondria have been observed in mice transgenic for mutant SOD1s and several mutant SOD1 proteins have been selectively recruited and accumulated in mitochondria in spinal cord. Studies transgenic for SOD1\textsuperscript{G93A} or SOD1\textsuperscript{D90A} do not develop ALS unless bred to homozygosity.\textsuperscript{42,43} Similarly, ALS-like phenotype is absent in SOD1\textsuperscript{A4V} transgenic mice, but evident when the levels of total SOD1 protein are raised by co-expression of wild-type (WT) human SOD1,\textsuperscript{39} suggesting that mutant SOD1 entraps WT SOD1 and form toxic aggregates. Supporting this hypothesis, it has been reported that overexpression of wild-type human SOD1 hastens the onset of disease without prolongation of the disease duration in the case of SOD1\textsuperscript{G93A}, SOD1\textsuperscript{L126Z} and SOD1\textsuperscript{G85R} transgenic mice, although the results may differ between the transgenic strains.\textsuperscript{31,39,44} In addition, severer dose-effect phenotypes have been reported in the rare consanguineous human cases carrying G27AGP, L84F, N85S, or L126S homozygous SOD1 mutant alleles.\textsuperscript{7}

Among SOD1 mutations identified in FALS cases, G93A mutant (SOD1\textsuperscript{G93A}) seems particularly vulnerable and gives rise to a shorter disease duration between the onset and death (2.2 ± 1.5 years) compared with an average duration (10 ± 5–6 years) in other SOD1 mutant causing FALS.\textsuperscript{3,7,30,31} Mice hemizygous for human SOD1\textsuperscript{G93A} transgene are viable and fertile while expressing human SOD1\textsuperscript{G93A} proteins. This SOD1\textsuperscript{G93A} mouse, often referred to as G1H founder line, contains the high transgene copy number up to 25 copies and recapitulates phenotypes similar to those observed in human ALS caused by the same mutant.\textsuperscript{30} Mice carrying the high copy number SOD1\textsuperscript{G93A} transgenes show aggregate formation in motor neurons, muscle weakening and motor neuron loss, and die at 135 days of age where normal life span of WT mice is two years.\textsuperscript{33} In contrast, a mouse subtype SOD1\textsuperscript{G93AGHCL} with low SOD1\textsuperscript{G93A} transgenic copies (~eight copies) delays onset and lengthens duration of the disease.\textsuperscript{45} Moreover, mitochondrial abnormalities in spinal cords is minimal in a low-copy SOD1\textsuperscript{G93AGHCL} subtype,\textsuperscript{45,46} indicating that higher expression levels of SOD1\textsuperscript{G93A} lead to an earlier disease onset and a shorter duration. Johnston et al. reported that soluble type of high molecular weight (HMW) SOD1-containing aggregates were detected in SOD1\textsuperscript{G93A} mice prior to the formation of insoluble heat- and reducing agent-resistant HMW SOD1 aggregates in motor neuron cells.\textsuperscript{47} This is also observed in transgenic mice carrying FALS-linked mutations A4V and G85R in SOD1.\textsuperscript{31,48} Collectively, overexpression of mutant SOD1 such as SOD1\textsuperscript{G93A}, SOD1\textsuperscript{L126Z} and SOD1\textsuperscript{G85R}
predominantly affect motor neurons and trigger ALS-like symptoms in mice, regardless of their ability to bind catalytic Cu$^{2+}$ and dismutase activity.

Transgenic ALS mice are powerful tools to unravel the complicated in vivo biological events in the disease course, especially when crossbred with mice carrying other genes of interest. Functional interactions between ALS-causing mutant SOD1 and other ALS-associated genetic modifiers are described below. Details of mouse ALS models and pathological features are summarized in Table 9.1.

# 9.4 Genetic Modifiers of ALS Mouse Model

## 9.4.1 Glutamate Excitotoxicity

### 9.4.1.1 Glial Glutamate Transporter

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, exerting its effects through a wide variety of receptors that can be either ionotropic or metabotropic. Glutamate excitotoxicity in motor neurons is generally induced when excessive glutamate signalling via an ionotropic glutamate receptor AMPA receptor leads to intracellular calcium-overload and generation of reactive oxygen species.

In motor neurons, a glial glutamate transporter EAAT2 removes excess glutamate from the synapse and protects motor neurons from the glutamate-mediated excitotoxicity.$^{49,50}$ In a subset of ALS patients, expression of EAAT2 is significantly reduced and is accompanied by decreased glutamate uptake in the motor cortex and spinal cord.$^{50-52}$ Consistent with human ALS cases, reduction of EAAT2 expression in astrocytes is observed in spinal cords of symptomatic mutant SOD1$^{G85R}$ mice.$^{32}$ In SOD1$^{G93A}$ mice, however, the EAAT2 protein level is progressively decreased in the ventral horn, but not in the dorsal horn, of lumbar spinal cords. This becomes prominent only at late-stage or not at all$^{53,54}$ and, basically, no change in EAAT2 level occurs before the SOD1$^{G93A}$-related ALS symptoms appear. Despite this, oxidative or caspase-3-mediated inactivation of EAAT2 was observed in spinal cords of mutant SOD1 mice, suggesting a link between apoptotic signals and glutamate excitotoxicity in ALS pathogenesis.$^{55,56}$

The diversity in glutamate handling in mutant SOD1 transgenic mouse models suggests that, in at least some cases, glutamate toxicity maybe a secondary event rather than a primary cause of disease. To further explore the glutamate uptake in astrocytes, SOD1$^{G93A}$ mice expressing human EAAT2 under the control of glial fibrillary acidic protein (GFAP) promoter were generated by crossing SOD1$^{G93A}$ mice with GFAP promoter-driven EAAT2 transgenic mice. These double transgenic EAAT2/SOD1$^{G93A}$ mice replenishing EAAT2 in astrocytes show a significant delay in motor neuron degeneration and associated motor functions at early-stage of the disease progression compared with observations in SOD1$^{G93A}$ mice, but no change in the disease duration. These results indicate that defects in astrocyte-mediated glutamate uptake may be critical for the disease progression from onset to early-stage,
Table 9.1  Mouse ALS models and pathological features.

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<tr>
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<th>Inclusions in MN</th>
<th>Axonal transport</th>
<th>Development of MND</th>
<th>References</th>
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<tr>
<td>Mutant hSOD1</td>
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<td>SOD1&lt;sup&gt;G93A&lt;/sup&gt;</td>
<td>SOD1, IF</td>
<td>Reduced</td>
<td>Yes</td>
<td>10, 33, 66, 140</td>
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<tr>
<td>SOD1&lt;sup&gt;G37R&lt;/sup&gt;</td>
<td>SOD1, IF</td>
<td>Reduced</td>
<td>Yes</td>
<td>39, 42, 140</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;G85R&lt;/sup&gt;</td>
<td>SOD1, IF</td>
<td>Reduced</td>
<td>Yes</td>
<td>31, 66, 140, 152</td>
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<tr>
<td>SOD1&lt;sup&gt;D90A&lt;/sup&gt;</td>
<td>SOD1, IF</td>
<td>Reduced</td>
<td>Yes</td>
<td>41, 43</td>
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<tr>
<td>SOD1&lt;sup&gt;H46R&lt;/sup&gt;</td>
<td>SOD1, IF</td>
<td>Reduced</td>
<td>Yes, but moderate</td>
<td>115, 141, 195</td>
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**NF deletion and mutant**

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<th>Development of MND</th>
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<tr>
<td>hNF-H</td>
<td>Perikaryal NF</td>
<td>ND</td>
<td>Moderate axonopathy</td>
<td>23, 155, 152</td>
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<td>hNF-H (KSP mutation)</td>
<td>Yes</td>
<td>Blocked</td>
<td>Yes, severe phenotype</td>
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<td>NF-L&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Perikaryal NF-M and NF-H</td>
<td>ND</td>
<td>Yes, no MN death</td>
<td>62, 118, 152</td>
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<td>hNF-L</td>
<td>Perikaryal NF</td>
<td>ND</td>
<td>Yes, no MN death</td>
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<td>Peripherin</td>
<td>IF</td>
<td>Reduced</td>
<td>Slow MN death</td>
<td>20, 28, 157,158</td>
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**Kinesin deletion**

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<td>KIF1B&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Yes</td>
<td>Blocked</td>
<td>Neuropathy</td>
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<tr>
<td>KIF1B&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Yes</td>
<td>Blocked</td>
<td>Neuropathy</td>
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<tr>
<td>KIF5A&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>Reduced</td>
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**Dynein mutations**

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<td>Loa&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>ND</td>
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<td>Yes, but normal lifespan</td>
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<td>Cral&lt;sup&gt;+/−&lt;/sup&gt;</td>
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**Others**

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<td>hVAPB&lt;sup&gt;P56S&lt;/sup&gt;</td>
<td>Aggregates in ER</td>
<td>ND</td>
<td>Yes</td>
<td>179, 180, 181</td>
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<td>Als&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>Yes, but moderate</td>
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<td>hTDP-43&lt;sup&gt;M337V&lt;/sup&gt;</td>
<td>TDP-43, SOD1, htt</td>
<td>ND</td>
<td>Yes</td>
<td>204</td>
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SOD1, human Cu-Zn superoxide dismutase 1; NF-H, high molecular weight neurofilament; NF-L, low molecular weight neurofilament; KIF, kinesin family; Loa, legs at odd angle, vesicle-associated membrane protein B; Cral (cramping 1), dynein heavy chain 1 gene mutants; VAPB, vesicle associated membrane protein-associated protein B; TDP-43, transactive response DNA-binding protein; MND, motor neuron disease; MN, motor neuron; htt, huntingtin; ER, endoplasmic reticulum; ND, not determined.

although it is still unclear whether loss of functional EAAT2 is the primary cause for neuronal degeneration or is the secondary.

**9.4.1.2 AMPA Receptor**

Motor neurons expressing SOD1<sup>G93A</sup> mutant are more susceptible to glutamate toxicity than wild type cells and show changes in expression of glutamate receptor subunits, suggesting mutant SOD1 affects glutamate transmitter signalling pathways as well as glutamate levels. Glutamate toxicity in motor
neurons is primarily mediated through AMPA receptors and in fact, administration of AMPA receptor antagonists (i.e. NBQX) to SOD1<sup>G93A</sup> mice significantly reduced symptoms and increase survival.<sup>59,60</sup> AMPA receptors can exert functional diversity as composed of four subunits, designated GluR1-4.<sup>61</sup> For example, permeability for Ca<sup>2+</sup> ions of a particular AMPA receptor is determined by its subunit composition. The GluR2 subunit is widely expressed and included in most of Ca<sup>2+</sup>-impermeable AMPA receptors.<sup>61</sup> This property is rendered by RNA editing of GluR2 transcripts. Investigations into changes in AMPA receptor subunit expression in ALS have identified reduced RNA editing and expression of GluR2 subunit in motor neurons compared to other neuronal populations,<sup>62-64</sup> but failed to find clear differences between subunit expression in motor neurons from ALS and that from control subjects.<sup>62,64</sup> These differences in glutamate receptor profile between motor neurons and other neuronal populations may partly explain the selective vulnerability of motor neurons to glutamate toxicity, but do not suggest changes in subunit expression lead to ALS. A study on AMPA receptor properties has shown no changes in Ca<sup>2+</sup> permeability of AMPA receptor in SOD1<sup>G93A</sup> expressing motor neurons, but an increase in Na<sup>+</sup> and K<sup>+</sup> permeability.<sup>58</sup> In this way, Ca<sup>2+</sup> influx is triggered through voltage-gated calcium channels.

### 9.4.1.3 Calcium Binding Proteins

Calcium signalling is usually attenuated by rapid buffering of free Ca<sup>2+</sup>, either by binding to cytosolic calcium binding proteins (CaBP) or by uptake into organelles. Motor neurons express only low levels of CaBP and thus there appears to be correlation between low CaBP expression and susceptibility to ALS whereas GABAergic neurons have marked expression of seven different CaBP such as calbindin 28K, calrecept and paralbumin.<sup>65</sup> Conversely, the effects of glutamate toxicity in SOD1<sup>G93A</sup> mutant-expressing motor neurons can be reduced by increased expression of CaBP or calbindin.<sup>66</sup> Selective motor neuron degeneration in ALS may be due to the low CaBP level in motor neurons. Therefore, in human ALS cases and ALS-causing rodent models, AMPA receptor activation causes greater mitochondrial calcium uptake than in GABAergic neurons and results in mitochondrial ROS generation leading to neuronal degeneration.<sup>67</sup> Taken together, calcium loading and handling should be a key modifier for the ALS phenotypes.

### 9.4.2 Mitochondrial Dysfunction and Apoptosis

Mitochondria are not only responsible for production of ATP, but also the major site for the generation of reactive oxygen species (ROS) as a by-product in the consequence of the energy production. Mitochondria also have roles in calcium buffering and initiation of apoptotic cell death, all of which are considered to be important in ALS pathogenesis.<sup>59</sup> Morphologically abnormal mitochondria have been described in motor neurons from ALS patients,<sup>68,69</sup> mutant SOD1 transgenic mice (SOD1<sup>G93A</sup>, SOD1<sup>G37R</sup> and SOD1<sup>G85R</sup>) and a
motor neuron cell expressing mutant SOD1. In SOD1<sup>G93A</sup> mice, mitochondrial abnormalities such as mitochondrial swelling, membrane fragmentation and dilatation of cristae appear before onset of muscle weakness. Massive mitochondrial vacuolation is also occurred with the onset of symptoms, suggesting that mitochondrial dysfunction may be an important early event in disease pathogenesis and constitute part of the toxic gain of function by ALS-causing SOD1 mutants (i.e. SOD1<sup>G93A</sup>). Biochemical studies of mitochondria from ALS patients and ALS cell culture models have consistently shown reduced electron transport chain activity, decreased mitochondrial membrane potential, disrupted calcium homeostasis and altered mitochondrial proteome.

The elevated levels of reactive oxygen species have been proposed for causing the increased frequency of mitochondrial DNA mutations in the motor cortex and spinal cord of ALS patients. Several studies have shown mitochondria damages in mutant SOD1 transgenic ALS mouse models. It has been reported that translocation of cytochrome C from mitochondria into cytoplasm, which is considered as a trigger of apoptosis, occurs and increases during the disease progression in SOD1<sup>G93A</sup> mice. The translocation of mitochondrial cytochrome C is not observed in age-matched non-transgenic littermates.

Age-dependent increase in mutant mitochondrial SOD1 accumulation has been shown to precede onset of human ALS. In mutant SOD1<sup>D90A</sup> and SOD1<sup>G93A</sup> transgenic mice, exogenously expressed mutant SOD1<sup>D90A</sup> and SOD1<sup>G93A</sup> also enter mitochondria and compete out intracellular cupper availability over endogenous WT SOD1 by forcing disulfide reduction. Age-dependent increase in mutant mitochondrial SOD1 accumulation has been shown to precede onset of symptoms. The accumulation of mutant SOD1 in transgenic mice coincides with increased oxidative damages, decreased respiratory activity of mitochondria, and appearance of mitochondrial swelling and vacuolization. However, the role for mutant mitochondrial SOD1 in ALS pathogenesis is still equivocal as the mutant SOD1 accumulation is not selectively occurred in spinal cords.

Pasinelli et al. have first shown a link between mitochondria dysfunction and apoptosis in ALS-affected motor neurons. In SOD1<sup>G93A</sup> mice, both dimeric and high molecular weight SOD1-containing aggregates, both of which are thought to be toxic to mitochondria, directly bind an anti-apoptotic protein Bcl-2 and trigger apoptosis. Piles of evidence have indicated that neuronal apoptosis in ALS patients and FALS mouse models is mediated by deregulation of Bcl-2, death signalling p75 neurotrophin receptor (p75NTR), Fas and ER stress, however none of them is conclusive.

### 9.4.3 Effects of Non-neuronal Cell Activation in ALS

#### 9.4.3.1 Effects of Microglia Activation

It is generally accepted that motor neuron neurodegeneration in FALS is non-cell autonomous, with ALS-causing mutant SOD1 affects multiple cell types. Non-neuronal cells in the spinal cord may contribute to motor neuron
protection, rather than degeneration, in mutant SOD1-associated ALS (ALS1). This concept is based on numerous studies of mutant SOD1 transgenic mice in which selective overexpression of mutant SOD1 in neurons, microglia and astrocytes was achieved by cell type-specific promoters (Table 9.2).

Selective expression of mutant SOD1 in astrocytes induced reactive gliosis without ALS-symptoms in mice, indicating that astrocytes do not contribute to the disease. Expression of mutant SOD1 in neurons, astrocytes and muscle but not in microglia driven by the prion promoter could cause motor neuron degeneration in mice. Effects of neuron-specific expression of SOD1G93A were investigated in Thy1 promoter-driven SOD1G93A transgenic mice (Thy1-SOD1G93A). Thy1-SOD1G93A mice did not develop motor neuron degeneration unless bred to homozygosity. Another targeted mutant SOD1 expression using the Cre-loxP system was able to show that Cre-mediated

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SOD1 transgenic</th>
<th>Target</th>
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<td>SOD1G93A + SOD1G37R BMT (+ SOD1G93A microglia)</td>
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<td>microglia (+ WT-microglia)</td>
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<tr>
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<td>microglia</td>
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<td>microglia</td>
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ND, not determined; ns, not sensitive; BMT, bone marrow transplant
activation of mutant \textit{SOD1}^{G85R} transgene in subpopulations of spinal motor neurons and interneurons could trigger selective degeneration of motor neurons.\textsuperscript{101} These results indicate that motor neuron degeneration is an intrinsic event in SOD1-associated ALS.

Selective expression of mutant SOD1 within motor neurons is a determinant of the disease initiation, whereas mutant expression within neighbouring non-neural cells (\textit{i.e.} microglia, astrocyte) may accelerate disease progression. To test the beneficial effect of microglia on ALS, novel mice lines carrying Cre gene driven by the myeloid CD11b promoter were generated and bred with floxed \textit{SOD1}^{G37R} mice.\textsuperscript{102} The specific transgene inactivation in CD11b-positive cells (\textit{i.e.} myeloid-derived microglia) dramatically prolonged lifespan by approximately three months in Cre-excised mice without causing microgliosis in the spinal cord.\textsuperscript{102} \textit{SOD1}^{G93A} mice, in which myeloid cells were ablated by deletion of PU.1 and restored by transplant of wild-type bone marrow grafts giving rise to microglia with normal functions, showed disease onset but delayed progression of the disease.\textsuperscript{103} In support of this, it has been demonstrated that deletion of myeloid-differentiation factor 88 (MyD88) in microglia derived from bone marrow worsens disease outcomes in \textit{SOD1}^{G37R} ALS mice.\textsuperscript{104} These results strongly suggest a neuroprotective role for microglia during ALS progression rather than initiation of the disease.

\subsection{9.4.3.2 Effects of Schwann Cells in Peripheral Nervous System}

Schwann cells, the myelinating glia of the peripheral nervous system, could be damaged by SOD1 mutant. Schwann cells are associated with almost the entire surface of peripheral axons and are essential for the survival of motor neurons during neuronal development, and again become important during neuronal regeneration. Lobsiger et al. have assessed the non-cell-autonomous contribution of Schwann cell-expressed mutant \textit{SOD1}^{G37R} to ALS.\textsuperscript{105-107} They mated mice heterozygous for human \textit{SOD1}^{G37R} transgene flanked by loxP sites (Lox\textit{SOD1}^{G37R} mice) with Cre-mice in which Cre recombinase was expressed under the control of mouse myelin-protein-zero (\textit{P}o) regulatory sequences.\textsuperscript{102} In this system, Cre-mediated excision of Lox\textit{SOD1}^{G37R} can remove \textit{SOD1}^{G37R} specifically from Schwann cells.\textsuperscript{107} Unexpectedly, reduction of a fully dismutase-active \textit{SOD1}^{G37R} within Schwann cells significantly accelerates disease progression.\textsuperscript{107}

\subsection{9.4.4 Axonal Transport Blockade}

\subsection{9.4.4.1 Cytoskeleton Networks in Motor Neuron Axons}

Motor neurons are highly specialized cells with extensive dendrite arbors and axonal processes that can extend up to one metre from the cell body. This particular morphology is maintained by cytoskeletal networks comprised of microtubules, actin filaments and neurofilaments, and continuous transport of proteins and organelles to and from the cell body. Many studies have shown that defects of axonal transport are one of the causes for motor neuron
degeneration in ALS. Here we summarize recent data showing a close link between axonal transport defects in motor neurons and ALS aetiology.

9.4.4.2 Microtubule-dependent Retrograde Transport

Microtubules provide stability and polarity to the axonal compartment of the neuron as they are polarized with a slow-growing minus end directed toward the cell body and a fast-growing plus end directed peripherally in the axon, while actin contributes mainly to the integrity of the cell periphery. In addition to their structural roles, the microtubules provide mainly two long-range transport paths, the fast anterograde movement (away from cell body) and the retrograde movement (toward cell body) mediated by kinesin motor proteins and dynein motor complex, respectively. In mice with heterozygous disruption of a kinesin family member KIF1B gene show defects in the anterograde axonal transport and provoke neurodegeneration similar to human neuropathies. In accordance with this mice phenotype, a loss-of-function mutation in the motor domain of the KIF1B gene has been found in patients with an inherited form of peripheral neuropathy, Charcot–Marie–Tooth disease type 2A. Missense mutations in a conventional kinesin KIF5A have also been found responsible for an inherited form of spastic paraplegia and disruption of KIF5A gene in mice has been known to impair the transport of neurofilament proteins.

In SOD1 transgenic mice, a considerable inhibition of retrograde axonal transport was observed at a very early stage of disease before animals became symptomatic. However, the mechanisms by which mutant SOD1 affects axonal transport have not been uncovered. Compared to WT SOD1, several SOD1 mutants have been shown to interact more stably with the dynactin–dynein complex. Moreover, the dynein has been reported to be colocalized with protein inclusions formed by mutant SOD1 in ALS. Mutant SOD1 may influence neuronal survival by impairing the dynein-dependent retrograde transport of neurotrophic factors and mitochondria.

Both anterograde and retrograde axonal transport have been shown to be disrupted in mutant SOD1 transgenic mice. Different motor proteins are believed to be involved in controlling anterograde and retrograde transport. While ALS-causing mutations in proteins engaged in anterograde transport have not been identified, a mutation in dynactin, a protein involved in dynein-mediated retrograde transport, caused a progressive lower motor neuron disorder. Two point mutations in a dynein subunit caused progressive motor neuron degeneration in heterozygous mice. Similarly, disruption of the dynactin complex (thereby reducing activation of cytoplasmic dynein) inhibited retrograde transport and triggered late-onset motor neuron degeneration in genetically engineered mice.

9.4.4.3 Deficits in Dynein-mediated Axonal Transport

As described above, decreased dynein-mediated retrograde axonal transport affects the disease course both in ALS patients and in transgenic ALS animal
models. To investigate the genetic modifying effects of dynein, a genetic crossing between ALS-causing transgenic and non-lethal dynein mutant mice may provide informative outcomes.

There are several mouse lines with point mutations in cytoplasmic dynein heavy chain (DHC) 1 available, including Legs at odd angles (Loa) and Cramping 1(Cral) mice.\textsuperscript{120,121} Loa and Cral mice not only develop late-onset, non-fatal motor neuron degeneration with impaired retrograde axonal transport, but also suffer from sensory neuropathy that occurs prior to the onset of motor symptoms. Interestingly, crossing heterozygous Loa and Cral mice with SOD1\textsuperscript{G93A} ALS mice ameliorated the transport defect and delayed disease onset, and slowed disease progression instead of showing an additive phenotype.\textsuperscript{122–124} The precise mechanisms underlying this effect remain unclear, though several explanations have been proposed: First, the dynein mutations alter intracellular transport and thereby change the subcellular localization of SOD1 with other proteins or organelles. For example, it is possible that decreased interaction of mutant SOD1 with mitochondria could improve cell survival by reducing apoptosis or other downstream consequences. SOD1-positive aggregates have been observed in homozygous Loa mice suggesting that the dynein mutation can affect WT SOD1 distribution in the cell. Secondly, the decreased rates of retrograde transport by the Loa or Cral mutants might counterbalance an inhibition of anterograde transport caused by SOD1\textsuperscript{G93A}, thereby restoring the balance between anterograde and retrograde transport.

Lai et al. reported that mice with a G59S amino acid substitution in a dynactin 150 kDa subunit (p150\textsuperscript{Glued}) showed signs of reduced motor neuron axonal transport and developed motor neuron disease in a similar way to phenotypes of Loa and Cral mice.\textsuperscript{125} However, when heterozygous G59S-p150\textsuperscript{Glued} knock-in mice were crossed with SOD1\textsuperscript{G93A} mice, no improvement of the SOD1\textsuperscript{G93A} phenotype was observed\textsuperscript{126,127} Sprawling (Swl) heterozygous mice harbouring another DHC mutation display proprioceptive sensory neuropathy without causing motor neuron deficits; however, they do not show late-onset motor neurons loss unlike Loa or Cral.\textsuperscript{128} Crossing Swl mice with SOD1\textsuperscript{G93A} mice did not affect ALS disease onset or progression.\textsuperscript{129}

### 9.4.4.4 Effects of Mutant SOD1 Aggregation on Axonal Transport

In ALS transgenic mice, dynein colocalizes with mutant SOD1 and forms aggregates in motor neuron axons.\textsuperscript{116,121} It is possible that mutant SOD1 aggregates could directly hinder dynein transport on microtubules in axons.

Recent studies have demonstrated that interaction of mutant SOD1 with dynein is more stable than that of WT SOD1 in both ALS cell culture and animal models.\textsuperscript{115} Despite no changes in the dynein–dynactin subunit interactions, the amount of mutant SOD1 that interacted with dynein increased as the disease progressed. Knowing the increased association of mutant SOD1 with dynein in mouse ALS models, the transport capacity of dynein might be
saturated by mutant SOD1 over time. Supporting this, a study in monkeys showed that the dynein–dynactin complex undergoes age-related changes including increased amounts of dynein in nerve endings and a decrease in the dynein–dynactin interaction. These changes suggest that less functional dynein–dynactin complexes may be available during aging. Either the increase in dynein–mutant SOD1 association or the decrease in functional dynein–dynactin while aging could contribute to reduced retrograde transport to levels that are no longer able to sustain neuronal survival, thereby producing a phenotype of adult-onset motor neuron degeneration.

9.4.5 Depletion of Neurotrophic Factors

A homozygous mutation in a splice site of ciliary neurotrophic factor (CNTF) is present among ~2% of European and Japanese populations. The mutation resulting in production of an inactive protein causes a 15–20% reduction in motor neuron number, but does not by itself cause neurodegenerative disease. One individual in a family carrying SOD1V148G mutation developed the disease at an early age; subsequent analysis showed the disease is sporadic and that the individual has the homozygous mutation in CNTF. Genetic reduction of CNTF in ALS was investigated by crossing SOD1G93A mice with CNTF−/− mice, which resulted in significantly earlier onset and increased severity of the disease compared to SOD1G93A (CNTF+/+) mice. This result supports a hypothesis that homozygous CNTF-null mutation may be a risk factor for early onset of disease.

The role of angiogenic factor vascular endothelial growth factor (VEGF) is implicated in ALS since hypoxic induction of VEGF increased disease severity of SOD1G93A mice. Importantly, subsequent overexpression of VEGF ligand and receptor prolonged survival in SOD1G93A mice, implicating VEGF as a modifier of SOD1-related ALS and a potential neuroprotective factor.

9.4.6 Protein Misfolding and Aggregation

Protein aggregates formed from misfolded mutant proteins are a common feature in many neurodegenerative diseases, but it has been largely unknown whether or not they are a primary cause of disease pathogenesis, a harmless byproduct or even a cellular defence mechanism to sequester potentially toxic proteins. Protein aggregates that are immunoreactive to antibodies against ubiquitin, a protein tag that targets proteins for proteolytic degradation, are present in all ALS cases tested. SOD1 forms protein aggregates in human FALS linked to SOD1 mutations and also in a population of sporadic cases. Analogous cytoplasmic aggregates consisted of ubiquitinated mutant SOD1 have also been observed in spinal cords of mouse ALS models during the symptomatic disease. The number of aggregates increased with age in SOD1G93A and SOD1G85R mice consistent with proteasome-mediated turnover. Most aggregates were positive for SOD1 in SOD1G85R
mice, whereas there was more variability in the presence of SOD1-positive aggregates in SOD1^{G93A} mice. Protein aggregates were not detected in control mice and rarely seen in SOD1^{G37R} mice.\textsuperscript{99,141}

Protein aggregation has been shown to inhibit the ubiquitin–proteasome system. In support of this hypothesis, reduced chaperone and proteasome activity was reported in mutant SOD1 transgenic mouse.\textsuperscript{140,142–145} Over-expression of a molecular chaperone HSP-70 and E3 ubiquitin ligase partly protected against mutant SOD1 toxicity in cell culture models and HSP-70 was chronically upregulated in several mutant SOD1 models.\textsuperscript{146} However, elevated levels of HSP-70 in mutant SOD1 models did not change disease onset or survival.\textsuperscript{147} In genetic cross experiments to assess the role of the ubiquitin–proteasome system, SOD1^{G93A} mice were bred with mice lacking the LMP2 subunit of 20S core proteasome;\textsuperscript{148} these mice were essentially not affected, although overall proteolysis in spinal cords was reduced. Therefore, genetic induction of chaperone proteins and proteasomes in transgenic mouse ALS models fails to change the disease onset, suggesting that the primary insult in the disease is upstream of refolding and degradation of SOD1 mutants.

### 9.4.7 Neurofilament Defects

Neurofilaments (NFs), which assemble from three subunits NF-L, NF-M and NF-H, are particularly abundant in motor neurons and regulate myelinated large calibre axons that conduct electrical impulses. One of the major components of neurofilaments, heavy chain form NF-H subunit, was identified as a causal gene for SALS.\textsuperscript{22,24} In transgenic mouse overexpressing human NF-H protein, both transcript and protein levels of NF-L and NF-M are increased.\textsuperscript{149–151} In contrast, expression of NF-L is reduced in cases of both SALS and FALS, and in SOD1^{G93A} ALS mice,\textsuperscript{152} causing changes in stoichiometry of NF subunits. These model mice with neurofilament deficit progressively developed ALS-like symptoms and defects in axonal transport, but no significant loss of motor neurons.

Neurofilaments are particularly important in motor neurons with their large size and long axons. There is much evidence supporting a role for abnormal NF assembly in ALS pathogenesis: aggregation of neurofilaments is a pathological feature of many neurodegenerative disorders including ALS and is also seen in SOD1 transgenic mice.\textsuperscript{2,30} Transport along neurofilaments was also shown to be disrupted in mutant SOD1 transgenic mice.

Although it is not known how mutant SOD1 causes neurofilament changes, it has been shown that mutant SOD1, but not wild-type SOD1, binds directly to 3' UTR of NF-L mRNA, thereby leading to destabilization and subsequent degradation of the mRNA.\textsuperscript{153,154} Since NF-L is required for NF-M and NF-H to assemble into filaments, changes in expression may disrupt neurofilament assembly and trigger neurofilament aggregation. In fact, deletion of NF-L in SOD1^{G85R} mice significantly delays onset and progression of the motor neuron disease, but accelerates degeneration of other neurons and reduces the selective
toxicity of the SOD1 mutant to motor neurons. Another study has demonstrated that SOD1\textsuperscript{G93A} mice overexpressing either NF-H or NF-L show late onset of the disease and survive longer than SOD1\textsuperscript{G93A} mice. Overexpression of human NF-H in SOD1\textsuperscript{G85R} mice also has beneficial effects resulting in increased survival, with lifespan extended by up to 65% compared with SOD1\textsuperscript{G85R} mice. In these transgenic models, an increase in perikaryal NF and a decrease in axonal NF inclusions are observed. However, there is minor controversy about whether extra neurofilaments can rescue the motor neuron disease caused by mutant SOD1. Couillard-Despres \textit{et al.} have reported that overexpression of human NF-L proteins resulting in extra axonal filaments does not shorten the lifespan of transgenic mice expressing mutant SOD1\textsuperscript{G37R} despite its very modest neuroprotective effects on spinal cord and ventral roots. Their results indicate that axonal neurofilaments may not be an exacerbating factor in motor neuron disease caused by mutant SOD1 but that an increase in perikaryal NF may play a neuroprotective role. In either model, neurofilaments are determinants for the selective vulnerability of motor neurons to ALS-causing SOD1 mutants.

Another intermediate filament protein, peripherin, has been identified in intermediate filament inclusions in ALS cases and SOD1 transgenic mice. Overexpression of peripherin in mice causes a late-onset selective motor neuron disease with intermediate filament aggregates containing peripherin. Deletion of \textit{peripherin} gene has no effect on the disease progression in SOD1\textsuperscript{G93A} mice. It was subsequently shown that one of the known splice form variants of peripherin, the 61 kDa isoform (peripherin-61) was responsible for this toxicity. Peripherin-61 has been reported to be detected in motor neurons from SOD1\textsuperscript{G37R} mice and human sporadic ALS patients. Although neither upregulation nor suppression of \textit{peripherin} gene had any effect on disease onset, severity and progression of SOD1\textsuperscript{G37R}-ALS mice, peripherin may be responsible for the pathogenesis in a small population of ALS cases.

### 9.4.8 Inflammatory Cytokines

Several inflammatory cytokines and enzymes including various interleukins, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) have been shown to be upregulated in the spinal cord of ALS patients and mutant SOD1 mice. Microglia, the resident macrophage population in the central nervous system, become activated by these inflammatory cytokines and reactive oxygen species.

There is growing evidence to suggest that inhibition of microglia activation is protective in ALS. For example, microglia cultured from adult pre-symptomatic mutant SOD1 mice showed increased TNF\(\alpha\) release upon stimulation compared to wild-type microglia and TNF\(\alpha\) antagonists significantly increased survival in mutant SOD1 mice. Similarly, reduction of PGE2 levels by inhibition of COX2 protected against motor neuron loss and increased survival in mutant SOD1 mice.
9.5 Protein Degradation in ALS Transgenic Mouse Model

Dynein-mediated transport is a key mechanism in protein degradation and accumulation of misfolded proteins in cells. Interaction between mutant SOD1 and dynein may directly influence SOD1 aggregation and degradation. Proteins are mainly degraded by two pathways—the ubiquitin–proteasome system (UPS) and autophagy.\textsuperscript{167,168}

Proteins destined to be degraded by the UPS are marked by the covalent attachment of ubiquitin and then transported to the proteasome for proteolytic cleavage. It has been widely accepted that dynein-mediated retrograde transport could be involved in shuttling ubiquitin-tagged proteins to the proteasome.

Autophagy is another intracellular process for the degradation of proteins, organelles and protein aggregates.\textsuperscript{168} Autophagy is important, particularly for neurons, as mice lacking the autophagy-related gene Atg7 develops axonal dystrophy characterized by distal accumulation of membrane structures and swelling of axonal terminals.\textsuperscript{169} Dynein-mediated transport is involved in autophagic clearance of aggregated proteins. In the autophagy process, the dynein–protein transporting complex collects misfolded proteins from the cell periphery and transports them to perinuclear region of the cells where they form intracellular inclusions called aggresomes. Dynein has also been implicated in lysosomal transport and in mediating the fusion of the autophagosome and the lysosome.

Mutant SOD1 is believed to be degraded by both UPS and autophagy.\textsuperscript{170} It has been shown that mutant SOD1 interacts strongly with dynein while WT SOD1 does not, or to a very little extent.\textsuperscript{108,114} Consistent with this view, mutant but not WT SOD1 interacts with p62/sequestosome,\textsuperscript{171} a protein that has been linked to autophagy.\textsuperscript{169} Moreover, it has been demonstrated that overexpression of p62/sequestosome 1 increased the formation of large mutant SOD1 inclusions resembling aggresomes.\textsuperscript{171} These data suggest that cells utilize dynein-mediated transport to collect mutant SOD1 as aggresomes and target them for autophagic degradation.\textsuperscript{108,167} In this context, the interaction between mutant SOD1 and dynein might be of beneficial effect to the cell. Supporting the importance of dynein in autophagic clearance of aggregates in preventing neuronal toxicity, \textit{Loa} mutation in fact decreased autophagic clearance of huntingtin protein resulting in increased inclusions and toxicity in Huntington’s disease model mice.\textsuperscript{172} This result may or may not be relevant to SOD1-mediated ALS as the negative effect of \textit{Loa} is not observed in SOD1\textsuperscript{G93A} mice.\textsuperscript{117} Both \textit{Loa} and \textit{Cral} ameliorate the ALS symptoms of SOD1\textsuperscript{G93A} mice and thus impaired autophagy can be a positive effect in ALS.\textsuperscript{122}

9.6 SOD1 Transgenic Rat Model of ALS

Howland \textit{et al.} reported that they developed transgenic rats expressing mutated human SOD1\textsuperscript{G93A} displaying ALS-like phenotypes, including motor neuron
degeneration in spinal cord.\textsuperscript{33} Despite the intense studies on genetic ALS mouse models,\textsuperscript{1,7} rats transgenic for ALS-causing SOD1 mutations render great advantages to biochemical analysis and surgical manipulation due to their size.

To date, rat transgenic strains overexpressing two different human SOD1 mutants have been established as ALS rat models. One is a rat transgenic for SOD1\textsuperscript{H46R} with prominent protein cytopathology in the spinal cord.\textsuperscript{36} Another is transgenic for ALS-causing SOD1\textsuperscript{G93A} with predominant vacuolar neurodegeneration.\textsuperscript{173} The main difference between mouse and rat ALS models lies in the prominence of the forelimb onset, which can predict rapid progression of ALS in the rat model but which may contrast to human situations.

In SOD1\textsuperscript{G93A} rats, a pronounced loss of a predominant glutamate transporter EAAT2 (GLT-1) protein is observed in the ventral horn at the end stage of the disease,\textsuperscript{33} reminiscent of a selective loss of GLT in spinal cord and motor cortex from ALS patients. Consistently, the SOD1\textsuperscript{G93A} rat ALS model shows marked reduction of glutamate uptake in the spinal cord but not in the brainstem or other brain areas in which levels of a neuronal glutamate transporter EAAC1 are not affected by the disease.\textsuperscript{173} The reduced glutamate uptake capacity in spinal cord is accompanied by decreased expression of astrocytic GLT-1 (or EAAT2) and glial high affinity glutamate transporter (GLAST); thus significant deficits in glutamate uptake are most likely mediated by glial cells in the SOD1\textsuperscript{G93A} transgenic rat.\textsuperscript{173}

Rat ALS models have several advantages over mice ALS models. Generally speaking, rat ALS models allow easier experimental manipulation for a large-scale biochemical analysis such as protein extraction, detection of neurotransmitters, synaptosome preparation or intrathecal injection of new drugs.\textsuperscript{31,36,173-177} The large rat spinal cord confers much easier access for microsurgery, which is required for testing of the gene or stem cell therapies.

9.7 VAPB Transgenic Mouse

Vesicle-associated membrane protein-associated protein A (VAPA) and vesicle-associated membrane protein-associated protein B (VAPB) interact with lipid-binding proteins carrying a short motif containing two phenylalanines in an acidic tract (FFAT motifs) and target them to the cytosolic surface of the endoplasmic reticulum (ER). A genetic mutation by which one of the phenylalanines in the conserved major sperm protein homology domain of VAPB is substituted with serine (P56S) has been linked to motor neuron degeneration in affected amyotrophic lateral sclerosis 8 (ALS8) patients.\textsuperscript{178-181} It has been reported that VAPB is abundant in motor neurons and that the P56S substitution causes aggregation of mutant VAPB in immobile tubular ER clusters, perturbs FFAT-motif binding and traps endogenous vesicle-associated membrane protein-associated protein (VAP) in mutant aggregates; thereby VAPB with P56S substitution (P56S-VAPB) causes motor neuron degeneration via a dominant negative mechanism.\textsuperscript{179,181} Expression of mutant VAPB or reduction of VAP by short hairpin RNA causes Golgi dispersion and neuronal cell death in vitro.
Both VAPA and VAPB are reduced in human ALS patients and mouse ALS models\textsuperscript{180,181}, suggesting that VAP family proteins may be involved in the pathogenesis of sporadic and SOD1-linked ALS. Enforced expression of wild-type VAPB, mainly localized in the endoplasmic reticulum, induced activation of one of the main UPR pathways, the IRE1/XBP1 pathway. P56S-VAPB mutant forms cytosolic aggregates and loses the ability to be involved in the activation of the IRE1/XBP1 pathway\textsuperscript{179} in which deficient VAP family protein levels result in decreased ER anchoring of lipid-binding proteins.

Animal models carrying VAPB mutations will provide additional insight into the cellular mechanisms by which a reduced level of functional VAP proteins in ER can result in specific degeneration of motor neurons in vivo. Loss of activity of IRE1/XBP1 pathway by mutant VAPB would lead to the accumulation of unfolded proteins in motor neurons which eventually increase sensitivity to ER stress-induced death. Supporting this idea, several reports have demonstrated that ER stress contributes to the development of sporadic ALS-related motor neuron cell death.\textsuperscript{182,183}

## 9.8 Alsin Transgenic Mouse

### 9.8.1 Alsin

The recessive form of juvenile-onset familial ALS designated as ALS2 has been linked to chromosome 2q33.\textsuperscript{1,4} This locus was overlapped with a familial juvenile primary lateral sclerosis (JPLS) locus.\textsuperscript{184,185} ALS2 is an early onset and slowly progressive disease: onset of ALS2 varies from one to 10 years of age where the duration of the disease may be as long as 50 years. This ALS2 gene encodes short and long forms of alsin protein, which is a novel guanine nucleotide exchanging factor (GEF) for the small GTPase Rab5 involved in a macropinocytosis-associated endosome fusion, and trafficking and neurite growth.\textsuperscript{184,185}

Alsin contains a pleckstrin homology domain that targets host proteins to membrane by binding to phosphoinositides as well as seven MORN (Membrane Occupation and Recognition Nexus) repeats that are usually found in phosphatidylinositol signalling proteins.\textsuperscript{184,185} Mutations in alsin gene cause chronic juvenile ALS (ALS2), juvenile primary lateral sclerosis (JPLS) and infantile-onset ascending spastic paralysis—all of which are neurodegenerative conditions—depending on the location of the mutations.\textsuperscript{186} Among 12 mutations reported for ALS2, two missense mutations (A46fsX50 and T185fsX189) have been revealed to cause familial ALS2, while the others seem to result in upper motor neuron syndromes.\textsuperscript{184,185} There is a report that the long form of alsin protects motor neurons from the toxicity induced by mutant SOD1 through the Rac1/PI3K/Akt3 pathway.\textsuperscript{187} This study has raised a possibility that loss of alsin is a upstream signalling pathway of motor neuron degeneration occurring both in ALS1 and ALS2, or that loss of alsin may contribute to development of ALS1.
9.8.2 Alsin Knock-out Mice

The major pathology in ALS2 patients is prominent in the upper motor neuron system, including upper motor neurons in the cortex, corticobulbar and corticospinal tracts (CST), with lesser involvement of the lower motor neuron system. Alsin-deficient mice show variable phenotypes of motor impairment and degenerative pathology in CST without apparent motor neuron pathology. Alsin−/− mice do not display any apparent neurological phenotype even in mice over 400 days old. In a Rota-Rod test, both male and female Alsin−/− mice showed a significantly reduced motor activity compared with age-matched controls. Alsin−/− mice do not display any apparent neurological phenotype even in mice over 400 days old. In a Rota-Rod test, both male and female Alsin−/− mice showed a significantly reduced motor activity compared with age-matched controls.

9.8.3 Phenotypes of Alsin Knock-out Mice

Alsin has two transcriptional forms with two distinct poly (A) signals and encodes one short and one long form of protein product. The short form of alsin gene has four exons while the long form of alsin gene has 34 exons sharing the first four exons. A 1-bp deletion in exon 3 causes frame shift and premature stop codon (A46fsX50), which is verified in the lymphoblasts of human ALS2 patients. A homozygous deletion mutation A46fsX50 that interrupts both forms of alsin proteins leads to ALS2, whereas a homozygous L623fsX647 which only interrupts the long form, leaving the short form intact, leads to JPLS.

The major pathology in ALS2 patients is prominent in the upper motor neuron system, including upper motor neurons in the cortex, corticobulbar and CST, with lesser involvement of the lower motor neuron system. It has been well-documented that transgenic mice overexpressing SOD1G93A show massive ubiquitinated SOD1-containing aggregates in neurons and neurite processes in the spinal cord sections. In contrast, Alsin−/− mice do not show substantial neuron loss in cortex and spinal cord despite apparent SOD1-positive ubiquitinated aggregates. These data indicates that alsin may not be indispensable for motor neuron survival. In fact, alsin-mediated ALS2 is primarily a distal axonopathy rather than a neuropathy, because the CST around and above the pyramidal decussation is more affected than the CST in the spinal cord. Recently increased susceptibility to glutamate excitotoxicity was observed in the cultured spinal cord slice of alsin-deficient mice.
The transgenic lines with high expression of mutant SOD1\textsuperscript{G93A} develop ALS around 100 days of the age.\textsuperscript{3,9} However, Alsin \textendash/ mice do not display any motor abnormality by the age of one year. This suggests that alsin deficiency is not responsible for the development of ALS1, although alsin levels are decreased in ALS1 cases and SOD1\textsuperscript{G93A} mice. In addition, SOD1\textsuperscript{G93A} mice on the Alsin \textendash/ background did not change progression or severity of the disease, suggesting that loss of alsin does not trigger motor neuron degeneration in ALS1.\textsuperscript{134} Therefore, it seems likely that the signalling pathways triggering motor neuron degeneration in ALS1 and ALS2 are independent or that the extremely rapid progression of motor dysfunction observed in the high copy number of SOD1\textsuperscript{G93A} could overwhelm the modest symptoms by loss of Alsin.

In contrast, alsin-deficiency in SOD1\textsuperscript{H46R} mice has been shown to cause widespread axonal degeneration with slowly progressive motor neuron degeneration in the spinal cord.\textsuperscript{195} Alsin-deficient SOD1\textsuperscript{H46R} mice show enhanced accumulation of SOD1 and polyubiquitinated proteins, and macroautophagy-associated proteins such as polyubiquitin-binding protein p62/SQSTM1 and a lapidated form of light chain 3 (LC3-II).\textsuperscript{195} Alsin is colocalized with LC3 and p62 and partly with SOD1 on autophosome/endosome hybrid compartments, and loss of alsin significantly lowered the lysosome-dependent clearance of LC3 and p62 \textit{in vitro}.\textsuperscript{195} Alsin-deficiency impairs the endosomal system and may exacerbate SOD1\textsuperscript{H46R} mediated neurotoxicity by accelerating the accumulation of immature vesicles and misfolded proteins in the spinal cord.

Human ALS2 pathology is not always replicated in mouse models. For example, Alsin \textendash/ mice do not show apparent locomotion deficits in their lifetime, while human ALS2 patients are forced to become bedridden before the age of \textasciitilde 59 years. This may be due to the anatomical and functional differences between humans and mice. In humans, a substantial proportion of the CST axons are located in the middle portion of the lateral column. In contrast, the main contingent of the CST axons in mice is located in the ventral part of the dorsal column and only a small portion of the CST descends in the dorsal portion of the lateral column.\textsuperscript{8,192} Unlike humans, the input from the CST seems not to be essential for normal overground locomotion in rodents, possibly due to the lack of direct cortico-motoneuronal synaptic connections between corticospinal axon boutons and motor neurons in anterior horns.

### 9.9 Transgenic Mutant TDP-43 Rodent

#### 9.9.1 TDP-43 in ALS

TDP-43 (TAR DNA binding protein 43 kDa) is encoded by \textit{TARDBP}, a highly-conserved gene on human chromosome 1. TDP-43 was initially identified as a transcriptional repressor of HIV-1 gene expression and later found to be a multifunctional protein involved in transcription, splicing and mRNA stabilization. Recent studies show that TDP-43 is a major protein component
of neuronal inclusion bodies in the affected tissues in a range of neurodegenerative disorders, including ALS, FTLD, Alzheimer's disease (AD) and other types of dementia. 196 Neuropathology related TDP-43 has currently been identified in a wide spectrum of neurodegenerative diseases collectively termed as TDP-43 proteinopathy, including ALS and FTLD. Decreased protein solubility, hyperphosphorylation, abnormal cleavage and cytoplasmic mislocalization of TDP-43 have been associated with TDP-43 proteinopathy. 196 It is not clear whether TDP-43 proteinopathy is caused by 'loss-of function' or 'gain-of-function' of TDP-43.

In ALS and FTLD patients, TDP-43-immunoreactive inclusions are observed in the cytoplasm and nucleus of both neurons and glial cells, suggesting that the two disorders share the common underlying mechanism 11,12,196 TDP-43 neuropathy induces characteristic abnormal hyperphosphorylation and ubiquitination of TDP-43 and production of ~25kDa C-terminus fragments lacking the nuclear targeting domains in the brains and spinal cords of patients. TDP-43 is also partly cleared from the nuclei of neurons containing cytoplasmic aggregates, supporting a view that pathogenesis of ALS in these cases may be driven, at least in part, by loss of normal TDP-43 function in the nucleus. Combined with subsequent reports, TDP-43 inclusions are now considered a common characteristic of most ALS patients except those with FALS caused by SOD1 mutations (ALS1). 1,4 However, the pathology alone leaves it unclear as to whether aggregation of TDP-43 is a primary event in ALS pathogenesis or whether it is a by-product of the disease process.

Dominant mutations in the TARDBP gene were reported by several groups as a primary cause of ALS (now designated ALS10). 11,12,197-200 A total of 30 different mutations are now known in 22 unrelated families (~3% of FALS cases) and in 29 sporadic cases of ALS (~1.5% of sporadic cases). 201 Interestingly, all but one of the mutations identified so far are localized in the C-terminal region encoded by exon 6 of TARDBP. 199,201 All these mutations are dominantly inherited missense mutations except a truncating mutation (Y374X) at the extreme C-terminus of the protein. 201 These missense mutations affect amino acids that are highly conserved during evolution.

FTLD and ALS are neurodegenerative diseases that show considerable clinical and pathologic overlaps. Approximately 20% of patients with ALS develop FTLD. FTLD is a relatively common cause of a dementia among patients with onset before 65 years of age, typically manifesting with behavioural changes or language impairment due to degeneration of subpopulations of cortical neurons in the frontal, temporal and insular regions.

A direct role for TDP-43 in neurodegeneration has been supported by recent findings of dominant missense mutations in TDP-43 in FALS patients. FALS-related mutations in TDP-43 were found in the C-terminal glycine-rich region, which is involved in protein–protein interactions between TDP-43 and other heterogeneous nuclear ribonuclear proteins (hnRNPs). 201 Furthermore, C-terminal fragments of TDP-43 are observed selectively in ALS and FTLD tissues, suggesting that proteolytic cleavage of TDP-43 may cause protein aggregation or another toxic property. 202
9.9.2 Mutant TDP-43 Transgenic Mice

Transgenic mice expressing a human TDP-43 construct containing the A315T mutation, which was identified in FALS patients, were generated under the control of the mouse prion promoter (Prp-TDP-43\textsuperscript{A315T}).\textsuperscript{203} Prp-TDP-43\textsuperscript{A315T} transgenic mice were born at normal Mendelian ratios, weighed the same as non-transgenic littermates and appeared normal up to three months of age. In Prp-TDP-43\textsuperscript{A315T} mice, the exogenous TDP-43\textsuperscript{A315T} was expressed highest in the brain and spinal cord, but also expressed at lower level in most other tissues, which is a typical pattern of PrP promoter-driven expression. TDP-43\textsuperscript{A315T} showed the nuclear localization in both neuron and glia throughout brain and spinal cord, similar to the endogenous TDP-43.

Although Prp-TDP-43\textsuperscript{A315T} mice initially appeared normal and weighed the same as their wild-type littermates, Prp-TDP-43\textsuperscript{A315T} mice developed a gait abnormality by 3–4 months of age. By approximately 4–5 months of age, TDP-43\textsuperscript{A315T} mice began losing weight and developed a swimming gait. At this stage, they were unable to hold their body off from the ground, but could use their limbs for propulsion to slide on their stomachs. During this end-stage, they either died spontaneously or had to be euthanized if they were unable to obtain food and water. Average survival of the TDP-43\textsuperscript{A315T} mice is 154 ± 19 days. Despite the universal expression of TDP-43\textsuperscript{A315T} protein in all layers of the cortex, cytoplasmic accumulation of ubiquitinated proteins was detected exclusively in layer 5 in cortex. Increased ubiquitination levels appeared in pyramidal cells and were prominent in motor cortex. Although Prp-TDP-43\textsuperscript{A315T} transgene was expressed in the nervous system including caudate/putamen, substantia nigra, thalamus and other structures, no ubiquitin aggregates were observed even at the late/end stage of TDP-43\textsuperscript{A315T} mice.\textsuperscript{203} In TDP-43\textsuperscript{A315T} transgenic mice, glial fibrillary acidic protein (GFAP) was also selectively increased in cortical layer 5, suggesting that neuronal degeneration led to local activation of astrocytes and microglia. Tau and α-synuclein were not present as aggregates, resembling FTLD brain pathology.\textsuperscript{202} TDP-43\textsuperscript{A315T} is quite selectively involved in certain neuronal subpopulations, including cortical upper motor neurons. Taken together with these results, TDP-43\textsuperscript{A315T} transgenic mice recapitulate key features of human ALS, including ubiquitin-positive aggregates or selective vulnerability of cortical projection neurons and spinal motor neurons, but without the presence of TDP-43-positive aggregates.\textsuperscript{202,203}

9.9.3 TDP-43 and FUS/TLS

The identification of TDP-43 mutations in ALS pathogenesis fuelled the discovery of ALS mutations in another DNA/RNA binding protein-encoding gene called FUS (fused in sarcoma) or TLS (translocated in liposarcoma).\textsuperscript{13,14} Vance et al.\textsuperscript{14} prioritized sequencing of genes within the linkage region identified in a large British family with familial ALS so as to target genes encoding DNA/RNA proteins like TDP-43. This strategy led to identification of a dominant missense mutation in the FUS/TLS gene on chromosome 16.
Subsequent survey of 197 familial ALS cases identified the same point mutation in four additional families, as well as two additional missense mutations in another four families.\textsuperscript{14} Kwiatkowski et al. conducted a linkage study in an ALS family originating from the Cape Verde islands in which disease transmission was compatible with an autosomal recessive inheritance pattern.\textsuperscript{13} A region of homozygosity shared by all affected members of this family was overlapped with previously reported FALS (ALS6) locus on chromosome 16.\textsuperscript{4}

Interestingly, TDP-43 mutant is partially cleared from the nucleus of either neuronal or glial cells when TDP-43 proteins aggregate in the cytoplasm.\textsuperscript{11,12} In a minority of neurons from ALS patients with FUS/TLS mutations or cells overexpressing symptomatic FUS/TLS mutants, FUS/TLS aggregates are observed in the cytoplasm.\textsuperscript{13,14} Cytoplasmic inclusions containing TLS/FUS protein are absent in normal unaffected individuals, in FALS patients with SOD1 mutations, and in SALS patients who presumably are positive for TDP-43 aggregates.\textsuperscript{200,202} In contrast, TDP-43-positive inclusions are absent in ALS patients with FUS/TLS mutations,\textsuperscript{13,14} implying that neurodegenerative processes driven by FUS/TLS mutations are independent of TDP-43 aggregation. It will necessary to assess FUS/TLS accumulation and localization in ALS patients with TDP-43 mutations, as well as in patients with other neurodegenerative diseases, especially those with mislocalized TDP-43.

### 9.9.4 Mutant TDP-43 Transgenic Rats

Transgenic rats expressing normal human TDP-43 or mutant form of human TDP-43 with a M337V amino acid substitution (TDP-43\textsuperscript{M337V}) have recently been developed.\textsuperscript{204} TDP-43\textsuperscript{M337V} transgenic rats manifest ALS phenotypes such as progressive degeneration of motor neurons and denervation atrophy of skeletal muscles.\textsuperscript{204} TDP-43\textsuperscript{M337V} transgenic rats also recapitulate major pathological features of ALS and TDP-43 proteinopathies such as formation of TDP-43 inclusions, cytoplasmic accumulation of phosphorylated TDP-43, and fragmentation of TDP-43 protein.\textsuperscript{202}

To fully understand how TDP-43 mutants cause motor neuron degeneration, more sophisticated transgenic models will be required, although rats transgenic for TDP-43\textsuperscript{M337V} have provided evidence that TDP-43 mutation is neurotoxic and develops ALS in rodent models.

### 9.10 Conclusions

The numerous studies carried out to understand the mechanisms of motor neuron degeneration in ALS by genetic and pathohistological approaches have revealed that ALS can be a multifactorial disease caused by various neurotoxic insults such as mutations in a gene encoding ubiquitously expressed enzymes or
defects in the cytoskeleton-dependent intracellular trafficking, all of which lead the selective degeneration of motor neurons.\textsuperscript{1,3,4}

ALS is a progressive neurodegenerative disease with limited survival, though promising therapeutic trials are underway. Nonetheless, animal models play a key role in ALS research, though their preclinical application requires extra caution. One of the long-standing arguments is how and when the disease begins and progresses. This problem often recurs even in the use of well-established mutant SOD1 transgenic rodent models. There cannot be a single standard ALS animal model for human ALS because of intrinsic differences between the two organisms. Knowing the various neurodegenerative mechanisms implicated in ALS pathogenesis, researchers must carefully select the disease model based on its applicability to what they are aiming to examine and clarify.

Enormous efforts have been made over the past 15 years, mainly in the study of ALS-related mutations in SOD1, to understand ALS pathophysiology.\textsuperscript{3} No consensus has yet been reached as to how SOD1 mutations lead to selective and premature death of motor neurons.\textsuperscript{205} Identification of genetic modifiers in ALS has provided potential therapeutic targets in ALS. Nonetheless, the transgenic approach is a robust tool for the identification and evaluation of the effects of genetic modifiers. A recent discovery of ALS-associated mutations in TDP-43 and FUS/TLS, both of which are DNA/RNA-binding proteins implicated in RNA processing,\textsuperscript{201,206,207} has shed light on RNA metabolism in ALS pathogenesis.\textsuperscript{11–14} Furthermore, a new causal gene product optineurin (OPTN), an adaptor protein which was already known to bind with multiple proteins, has recently been discovered.\textsuperscript{15} OPTN may share commonality with TLS/FUS or TDP-43 because, for example, TLS/FUS binds to actin motor protein, myosin Va and VI,\textsuperscript{208,209} whereas OPTN also binds to myosin VI.\textsuperscript{210} Various animal models of TLS/FUS and OPTN will provide further insights to understanding the pathophysiology of ALS.

There are limitations in the application of the animal models to human ALS due to intrinsic differences in genetics and anatomy. To compensate for these, multiple ALS models should be incorporated into preclinical trials. Translational research has made great progress with the development of ALS rodent models for the past 15 years. However, none of them has been successful yet. Additional breakthroughs in technologies and therapeutic approaches are still demanded to successfully translate animal ALS models to human patients, or vice versa.

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References


