Expression of wild-type human superoxide dismutase-1 in mice causes amyotrophic lateral sclerosis

Karin S. Graffmo1,†, Karin Forsberg1,†, Johan Bergh1, Anna Birve2, Per Zetterström1, Peter M. Andersen2, Stefan L. Marklund1,* and Thomas Brännström1

1Department of Medical Biosciences and 2Department of Pharmacology and Clinical Neuroscience, Umeå University, SE-901 85 Umeå, Sweden

Received June 19, 2012; Revised August 21, 2012; Accepted September 18, 2012

A common cause of amyotrophic lateral sclerosis (ALS) is mutations in the gene encoding superoxide dismutase-1. There is evolving circumstantial evidence that the wild-type protein can also be neurotoxic and that it may more generally be involved in the pathogenesis of ALS. To test this proposition more directly, we generated mice that express wild-type human superoxide dismutase-1 at a rate close to that of mutant superoxide dismutase-1 in the commonly studied G93A transgenic model. These mice developed an ALS-like syndrome and became terminally ill after around 370 days. The loss of spinal ventral neurons was similar to that in the G93A and other mutant superoxide dismutase-1 models, and large amounts of aggregated superoxide dismutase-1 were found in spinal cords, but also in the brain. The findings show that wild-type human superoxide dismutase-1 has the ability to cause ALS in mice, and they support the hypothesis of a more general involvement of the protein in the disease in humans.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by progressive loss of upper and lower motor neurons, which results in paralysis and finally death from respiratory failure. While most of the cases appear to be sporadic, at least 10% of the patients show a familial predisposition. More than 10 ALS-associated genes have been identified in families so far; the most common being C9ORF72, superoxide dismutase-1 (SOD1), TAR-DNA binding protein-43 (TDP43) and fused in sarcoma (FUS). Mutations in these are also occasionally found in apparently sporadic patients (1). The underlying causes of the remaining ALS cases are unknown. In several other neurodegenerative conditions such as Alzheimer’s, Parkinson’s and Creutzfeldt-Jacob’s diseases, some of the proteins found mutated in families are also thought to be involved in the pathogenesis in patients lacking such mutations (2). Could the same situation pertain to ALS? Neuronal cytosolic inclusions containing TDP43 are found in cases carrying mutations in the gene, but usually also in apparently sporadic ALS patients and patients carrying some other ALS-linked mutations (3). Based on such findings, it has been suggested that TDP43 might more generally be involved in ALS pathogenesis. The disease caused by mutations in SOD1 has been regarded as an entity separate from ALS in general, since TDP43 inclusions are not found in the motor neurons (3).

There is, however, circumstantial evidence suggesting that the wild-type (wt) human SOD1 (wt-hSOD1) might commonly be involved in ALS. Inclusions containing aggregated SOD1 are considered hallmarks of ALS caused by mutant SOD1s (4–6), but can with a set of antibodies specific for misfolded hSOD1 species also regularly be demonstrated in motor neuron somas (7) and glial cell nuclei (8) of ALS patients lacking SOD1 mutations. Other antibodies reactive with an epitope in mutant SOD1 or in misfolded SOD1s (4–6) have been found to stain motor neuron somas (9) and axons (10) in some patients with sporadic ALS. Astrocytes generated from sporadic ALS patients are toxic to cocultured motor neurons, an effect that is attenuated by SOD1 knockdown (11). In old mice expressing wt-hSOD1 (12), a loss of motor neurons...
can be demonstrated, although the lifespan is not shortened (13,14). Wt-hSOD1 expression in mice also exacerbates disease caused by mutant hSOD1s (13,15–17). Although most ALS-linked mutant hSOD1s show low structural stabilities, some are close and even equal to that of wt-hSOD1, which in turn is much less stable than the murine SOD1 (18–20). Accordingly, misfolded mutant hSOD1s can in cultured cells induce misfolding of the moderately stable wt-hSOD1 and the process thereafter seems to propagate independent of the mutant hSOD1s (21).

To examine the ALS-causing ability of wt-hSOD1 more directly, we here studied the effects of overexpressing the protein in mice. To cause disease within the short lifespan of mice, mutant hSODs have to be expressed at rates around 25-fold higher than that of the endogenous murine SOD1 (14). Furthermore, the toxicity in the transgenic ALS models is highly dependent on the expression rate: for a given mutant, a doubling broadly halves the lifespan (6,14,19). In the most commonly studied murine model, G93A mutant hSOD1 is expressed at a very high rate, around double that seen in many other current models including mice expressing wt-hSOD1 (12,14,19). In this study, we aimed at expressing wt-hSOD1 in mice at a rate close to that seen in the G93A model. The mice generated developed a fatal ALS-like disease, mimicking that seen in mice expressing mutant hSOD1s. The findings lend further support to the idea that wt-hSOD1 may generally be involved in ALS in humans.

RESULTS

Mice expressing wt-hSOD1 at a high-level develop deadly motor neuron disease

To increase the expression of wt-hSOD1, we initially crossed the hemizygous N1029 mice (12) on C57Bl/6 background to generate homozygotes for the transgene insertion. Only few homozygous pups were born, however, which is why we instead tested mice with the CBA genetic background. On that background homozygous, pups were born at apparently Mendelian rates: among 79 pups 21.5% were homozygotes, 59.5% hemizygotes and 19.5% non-transgenic. The expression rate of wt-hSOD1 in the homozygotes was assessed by northern blots and found to be close to that of mutant hSOD1 in G93A mice (Table 1). The wt-hSOD1 homozygous mice showed a slower weight development than hemizygous and non-transgenic CBA mice, but they appeared otherwise normal as pups and young adults (Fig. 1).

Table 1. Expression of hSOD1 mRNA in the brain of transgenic strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Percent of level in G93A mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>G93A hemizygous</td>
<td>C57Bl/6</td>
<td>100</td>
</tr>
<tr>
<td>Wt-hSOD1 hemizygous</td>
<td>C57Bl/6</td>
<td>54.4 ± 10.1</td>
</tr>
<tr>
<td>Wt-hSOD1 homozygous</td>
<td>CBA</td>
<td>45.0 ± 9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>76.3 ± 12.0</td>
</tr>
</tbody>
</table>

Messenger RNA was analyzed by northern blot. The results were normalized against β-actin. The values are means ± SD of results for three 100-day-old mice of each strain which were analyzed twice. They are expressed as percentages of values for three G93A mice which were analyzed in parallel.

Figure 1. Weight development of the mice. (A) Female mice: CBA control (filled squares, n = 14), wt-hSOD1 hemizygous (open circles, n = 11) and wt-hSOD1 homozygous mice (filled triangles, n = 11). (B) Male mice: CBA control (filled squares, n = 19), wt-hSOD1 hemizygous (open circles, n = 20) and wt-hSOD1 homozygous mice (filled triangles, n = 19). The mice were weighed once a week.

Figure 2. Kaplan–Meyer diagram showing the cumulative survival of homozygous wt-hSOD1 mice. The median survival time was 367 days.
At an average of 253 ± 46 (SD) days of age, the mice started showing evidence of hindleg paresis in the form of deficient leg splaying. At a mean age of 367 ± 56 (SD; n = 25) days of age, the mice were deemed terminally ill and were killed (Fig. 2). For comparison, the G93A mice currently live 155 ± 9 (SD; n = 170) days in our laboratory. The principal symptoms were progressive hindleg paresis similar to that seen in the G93A and D90A hSOD1 transgenic models (14,19). The weights did not reach normal values at any age, and a weight loss was seen during the final 100 days (Fig. 1). Hemizygous wt-hSOD1 CBA mice had a normal lifespan and no obvious motor neuron phenotype. Their weight development was similar to that observed in non-transgenic CBA mice (Fig. 1).

A deviant feature in the homozygous mice is an ataxic staggering gait, which appears at around 200 days. This staggering is also seen in hemizygous wt-hSOD1 transgenic mice on both CBA and C57Bl/6 background starting at ~450 days, but has not been observed in any of the four mutant hSOD1 transgenic ALS models [G85R, D90A, G93A, G127insG] kept in our laboratory.

CNS histopathology

In the spinal cord, diffuse staining of misfolded hSOD1 was seen in motor neurons already at 100 days of age in wt-hSOD1 homozygous mice and was aggravated in terminally ill mice. The staining was mostly cytoplasmic, although in some cases it could also be seen in the nuclei (Fig. 3B and G). Terminally ill mice showed severe loss of motor neurons and the remaining cells were pyknotic and showed the disruption of integrity. In addition, cytoplasmic staining of misfolded
Table 2. Numbers of neurons in the thoracic spinal cord ventral horns of transgenic and non-transgenic control mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt-hSOD1 tg mice (n = 8)</td>
<td>134 700 ± 29 700 (mean age 340 ± 50 days)</td>
</tr>
<tr>
<td>Hemizygous</td>
<td>153 500 ± 25 600 (mean age 403 ± 7 days)</td>
</tr>
<tr>
<td>Wt-hSOD1 tg mice (n = 5)</td>
<td>229 800 ± 28 200 (mean age 335 ± 14 days)</td>
</tr>
<tr>
<td>CBA mice (n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

The homozygous wt-hSOD1 mice were terminally ill, and the hemizygous mice and CBA controls were examined at matching ages.

SOD1 was seen in brainstem motor neurons as well as in neurons in the motor areas of the cortex as defined in Tennant et al. (22). There was also vacuolization in the ventral horn neuropil, which became more pronounced as the disease progressed. The vacuoles were lined with material immunopositive for misfolded SOD1, and in terminal mice, this was the dominant pathology. Vacuoles were also seen in the ventral funiculus and ventral roots, indicating that at least some of the neuropil vacuoles represent axonal damage. Astrogliaosis was seen in the 100-day-old wt-hSOD1 mice and was aggrivated in the terminal mice. Some of the glial cells also showed intranuclear staining for misfolded SOD1 (Fig. 3B and G, arrowhead). Interestingly, age-matched wt-hSOD1 hemizygotes also had inclusions of misfolded SOD1 in the cytoplasm and also vacuolization in the neuropil, resembling the picture in 100-day-old wt-hSOD1 homozygotes (Fig. 3I).

To quantify the damage, ventral horn neurons were counted by stereology (Table 2). The thoracic part of the degenerating spinal cord was chosen for this study, since it can be precisely delimited generating high precision in the counting and calculations. Terminally ill homozygous wt-hSOD1 mice had lost 41% of the neurons. Remaining thoracic ventral horn neurons in end-stage G93A and D90A mice have previously been counted using the same protocol, yielding similar results (45 and 40% losses, respectively) (19). The loss in age-matched hemizygotes (33%) was somewhat less than that previously found at a later stage (570 days) in hemizygous wt-hSOD1 mice on C57Bl/6 background (38%) (19).

The terminal wt-hSOD1 homozygous mice and the age-matched wt-hSOD1 hemizygotes all had cytoplasmic SOD1-positive inclusions in the axons and neurons of the hippocampus. Vacuolization in the hippocampal area was found already at 100 days in wt-hSOD1 homozygotes and in the terminal homozygotes almost all neurons in the subiculum showed heavy vacuolization (Fig. 3I). For comparison, four terminally ill mice of our other ALS models were examined by SOD1 immunohistochemistry, as were used 600-day-old hemizygous wt-hSOD1 mice on C57Bl/6 background. Misfolded hSOD1 was seen in at least some hippocampal axons and neurons in all transgenic mice, most staining seen in the cornu ammonis 1 and 2 areas. As in the homozygous wt-hSOD1 mice, heavy vacuolization in the subiculum was seen in D90A and the hemizygous wt-hSOD1 mice, whereas this area was unremarkable in G93A, G85R and G127X mice.

In the cerebellum, Purkinje cells showed mostly cytoplasmic staining for misfolded hSOD1. This was seen already in 100-day-old wt-hSOD1 homozygotes and also in 350-day-old wt-hSOD1 hemizygotes. In terminally ill homozygotes, there was a loss of Purkinje cells, which was estimated to be around 25%. The remaining Purkinje cells were heavily surrounded by vacuoles and showed severe damage and disruption of integrity (Fig. 3I). In the surrounding neuropil as well as in the granular layer of the cerebellum, many hSOD1-positive vacuoles and aggregates were seen (Fig. 3H and I). In our other ALS model mice, the strongest staining for misfolded hSOD1 was likewise seen in Purkinje cells, which, however, did not show any cell loss, except in one G85R mouse in which a 5% loss was estimated. In the 600-day-old hemizygous wt-hSOD1 transgenes, however, there was vacuolization in the Purkinje cell layer as well as a cell loss estimated at 5–10%.

Expression of hSOD1 in tissues

The content of wt-hSOD1 was determined in the spinal cord, brain, liver and muscle at around 100 days and in terminal/350-day-old mice (Table 3). In the spinal cord and brain from 100-day-old wt-hSOD1 homozygotes, the levels were twice as high as in hemizygotes and around 4-fold as high as in G93A mice (14,19). The wt-hSOD1 accounted for 3–4% of the soluble protein in the central nervous system (CNS) tissues (Table 3). If the murine SOD1 in CBA controls is fully active and has the same specific activity as wt-hSOD1, the enzymatic activity data suggest that it accounts for 0.06% of the total soluble tissue protein. The levels of wt-hSOD1 protein in the homozygotes thus appear to be some 50-fold higher than the level of endogenous murine SOD1. In 100-day-old homozygous mice, the proportion of wt-hSOD1 with a reduced C57-C146 disulfide bond was higher in the spinal cord than in the brain and cerebellum but became lower in terminally ill mice (Table 3). As previously observed in G93A and D90A mice which also express high levels of hSOD1 (14), most of the protein lacked enzymatic activity. We calculated that the proportion active SOD1 in the homozygous spinal cord was ~15%, using the specific activity of native Cu-charged hSOD1 (18,23). The proportion active SOD1 was somewhat higher in the brain and cerebellum and much higher in the liver and muscle. This low enzymatic activity of the hSOD1 protein is caused by insufficient Cu-charging: addition of Cu²⁺ ions to an extract led to a 7-fold increase in SOD activity (Fig. 4A). Only single bands at the expected position of SOD1 monomers were seen in western immunoblots of extracts of the spinal cords from terminally ill mice (Fig. 4B).

As analyzed by a filter trap assay for aggregates, the spinal cords from terminal homozygous wt-hSOD1 mice contained large amounts of hSOD1 aggregates (Fig. 4C). The brain also contained significant amounts of aggregates, at around 40% of the level seen in the spinal cord (comparison in four mice). Despite similar expression of the hSOD1 protein (Table 3), no aggregates were detected in the liver nor in the skeletal muscle (Fig. 4C). No significant amounts of aggregates were found at 100 days in homozygous and hemizygous mice and 350 days in hemizygous wt-hSOD1 mice. At 600 days, aggregates were found in the spinal cords and brain also from hemizygous mice, at around 1/10th of the levels seen in terminal
Table 3. Analysis of hSOD1 content, percentage of C57–C146 disulfide bond reduction and SOD1 activity

<table>
<thead>
<tr>
<th></th>
<th>100 days</th>
<th>SOD1 (mg/g ww)</th>
<th>SOD1 % reduced</th>
<th>SOD1 activity (kU/g ww)</th>
<th>350 days/terminal</th>
<th>SOD1 (mg/g ww)</th>
<th>SOD1 % reduced</th>
<th>SOD1 activity (kU/g ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal cord</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>3.34 ± 0.94</td>
<td>11.4 ± 0.4</td>
<td>120.0 ± 5.4</td>
<td>4.14 ± 0.40</td>
<td>3.9 ± 0.2</td>
<td>141.0 ± 7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemizygous</td>
<td>1.35 ± 0.38</td>
<td>6.0 ± 0.4</td>
<td>115.0 ± 7.1</td>
<td>1.55 ± 0.69</td>
<td>6.8 ± 0.4</td>
<td>119.0 ± 9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA control</td>
<td></td>
<td>12.2 ± 0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>3.44 ± 0.81</td>
<td>8.4 ± 0.7</td>
<td>135.0 ± 9.7</td>
<td>4.67 ± 0.87</td>
<td>6.4 ± 0.2</td>
<td>142.0 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemizygous</td>
<td>1.60 ± 0.04</td>
<td>8.5 ± 0.7</td>
<td>125.0 ± 3.3</td>
<td>1.71 ± 0.32</td>
<td>7.0 ± 0.3</td>
<td>157.0 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA control</td>
<td></td>
<td>16.9 ± 6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>3.06 ± 0.71</td>
<td>8.5 ± 0.7</td>
<td>143.0 ± 8.5</td>
<td>4.54 ± 0.79</td>
<td>3.9 ± 0.4</td>
<td>169.0 ± 3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemizygous</td>
<td>1.64 ± 0.44</td>
<td>6.1 ± 0.6</td>
<td>139.0 ± 9.1</td>
<td>1.97 ± 0.42</td>
<td>7.3 ± 0.3</td>
<td>158.0 ± 10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA control</td>
<td></td>
<td>15.8 ± 6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>3.37</td>
<td>527</td>
<td></td>
<td>4.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemizygous</td>
<td>1.52</td>
<td>371</td>
<td></td>
<td>2.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA control</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>1.21</td>
<td>117</td>
<td></td>
<td>1.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemizygous</td>
<td>0.80</td>
<td>50</td>
<td></td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA control</td>
<td></td>
<td>7.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The spinal cord, brain and cerebellum were analyzed from three mice of all genotypes of both ages. Data presented are mean ± SD. At 100 days, the contents of the total soluble protein in the spinal cord, brain, cerebellum, liver and skeletal muscle were 83, 119, 105, 249 and 123 mg/g wet weight (ww), respectively.

homozygous mice. For comparison, terminal G93A mice were examined (Fig. 4C). In these, the highest levels of aggregates were also found in the spinal cord, but the levels in the brain were relatively lower: around 4% of those in the spinal cord. The mean value in the filter trap assay of the spinal cords from end-stage homozygous wt-hSOD1 mice was 65% (n = 4) of that in G93A mice (n = 5). In comparison, the results for D90A, G85R and G127X mice were 88% (n = 5), 119% (n = 3) and 36% (n = 4), respectively, of those found in G93A mice. The brain/spinal cord ratios were also low in the other mutant hSOD1-expressing mice: around 2, 4 and 10% in the D90A (n = 3), G85R (n = 3) and G127X (n = 4) models, respectively.

**DISCUSSION**

Our principal finding is that mice that express wt-hSOD1 at a rate close to that of the mutant enzyme in G93A transgenics develop a fatal ALS-like disease. In terminally ill mice, the loss of ventral horn neurons is similar in the two models (Table 2) (19). The vacuolization, gliosis and other pathological changes in the spinal cords (Fig. 3A, B, F and G) are also similar to those previously seen in G93A and D90A transgenic mice (14,19). There is also considerable aggregation of hSOD1 in the spinal cords, as in mice carrying mutant hSOD1s (Fig. 4C).

There are, however, also features different from those in mice expressing mutant hSOD1s. The mice develop an ataxic staggering gait, earlier in homozygous than in heterozygous wt-hSOD1 transgenics, which is seen both on CBA and C57Bl/6 background. The relative amounts of hSOD1 aggregates in the brain were higher than found in mice expressing G93A (Fig. 4C) and other mutant hSOD1s.

Although the motor system impairment is most prominent in ALS, other parts of the CNS can also become involved. This was first observed already in the 19th century (24). More recently, imaging (25,26) and histopathological studies (27–30) have established the occurrence of multisystem pathology in the CNS including the cerebellum in ALS patients with and without SOD1 mutations. Lending further support for the involvement of the cerebellum is the observation of overt cerebellar ataxia in some cases of ALS with SOD1 mutations (31). For these reasons, we examined the brain and cerebellum in the homozygous wt-hSOD1 transgenic mice and for comparison in our other hSOD1 transgenic models. The pathological findings were found to vary considerably between the ALS model mice. The distinct vacuolization of the subiculum of the homozygous wt-hSOD1 mice appeared also in terminal D90A mice and the 600-day-old hemizygous wt-hSOD1 transgenes but not in G93A, G85R and G127X mice. It has also previously been observed in hemizygous wt-hSOD1 transgenes (13,32). The single unique pathological change associated with wt-hSOD1 overexpression in the areas here examined was the disturbance and loss of Purkinje cells. Perhaps, the staggering gait of the mice is related to this pathology.

The amounts of hSOD1 in the CNS were high in homozygous wt-hSOD1 transgenic mice, four times those in G93A transgenic mice (14). The difference versus the latter is apparently explained by the lower stability of the G93A mutant (33–35), leading to greater population of un/misfolded molecular species which become targeted for degradation (19). The levels of the hSOD1 protein in the CNS vary widely between mutants, e.g. the ratios between D90A and G217X hSOD1 proteins are around 100 in humans and 40 in transgenic mice (6,14). This suggests that ALS is not caused by the bulk of essentially natively folded hSOD1 species, but rather by minute amounts of misfolded and probably disulfide-reduced hSOD1 species (6,36). Loss of the disulfide bond leads to a greater propensity of hSOD1 to aggregate in vitro...
and the hSOD1 present in spinal cord aggregates lacks the C57–C146 disulfide bond (39,40). The aggregates detected here should therefore be derived from misfolded subfractions of the large amounts of C57–C146 bond reduced hSOD1 present in the CNS of the homozygous wt-hSOD1 transgenic mice (Table 3). The proportion of disulfide-reduced wt-hSOD1 in the spinal cord was reduced in the terminal stage (Table 3). Perhaps, this can be explained by the terminal oxidative stress seen in the transgenic models (41). Whether SOD1 aggregates are the offenders in ALS pathogenesis or whether they should be seen as terminal markers for the presence of more toxic oligomeric or monomeric misfolded SOD1 species is currently unknown.

A word of caution is necessary because of the great overexpression of the hSOD1 protein in the current homozygous wt-hSOD1 mice. Such overexpression, although not as extensive, is also seen in mice expressing wild-type-like hSOD1 mutants, and it might produce artifacts unrelated to the core ALS-causing mechanisms. There is incomplete Cu-charging of the hSOD1 variants despite 3–5-fold increases in copper chaperone for SOD (CCS) (14). This CCS induction is probably insufficient to charge the hSOD1s which are overexpressed to much larger extents. It also suggests that there is a reduced availability of Cu ions in the tissue (42) which might cause adverse effects. Treatment of G93A mice with a cell-permeant Cu chelator has, however, been found to prolong the lifespan, arguing against a toxic effect of Cu deficiency in models expressing wild-type-like hSOD1s (43). Another effect is marked overloading of the mitochondrial intermembrane space (IMS) with hSOD1 (44), which is associated with vacuolization and other morphological changes (Fig. 3) (13,14,19,32). It is not with certainty known to which extent this artifact contributes to the disease phenotype of the mice. In this regard, a series of studies in mice overexpressing CCS suggest that IMS overloading at least with mutant hSOD1s can cause severe adverse effects (45–47).
The overexpression of CCS drastically shortened the lifespan of mice expressing the wild-type-like hSOD1 mutants G93A and G37R, concomitantly with markedly increased proportions of disulfide-reduced hSOD1 in the tissue as well as marked increases in mitochondrial IMS hSOD1 loading and vacuolization. Unlike in single hSOD1 transgenics, the terminal disease in the double-transgenic mice was not associated with hSOD1 aggregation. CCS overexpression also caused moderately increased loading of wild-type hSOD1 in mitochondria in N1029 hemizygous wt-hSOD1 mice, but in this case no adverse effects could be detected. Notably, in these mice, the proportion of disulfide-reduced hSOD1 was decreased by the CCS overexpression. Another phenomenon that seems to be related to the mitochondrial hSOD1 overloading and morphological changes is axonal transport deficits which are seen in both G93A and G37R mice (48). Such deficits are, however, also found in N1029 hemizygous wt-hSOD1 mice but not in G85R mice. This suggests that the transport deficits are tolerated and that hSOD1-induced ALS is caused by other mechanisms. To conclude, the wild-type and wild-type-like hSOD1 mutants might cause disease phenotypes which stem from a combination of ALS-relevant effects and artifacts caused by the large overexpression of the hSOD1 protein. From the current knowledge, there are no indications that the great overexpression of wild-type hSOD1 would cause more irrelevant effects than the wild-type-like mutants.

Related to expression rates (=mRNA), the D90A mutant hSOD1 has the lowest potential to cause ALS of the mutants expressed in the transgenic models kept in our laboratory (14,19). Homozygous D90A mice and hemizygous wt-hSOD1 mice have similar hSOD1 mRNA levels (14). Based on the loss of spinal ventral neurons and other evidence of pathology, wt-hSOD1 has been estimated to have a neurotoxicity between half of and equal to that of the D90A mutant (19). While hemizygous D90A mice have normal lifespans, the homozygous mice live around 430 days. According to our previous assessment, homozygous wt-hSOD1 mice should have a somewhat shorter lifespan than that, which is what we found here.

N1019 hemizygous wt-hSOD1 mice have commonly been used as controls for hSOD1 overexpression in studies of G93A and other hSOD1 transgenic ALS models. This is unfortunate for two reasons. (I) The relatively low hSOD1 expression rate, which was reported in the original paper (12), makes it a less than perfect control for the overexpression of presumed innocuous hSOD1. (II) It is here shown that it can exert ALS-provoking effects. Thus, alterations seen in the control and therefore disregarded may have been relevant.

An antibody reactive with misfolded wt-hSOD1 was recently found to label motor axons in a carrier of a FUS mutation as well as in sporadic ALS patients displaying cytosolic TDP43-immunoreactive inclusions (10). Moreover, cytosolic expression of human mutant TDP43 or FUS and wild-type TDP43 in cultured human neuroblastoma cells was found to induce misfolding of the endogenous wt-hSOD1 (10). Thus, misfolding of wt-hSOD1 might occur secondarily to TDP43 or FUS pathology. Since mutant hSOD1s owing to reduced structural stabilities are prone to misfold spontaneously, the findings might explain the absence of TDP43-immunoreactive inclusions in so far examined ALS patients carrying SOD1 mutations.

Hypothetically, toxicity caused by misfolded hSOD1s might be a final common pathway in ALS pathogenesis.

In conclusion, we here show that wt-hSOD1 has the ability to cause ALS, although it is lower than that of mutant hSOD1s. The variation in phenotypes and penetrance in families expressing mutant hSOD1s and the existence of recessive and dominant inheritance associated with the D90A mutant suggest that genetic, environmental and lifestyle-related factors influence the susceptibility to hSOD1-induced ALS (1). Possibly such factors occasionally induce the wild-type hSOD1 to cause the disease.

MATERIALS AND METHODS

Mice

The G1H G93A mice used (12) were backcrossed >30 generations in C57Bl/6 mice. Mice hemizygous for the insertion site of a wt-hSOD1 transgene (N1029) (12) in C57Bl/6 background were backcrossed 8–10 generations into CBA background. These mice were in turn crossed for the production of mouse homozygous for the wt-hSOD1 insertion. For comparison, mice expressing D90A (19), G127X (6) and G85R (49) mutant hSOD1s were also examined. All were backcrossed 10–30 generations into C57Bl/6 background. The mice were checked for symptoms at least every third day and they were weighed once a week. The initial ALS-like symptoms considered were deficient splaying of the hindlegs when held by the tail. The mice were deemed terminally ill if they could not right themselves within 5 s after being put on their side. The use and maintenance of the mice and the experimental protocols described in this article were approved by the Ethics Committee for Animal Research at Umeå University.

Antibodies

Antibodies to peptides corresponding to amino acids 24–39, 57–72 and 131–153 in hSOD1 were coupled to keyhole limpet hemocyanin and raised in rabbits as described previously (6,7). The antibodies were purified from sera using Protein A-Sepharose (GE Healthcare, Uppsala, Sweden) followed by Sulfolink gel with the respective peptides coupled (Pierce, Rockford, IL, USA). These antibodies only react with misfolded hSOD1 species and show no reactivity with the native protein (7,8).

Histopathology

Wild-type hemi- and homozygotic transgenic mice and non-transgenic control mice were anesthetized by an intraperitoneal injection of midazolam, fentanyl and furosamide and killed by perfusion fixation through the heart with 4% paraformaldehyde in phosphate buffer (pH 7.6). Animals were studied at ~100 and 350 days of age (if applicable) and at terminal disease as defined previously. Five to eight mice of each age and genotype were examined. After fixation, the central nervous system was dissected in situ and the cervicothoracic and thoracolumbar borders as well as the junction between the brainstem and the spinal cord were identified by guidance.
by the ventral roots. The cervical, thoracic and lumbosacral spinal cord was dissected free, and these parts were then divided equally into six blocks, which were embedded separately in paraffin wax. The uppermost blocks of the cervical, thoracic and lumbosacral spinal cord, as well as the second lowermost block of the lumbosacral spinal cord, were used for the histopathology investigations. Sections from each piece were stained with hematoxylin and eosin as well as with antibodies toward GFAP (Dako, Glostrup, Denmark), ubiquitin (Dako) and with the 57–72 and 131–153 anti-hSOD1 peptide rabbit antibodies described previously (7,8).

If not otherwise stated, the results presented in the Results section correspond to findings at all three levels of the spinal cord. The immunohistochemistry was performed using the Ventana ES immunohistochemistry system and the standard protocol, which was preceded by microwave irradiation of the sections in citric acid buffer for 5 min. The primary antibodies were detected with biotin-conjugated secondary antibodies coupled to an avidin-borseradish peroxidase conjugate. The complexes formed were visualized using amionoethylcarbazole as the precipitating enzyme product. Sections were counterstained with hematoxylin, washed and mounted with glycerin gelatin.

Stereology

From the upper end of each of the six paraffin blocks containing the thoracic spinal cord from terminally ill homozygous wt-hSOD1 mice and from around 350-day-old hemizygous and control CBA mice, 50 μm thick sections were cut on a sliding microtome, mounted on glass slides and stained with cresyl violet. Cells with identifiable nucleolus in the nuclei were counted and the total numbers of neurons in the thoracic spinal cords were calculated as previously described (19).

Tissue homogenization

Mice were killed by intraperitoneal injection of pentobarbital. The dissected tissues were homogenized with an Ultraturrax apparatus (IKA, Staufen, Germany) for 1 min and sonicated for 1 min at 4°C in 25 volumes of ice-cold pH 7.0 buffer solution composed of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 1.8 mM ethylenediaminetetraacetic acid, 1 mM dithithreitol, and the antiproteolytic cocktail Complete® (Roche Diagnostics, Basel, Switzerland).

Immunoblots and quantifications

Common western immunoblots were generally carried out as previously described (14) using the 24–39 antipeptide antibody which is specific for hSOD1. The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and analyzed with Quantity One software (Bio-Rad Laboratories). For quantification of hSOD1s in immunoblots, wt-hSOD1 with the concentration determined by quantitative amino acid analysis was used as the original standard (18).

For analysis of proportion of reduced C46-C157 disulfide bond, 40 mm of iodoacetamide was added to the homogenization buffer to alkylate-free thiol groups. Western immunoblots were carried out with sample buffer containing 40 mm iodoacetamide but no reductant. The proportion of reduced wt-hSOD1 was then determined using as standard stepwise diluted homogenates run in parallel with reductant in the sample buffer (14).

Filter trap assay for aggregates

The 1 + 25 tissue homogenates were further diluted 1 + 20 in homogenization buffer containing 1% NP40, sonicated for 30 s and then centrifuged at 200g for 10 min. The supernatants were then diluted stepwise 1 + 1 in homogenization buffer and 100 μl captured on a 0.22 μm cellulose acetate filter in a 96-well dot-blot apparatus (Whatman GmbH, Dassel, Germany). Following 3 × 300 μl washes with homogenization buffer, the filters were blocked and developed with the 57–72 anti-hSOD1 peptide antibody and subsequently quantified in the Chemidoc apparatus similarly to the western immunoblots. A homogenate of a spinal cord from a terminal G93A transgenic mouse, kept frozen in multiple aliquots, was handled in a similar way and always run in parallel on the filters as a standard.

Analysis of SOD activity

Enzymatic activity of SOD was determined with the direct spectrophotometric assay using KO2 (50). One unit is defined as the activity that brings about a decay of superoxide at a rate of 0.1 s−1 in 3 ml buffer. One unit corresponds to 4.3 ng fully Cu- and Zn-charged wt-hSOD1 (18).

Northern blot

RNA from the mouse brains was extracted using the Trizol reagent (Invitrogen) and was then subjected to northern blotting utilizing an Ambion® NorthernMax®-Gly Kit (Invitrogen), all according to the manufacturer’s instructions. The samples were normalized against β-actin, and the quantifications were carried out twice on three 100-day-old mice of each genotype. Probes were labeled using MegaPrime random labeling kit (Amersham Biosciences). As template for the β-actin probe, the DECA-template β-actin mouse supplied with the Ambion® NorthernMax®-Gly Kit was used. As template for the SOD1 probe a polymerase chain reaction (PCR) fragment was used, purified employing High Pure PCR Product Purification kit (Roche Diagnostics). The primers used were 5’- TGCTAGCTGTAGAAATGTATCC and 5’- TTCACAGGGCTGTTAGACC-3’, yielding a 306 bp amiplon of the 3’UTR region that is not homologous to the mouse transcript. Bands were visualized with a Storage phosphor screen (GE Healthcare) and scanned using a Typhoon 9400 variable mode imager (GE Healthcare). The amounts of SOD1 and β-actin mRNA were analyzed with Quantity One Software (Bio-Rad Laboratories).

ACKNOWLEDGEMENTS

We thank Eva Bern, Karin Hjertkvist, Ulla-Stina Spetz, Ann-Charloth Nilsson and Agneta Öberg for expert technical assistance.

Conflict of Interest statement. None declared.
FUNDING

This work was supported by the Swedish Science Council, the Hallström Foundation/The Brain Fund, the Swedish Association for Individuals with Neurological Disabilities, the Torsten Hållsten Foundation/the Brain Fund, the Swedish Association FUNDING

REFERENCES


