Lysosomal proliferation and distal degeneration in motor neurons expressing the G59S mutation in the p150Glued subunit of dynactin

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An increasing number of neurodegenerative diseases are being linked to mutations in genes encoding proteins required for axonal transport and intracellular trafficking. A mutation in p150Glued, a component of the cytoplasmic dynein/dynactin microtubule motor complex, results in the human neurodegenerative disease distal spinal and bulbar muscular atrophy (dSBMA). We have developed a transgenic mouse model of dSBMA; these mice exhibit late-onset, slowly progressive muscle weakness but do not have a shortened lifespan, consistent with the human phenotype. Examination of motor neurons from the transgenic model reveals the proliferation of enlarged tertiary lysosomes and lipofuscin granules, indicating significant alterations in the cellular degradative pathway. In addition, we observe deficits in axonal caliber and neuromuscular junction (NMJ) integrity, indicating distal degeneration of motor neurons. However, sciatic nerve ligation studies reveal that inhibition of axonal transport is not evident in this model. Together, these data suggest that mutant p150Glued causes neurodegeneration in the absence of significant changes in axonal transport, and therefore other functions of dynein/dynactin, such as trafficking in the degradative pathway and stabilization of the NMJ are likely to be critical in maintaining the health of motor neurons.

INTRODUCTION

Disruption of cellular transport is implicated in multiple neurodegenerative diseases, an increasing number of which are due to mutations in genes coding for motor and cytoskeletal proteins (1). Distal spinal and bulbar muscular atrophy (dSBMA) with vocal fold involvement is caused by an autosomal dominant point mutation in the p150Glued subunit of dynactin, an activator of the retrograde motor protein cytoplasmic dynein (2). The G59S point mutation occurs in the highly conserved, glycine-rich cytoskeleton-associated protein (CAP-Gly) domain of the p150Glued polypeptide, which is known to interact directly with microtubules (3). The predicted structure of the CAP-Gly domain suggests that the introduction of the G59S mutation induces a conformational change leading to an enhanced tendency for the polypeptide to misfold and aggregate (2,4). Indeed, aggregates composed of dynactin and dynein are detected in motor neurons of patients with dSBMA (5). In addition, both motor neuron loss and decreased neuropil density are observed in patient tissues. Clinically, patients with the G59S mutation present with a slowly progressing phenotype that begins with inspiratory stridor, followed by distal muscle weakness (5).

Analysis of fibroblasts cultured from patient tissue as well as of mammalian cells transfected with the G59S polypeptide have revealed defects that suggest that the mutation induces both an inhibition of dynein/dynactin function and a toxic gain of function (4). Mutant G59S p150Glued has a decreased affinity for microtubules and the microtubule plus-end binding protein EB1. Patient-derived fibroblasts showed delayed recovery after cellular stress induced by microtubule depolymerization, consistent with a loss of dynein/dynactin function. However, the presence of prominent dynein and dynactin-positive aggregates and entrapped mitochondria in transfected cells argues for a toxic gain of function that may disrupt degradative pathways and/or metabolic function (4). The effects of the G59S mutation are more pronounced in neuronal cell lines, suggesting that an in vivo model may reveal alterations to neuronal health not apparent in cell
culture and clarify the relative contributions of loss of normal dynein/dynactin function and toxic gain of function, due for example to protein misfolding/aggregation, to primary pathogenesis.

Numerous lines of evidence support the idea that defects in dynein function can lead to neuronal dysfunction and death. In Cra1 and Loa mice, point mutations in the heavy chain of the retrograde axonal motor cytoplasmic dynein (DHC) lead to a neurodegenerative phenotype (6). Disruption of the association between dynein and its activator dynactin also results in progressive neurodegeneration in mice (7). These phenotypes have been interpreted primarily as resulting from induced deficits in retrograde axonal transport, as cytoplasmic dynein is the major molecular motor driving transport from the cell periphery to the cell body. However, dynein and dynactin are essential for multiple cellular functions, including trafficking of endosomes, lysosomes and mitochondria (1). Dynactin has also been suggested to be essential to maintain the stability of the neuromuscular junction (NMJ) (8).

Here, we examine the cellular effects of mutant p150 Glued expression in a transgenic mouse model of dSBMA. This model is characterized phenotypically by slowly progressive muscle weakness. At the cellular level, we see the enlargement and proliferation of lysosomes and lipofuscin granules in comparison with littermate controls. In addition, we observe alterations in the axonal caliber of motor neurons and disruptions in the morphology of NMJs, indicating distal changes in motor neurons. Surprisingly, however, we do not see a significant inhibition of retrograde axonal transport, suggesting that other dynein/dynactin-driven processes are critical in maintaining neuronal health.

RESULTS

Low-level expression of mutant p150 Glued expression in a transgenic mouse model leads to slowly progressive muscle weakness

To examine the cellular consequences of mutant p150 Glued expression in a novel model of the human neurodegenerative disease dSBMA, we developed several lines of transgenic mice expressing human p150 Glued with the G59S point mutation, fused to a C-terminal Myc-tag and driven by the Thy 1.2 expression cassette, which drives postnatal, neuronal-specific expression primarily in motor neurons (7). Of the two transgenic lines identified that stably express the transgene both the endogenous and G59S forms of p150 Glued. The blots were also probed with antibodies to the myc-tag of the transgene, dynein (DIC), and actin. (C) Brain lysates from non-transgenic control mice (top) and Tg G59S mice (bottom) were fractionated on sucrose density gradients, and then analyzed by SDS–PAGE and western blot, using antibodies to dynein (DHC, DIC), dynactin (p150, p50), the myc tag, and actin. Dynein and dynactin co-sediment at ~20S from both control and Tg G59S mice, indicating that expression of the transgene does not disrupt endogenous dynein or dynactin. (D) Tg G59S female mice show mild, progressive declines in forelimb grip strength in comparison with age-matched non-transgenic littermate controls. Grip strength was measured monthly starting at 2 months of age and continuing until 18 months of age. Asterisks indicate a statistical difference determined by t-test at that time point, with P < 0.05 (n ≥ 12 per genotype); error bars represent SEM.
(Fig. 1A), we focused on the M20 line that displays higher levels of transgene expression in the spinal cord as determined by reverse transcription–polymerase chain reaction (RT–PCR) (data not shown), referred to here as the Tg\textsuperscript{G59S} line.

To verify that the transgenic protein was expressed in brain and spinal cord, western blots from Tg\textsuperscript{G59S} mice and non-transgenic littermate controls were probed with anti-myc antibody (Fig. 1B). These blots suggest that the transgene is expressed at low levels. Quantitative western blotting was performed to compare overall levels of p150\textsuperscript{Glu}ed expression in spinal cord extracts from transgenic and non-transgenic littersmates. Two different antibodies were used to differentiate between endogenous and transgenic protein: a monoclonal antibody that does not detect the mutant form of p150\textsuperscript{Glu}ed and a polyclonal antibody that detects both the normal and G59S forms (4). Overall levels of transgene expression were low in both spinal cord and brain. Using a recombinant myc-tagged p150\textsuperscript{Glu}ed polypeptide as a calibration standard, we estimate that in spinal cord extracts expression of the myc-tagged transgene was \~13\% of total levels of p150\textsuperscript{Glu}ed; due to the cell-specific expression of the Thy1.2 promoter, the relative expression level of the transgene is likely to be somewhat higher in motor neurons.

Subunits of dynein and dynactin isolated from wild-type tissue co-sediment at high S values (19–20S), while disruption to the cell-specific expression of the Thy1.2 promoter, the relative expression level of the transgene is likely to be somewhat higher in motor neurons.

To characterize the phenotype of Tg\textsuperscript{G59S} mice, we used grip strength assays to look for quantitative evidence of muscle weakness. Forelimb grip strength was assessed monthly from 2 months until 18 months of age in a cohort of age-matched transgenic and non-transgenic littermates. A modest but consistent decline in grip strength was seen as early as 2 months after birth in Tg\textsuperscript{G59S} females; this difference did not reach significance until 10 months of age (Fig. 1D). Deficits in male Tg\textsuperscript{G59S} mice were more subtle, perhaps because of increased body mass observed in male transgenic mice relative to age-matched controls (data not shown). Less quantitative measures, such as wire-hang tests and foot-printing analyses did not reveal statistically significant differences between Tg\textsuperscript{G59S} mice and non-transgenic littersmates, consistent with a relatively mild phenotype of progressive muscle weakness. Notably, lifespan was not shortened in Tg\textsuperscript{G59S} mice. Taken together, these data indicate a mild, slowly progressive, non-fatal motor impairment similar to that observed in human dSBMA patients expressing the G59S mutation (5).

G59S transgene expression does not disrupt axonal transport but does lead to the proliferation of lysosomes in motor neurons

Cytoplasmic dynein and dynactin drive retrograde axonal transport (1). To determine if the expression of the mutant transgene impairs axonal transport, we performed a double ligation assay on sciatic nerves of Tg\textsuperscript{G59S} mice and non-transgenic littersmates. If axonal transport was functioning normally, kinesin would accumulate proximal to the ligatures while dynein and dynactin would accumulate both proximally and distally (9). Proximal accumulation of kinesin was clearly seen in both Tg\textsuperscript{G59S} animals and controls (Fig. 2). We also observed that dynein and dynactin accumulated on both sides of the ligation site with no significant difference observed between Tg\textsuperscript{G59S} mice and controls (Fig. 2), indicating that expression of the G59S transgene does not significantly affect axonal transport in this model. In contrast to these observations, an \~50\% inhibition in retrograde transport has been observed in similar experiments in Loa or Tg\textsuperscript{dynamin} mice, both models of slowly progressive neuronal degeneration due to either a point mutation in dynein or overexpression of a dynamin transgene, respectively (Perlson and Holzbaur, submitted for publication). Thus, mouse models with somewhat similar phenotypes of mild motor impairment resulting from defects in related proteins demonstrate significantly different mechanisms at the cellular level.

We examined the expression and localization of the G59S polypeptide at the cellular level in tissue sections from the Tg\textsuperscript{G59S} model, in comparison with sections from age-matched controls. Low-level expression of the myc-tagged transgene was observed in motor neurons from the spinal cord in the Tg\textsuperscript{G59S} model; only background levels of staining are seen in age-matched controls (Fig. 3A). Double-label immunofluorescence using antibodies to the myc tag and p150\textsuperscript{Glu}ed show co-localization throughout the soma and processes of motor neurons (Fig. 3B). While prominent aggregates of dynein and dynactin are seen in neurons from dSBMA patients (5), we do not see consistent formation of aggregates in 9 month-old Tg\textsuperscript{G59S} mice. We also examined the localization of the dynactin subunit dynamitin and the dynein subunit DIC (Fig. 3C), which are uniformly distributed throughout the cell soma and processes in both Tg\textsuperscript{G59S} mice and non-transgenic controls.

Overexpression of mutant G59S p150\textsuperscript{Glu}ed in cell lines leads to the formation of perinuclear aggregates of dynein/dynactin not seen at the lower expression levels in the Tg\textsuperscript{G59S} model, as well as the sequestration of mitochondria within those aggregates (4). To examine the effects of expression of the mutant polypeptide on intracellular organelles in the Tg\textsuperscript{G59S} mouse model, spinal motor neurons were examined at higher resolution using electron microscopy. Motor neurons from transgenic mice were overtly normal, and abnormal sequestration of mitochondria to the perinuclear region was not observed in comparison with age-matched non-transgenic controls.
However, a prominent feature of motor neurons from TgG59S mice was an increase in the size and complexity of degradative organelles as compared with age-matched controls (Fig. 4A–D). Lipofuscin granules are a mature form of tertiary lysosome that proliferates with age (10). Quantification of this observation revealed a significant increase in the number of lipofuscin granules compared with non-transgenic littermate controls (t-test, \( P < 0.002 \); Fig. 4E). In addition, there is an increase in the average size of the granules compared with those found in cell bodies of motor neurons from age-matched controls, although the difference did not reach statistical significance (t-test, \( P < 0.07 \); Fig. 4F). Both the increased number and increased size of lipofuscin granules in TgG59S mice results in an almost two-fold increase in the total cell area occupied by these organelles.

In addition to their increased size, the lipofuscin granules in the TgG59S animals are more likely to cluster, display greater heterogeneity, and contain greater densities of lamellae (Fig. 4B and D), suggesting that these lipofuscin granules are more mature (10) than those in age-matched controls.

In cellular studies on the G59S mutation, it was suggested that both loss-of-function and toxic gain-of-function due to protein misfolding/aggregation may both contribute to the observed phenotype in patients expressing the dynactin mutation. The increased number, size, and complexity of lipofuscin granules, and the lack of an effect on axonal transport argue that the gain-of-function mechanism may be most relevant in this model. To further address this question, we compared the electron microscope (EM) analysis of TgG59S mice with a similar analysis of Loa mice, which express a hypomorphic allele of cytoplasmic DHC (6). As shown in Fig. 4G, analysis of motor neurons from heterozygous Loa/þ mice indicates that there is not a significant increase in the number of lipofuscin granules.
Reduced axonal caliber and disruption of NMJ morphology indicate distal neuronal degeneration in TgG59S mice

Cross-sections of spinal cord from TgG59S and non-transgenic mice were stained with hematoxylin in order to visualize neurons. No significant loss of motor neurons was observed in TgG59S mice as compared with age-matched non-transgenic controls (t-test, P > 0.25). Although loss of spinal cord motor neurons is not seen, there is a significant change in axonal caliber in mice expressing mutant p150Glued (Fig. 5A and B). In TgG59S mice, there is a clear reduction in the relative number of large caliber motor neurons (K–S test, P < 0.001; Fig. 5C). We did not see a significant increase in axonal demyelination in the TgG59S model (Fig. 5D). While not statistically significant, there is a decrease in the percentage of remyelinated axons in TgG59S mice (Fig. 5D), suggesting that expression of the transgene may lead to an impaired ability to remyelinate.

To further investigate the effects of expression of the G59S mutation on the integrity of the distal axon, we examined NMJ morphology in the sternomastoid and soleus muscles from age-matched transgenic and non-transgenic mice. The integrity of pre- and post-synaptic junctions were scored as intact, partially disrupted, or fragmented in 18-month-old mice (Fig. 6). In soleus muscle from non-transgenic control mice, a large proportion (45%) of pre-synaptic junctions were clearly intact, while 25% of the junctions were partially disrupted, and 30% were fragmented. In contrast, in NMJs from the soleus of TgG59S mice, only 31% of pre-synaptic junctions were intact, 23% were partially disrupted, and the majority (46%) were fragmented (Fig. 6). Similar observations were seen in sternomastoid muscles from transgenic TgG59S mice as compared with non-transgenic controls. In all cases, morphology of the post-synaptic junction mirrored that of the pre-synaptic junction. Also, whether intact or fragmented, all junctions examined remain innervated, as assessed by confocal microscopy of muscle preparations stained for neurofilaments.

While significant changes were observed in NMJ morphology in TgG59S mice, we observed no significant alterations in muscle histology. Hematoxylin staining of quadriceps, soleus, and EDL muscles from 18-month-old TgG59S mice revealed few central nuclei, regularly shaped fibers, and none of the clustered nuclei indicative of fiber drop out (data not shown). This lack of morphological alterations in muscle is consistent with the relatively mild and slowly progressive muscle weakness observed in grip strength assays, described above.

**DISCUSSION**

In this study we describe a mouse model of the human disease dSBMA caused by a G59S mutation in the cytoplasmic dynein interacting protein p150Glued. These mice model the disease seen in human patients in several key respects, including the relatively mild, slowly progressive phenotype and the underlying degeneration of motor neurons.

Cytoplasmic dynein and dynactin are pleiotropic cellular motors, with roles in intracellular trafficking including ER-to-Golgi motility, endocytosis, lysosome motility, and mitochondrial motility, as well as a critical function in retro-
grade axonal transport. Further specific roles for dynein and/or dynactin have been proposed, including aggresome formation, autophagy, and stability of the NMJ (1). Thus, while it is clear that defects in dynein or dynactin result in neuronal degeneration, gaining a mechanistic understanding of the basis for this degeneration is not straightforward.

Further, when expressed in cell culture, the G59S allele of p150Glued has effects indicative of both the loss of dynein/dynactin function and the toxic gain of function (4). The relative contribution of these effects to overall pathology may be deduced by comparisons among mouse models showing inhibition of dynein function, such as the Loa and Cra1 lines that express point mutations in DHC (6), as well as the previously characterized Tg\textsuperscript{dynamitin} mouse (7), and the Tg\textsuperscript{G59S} model described here. Pathology common to all of these models would suggest effects due to a loss of dynein/dynactin function while deficits seen only in the Tg\textsuperscript{G59S} model would likely be due to toxic gain of function induced by the mutation (Fig. 7).

Deficits in axonal transport have been proposed to function as a critical driver of pathology in a number of human neurodegenerative diseases; significant inhibition of axonal transport has been measured in multiple animal models of motor neuron disease, including the Tg\textsuperscript{dynamitin} mouse (7), the Loa/Loa mouse (6), and the SOD1\textsuperscript{G93A} mouse model of familial amyotrophic lateral sclerosis (11). Here, we used ligation experiments to show that low-level expression of the G59S mutation does not induce a measurable inhibition of axonal transport.

In contrast, other signs of neuronal dysfunction are apparent in the Tg\textsuperscript{G59S} mouse. Therefore it is possible that aberrant axonal transport is secondary to other cellular defects, and disruption of other functions of the dynein/dynactin complex, such as trafficking, are contributing to neuronal pathology. Alternatively, the lack of a measurable defect in axonal transport, coupled with biochemical data indicating that expression of mutant p150\textsuperscript{Glued} in this model does not disrupt the dynein/dynactin complex, suggests that the Tg\textsuperscript{G59S} mouse does not display a loss-of-function phenotype.

Instead, observations from this model are consistent with a toxic gain of function due to protein misfolding leading to enhanced degradation. Specifically, we noted an increase in the number, size, and complexity of lipofuscin granules in the Tg\textsuperscript{G59S} mouse, but not in mice carrying the Loa mutation,
which may be considered a hypomorphic dynein allele. Further indication that toxicity caused by misfolding of mutant p150\textsuperscript{Glued} is critical to the pathogenesis of dSBMA comes from observations of pronounced aggregate formation in motor neurons in vivo seen in immunocytochemistry on tissues from an affected individual (5), and biochemical and immunocytochemical evidence for aggregate formation in cellular models (4).

Despite the lack of a clear effect on axonal transport in this model, we see evidence for distal degeneration in motor neurons, including reduced axonal caliber, and fragmentation at the NMJ. Studies in Drosophila have suggested that dynactin has a direct role in maintaining NMJ stability (8), but a specific mechanism has not been explored. In our EM analysis of motor neurons from the Tg\textsuperscript{G59S} mouse, we found no indication of protein aggregates localized along neuronal processes. Given that axonal transport is unaltered in Tg\textsuperscript{G59S} mice, it is unlikely that axonal strangulation due to protein aggregation along the axon is contributing to peripheral abnormalities, such as loss of axonal caliber and disruption of the NMJ. Instead, expression of misfolded mutant protein may induce both the proliferation of degradative organelles and cellular stress responses that in turn could lead to distal degeneration. It is interesting that both the possible induction of cellular stress pathways and the distal degeneration that we observe are not sufficient to cause motor neuron loss, but instead may represent a chronic response. Again, this may serve as a useful paradigm to understand pathogenesis in dSBMA patients, since this is not a lethal disease in patients that are provided appropriate palliative care.

Recent description of a knock-in model of the G59S mutation by Lai et al. (12) now allows a useful comparison of multiple models of dSBMA. Heterozygous knock-in mice exhibit a subtle phenotype with no alteration in grip strength or performance on a rotorod test, but with some mild gait abnormalities. Some motor neuron loss per section is observed in the heterozygous knock-in model, as well as apparent destabilization of the NMJ. In comparison with the results from the transgenic model reported here, we can conclude that low-level expression of the transgene in motor neurons only is sufficient to induce a relatively similar phenotype to that observed when the G59S mutation is expressed ubiquitously.

In summary, we hypothesize that multiple factors contribute to the pathogenesis in dSBMA: toxicity of the G59S p150\textsuperscript{Glued} as well as destabilization of the neuronal periphery, and that both of these pathways are independent of deficits in axonal transport. It may be that more significant defects in axonal transport may lead to a more rapidly progressive motor neuron degeneration, and in the absence of these defects, a milder and more slowly progressive phenotype is observed.

**MATERIALS AND METHODS**

**Genotyping**

Observed patterns of inheritance indicate that the insertion site of the transgene is on the X-chromosome in the Tg\textsuperscript{G59S} (M20) mouse. Crude digests from mouse ear punches were diluted and used as template DNA for PCR. PCR was performed.

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**Figure 7.** Comparison of the pathological hallmarks of neurodegeneration in multiple mouse models. Various pathological features associated with motor neuron degenerative disease are listed in boxes. Experimental data on mouse models with disruptions in either dynein or dynactin are summarized; factors that have been observed in the various models are listed in green text; whereas mouse models experimentally shown to lack a particular feature are listed in red. Supporting data for this model was gathered from multiple references (2,4–7,16).
using the following primer sequences: 5′-CTGCTCCATCT
TCAATACCC-3′ and 5′-GGACTCCAGGCCCTCAAG-3′
(Invitrogen); PCR reactions were analyzed by agarose gel electrophoresis.

**RT–PCR**

Freshly dissected mouse tissue was homogenized in three volumes of buffer B (20 mM
Freshly dissected brains and spinal cords from individual mice
agarose gel electrophoresis. Similar levels of transgene
expression, as measured by antibody reactivity to the myc-tagged transgene
were analyzed by gel electrophoresis and immunoblot. Bands were quantified using ImageJ software. Similar levels of transgene expression, as
normalized to a GAPDH loading control. Detection was performed with Western Light-
Western blot analysis

Freshly dissected brains and spinal cords from individual mice
were homogenized in three volumes of buffer B (20 mM
Tris–HCl, pH 7.4, 1 mM ethylene diamine tetraacetic acid, 2 mM ethylene glycol tetraacetic acid) with protease inhibitors
phenylmethylsulphonyl fluorid (PMSF), leupeptin, pepstatin A,
and N-p-tosyl-L-arginine methyl ester hydrochloride (TAME).
Low speed supernatants were collected (10–30 min spin at 11 600×g) and analyzed by gel electrophoresis and western blotting. Blots were probed using antibodies against dynactin
subunit p150Glued [monoclonal antibody 610474 from BD
Biosciences or affinity purified polyclonal antibody UP502
(13)], DIC (DIC monoclonal MAB1618 from Chemicon),
DHIC (DHIC polyclonal R-325 from Santa Cruz), and the myc
tag (monoclonal R950-25 from Invitrogen R950-25); actin
(monoclonal MAB1501R from Chemicon) was used as a
loading control. Detection was performed with Western Light-
ning (Amersham Biosciences), visualized with the LAS 3000
system, and densitometry of the bands was performed with
ImageJ software. Similar levels of transgene expression, as
measured by antibody reactivity to the myc-tagged transgene
were seen in males and females by this assay (data not shown).

**Ligation experiments**

Six 18-month-old Tg<sup>G59S</sup> and six age-matched control mice
were anesthetized using an IP injection of ketamine/xylazine
and the sciatic nerve was exposed. Two ligations were
performed at site and were analyzed by gel electrophoresis and immunoblot.

**Sucrose density centrifugation**

Brain homogenates from 3-month-old Tg<sup>G59S</sup> and non-transgenic control mice in PIPES HEPES EGTA magnesium chloride buffer,
pH 7.4, plus protease inhibitors (PMSF, leupeptin, pepstatin A,
and TAME) were spun at 11 600×g for 10 min to obtain a low-speed
supernatant. High-speed supernatants were generated by a second centrifugation step at 80 000×g for 10 min. High speed
supernatant fractions were fractionated by centrifugation through
5–25% sucrose density gradient as previously described (7).
Fractions from the gradient were analyzed by SDS–PAGE
(sodium dodecyl sulfate polyacrylamide gel electrophoresis) and immunoblot, using antibodies to dynactin (DHIC and DIC), dynactin subunits (p150<sub>Glaed</sub> polyclonal UP502) and dynamitin
(p50 monoclonal 610003 from BD Biosciences), and actin. To
verify that the transgenic G59S p150<sub>Glaed</sub> myc-tagged protein
was co-fractionating with the other components dynactin/dynamitin,
the myc tag was identified on separate blots run with the same
sucrose gradient samples and probed with a monoclonal antibody
specific for myc.

**Behavioral analyses**

A grip strength meter (Columbus Instruments) measured the
force exerted by a mouse as it was pulled from a grid by the
tail while grasping the grid with its forepaws only. An
average of three trials per mouse was calculated on a monthly
basis. Significance of grip strength comparisons and of body
weight comparisons between genotypes were determined by
<sup>t</sup>-test at each time point. Gait analyses were performed by
measuring stride length of each foot. Significance between
genotypes for stride lengths was determined by student’s
<sup>t</sup>-test.

**Histology**

For motor neuron counts in spinal cord, tissues were
embedded in OCT (Tissue-Tek) and cooled on dry ice. For
muscle, tissues were embedded in OCT snap frozen with iso-
pentane cooled by liquid nitrogen. Frozen sections (10 μm)
were fixed in 4% paraformaldehyde for 10 min. Harris’s
hematoxylin staining (Vector) was applied for 7 min to visualize
neurons and 5–10 min to visualize muscle fibers. Slides were
dehydrated briefly in an ethanol series followed by a xylene series and mounted in Vectamount (Vector).

Quantification of motor neurons

Motor neuron number per section was counted in 10 μm sections of cervical spinal cord from 18 month-old mice. A minimum of five sections were counted per animal; scored sections were at least 50 μm apart. Statistical significance was determined using Student’s t-test.

EM of ventral roots and spinal cord

Eighteen month-old TgG59S and age-matched control mice were anesthetized using an IP injection of ketamine/xylazine. Mice were perfused with 50 ml of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The L5 ventral roots and cervical spinal cords were dissected following perfusion. Tissues were post-fixed in 1% osmium tetroxide for 20 min, and processed for embedding in Epon-Araldite resin. Ultrathin sections were cut, mounted on an open grid, and stained with 1% uranyl acetate and 0.5% lead citrate. Grids were analyzed with a 100CX Joel Electron Microscope at 2.5–75 kV. Axonal cross-sectional areas were measured from images taken at 2.5 kV using ImageJ software. At least 10 fields were analyzed per animal and three animals were analyzed per genotype. A K–S test was performed to determine statistical significance. The number of lipofuscin granules was counted and their area was measured from images taken at 5 kV using ImageJ software. At least 100 fields were analyzed per animal and three animals were analyzed per genotype. Student’s t-test was used to determine statistical significance. To determine the percentage of cell area composed of lipofuscin granules, the total area of lipofuscin granules per cell was taken as a ratio to the total area of that cell; averages were determined for each genotype.

Visualization and scoring of NMJs

Sternomastoid and soleus muscles from 18-month-old TgG59S mice and age-matched controls were dissected and stained as described (15) with minor modifications. Briefly, muscles were fixed in 4% paraformaldehyde, permeabilized, and stained with rhodamine-conjugated bungarotoxin to visualize post-synaptic AchR (Molecular Probes), antibodies against phosphorylated neurofilaments (SMI 31 monoclonal – Sternberger Monoclonals Incorporated) to visualize axons, and antibodies to SV2 (Developmental Studies Hybridoma Bank) to visualize pre-synaptic junctions. Primary antibodies were detected using fluorescein isothiocyanate-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch). Muscles were whole mounted in Vectashield mounting media (Vector). To visualize, count, and score NMJs, a Leica microscope (DMI1REB) with a 40× dry objective was used. Images were acquired with OpenLab software (Improvision) and an OrcaER camera (Hamamatsu). NMJ morphology was scored as intact, partially disrupted, or fragmented; a minimum of 150 NMJs were scored per animal. Three 18-month-old mice per genotype were scored.

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