Overexpression of wild-type androgen receptor in muscle recapitulates polyglutamine disease

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We created transgenic mice that overexpress WT androgen receptor (AR) exclusively in their skeletal muscle fibers. Unexpectedly, these mice display androgen-dependent muscle weakness and early death, show changes in muscle morphology and gene expression consistent with neurogenic atrophy, and exhibit a loss of motor axons. These features reproduce those seen in models of Kennedy disease, a polyglutamine expansion disorder caused by a CAG repeat expansion in the AR gene. These findings demonstrate that toxicity in skeletal muscles is sufficient to cause motoneuron disease and indicate that overexpression of the WT AR can exert toxicity comparable with the polyglutamine expanded protein. This model has two clear implications for Kennedy disease: (*i*) mechanisms affecting AR gene expression may cause neuromuscular symptoms similar to those of Kennedy disease and (*ii*) therapeutic approaches targeting skeletal muscle may provide effective treatments for this disease.

Kennedy disease | neuromuscular | skeletal muscle | spinal and bulbar muscular atrophy | axonopathy

A polymorphism in exon 1 of the androgen receptor (AR) gene, consisting of a variable number of glutamine (Q) repeats, affects AR function. Very long polyglutamine repeat (polyQ) tracts are associated with a progressive neuromuscular disease known as Kennedy disease (KD, or spinal bulbar muscular atrophy) (1). The etiological mechanism mediating polyQ toxicity is uncertain, but is generally thought to confer novel toxic functions to the protein, because expansion of polyQ tracts beyond 40 repeats in other proteins also cause neurodegenerative disease, including Huntington's disease (HD), and several autosomal dominant forms of spinocerebellar ataxia (SCA) (2). Histopathological studies of KD patients suggest "neurogenic" responses to denervation, and the etiology of this disease is therefore generally thought to begin with motoneuron pathology (3).

Explicit mouse models of KD, in which polyQ AR alleles containing 60 CAG repeats or more are expressed, develop a disease phenotype that includes a marked reduction in body weight, kyphosis, and striking deficits in muscle strength and motor coordination (4–8). Androgen dependence, motoneuron and muscle pathology, and/or inclusions containing AR are also observed in these models (4, 5, 7, 8). Our studies of AR in skeletal muscle (9, 10) led us to generate transgenic (Tg) mice in which AR is overexpressed solely in skeletal muscle fibers using an expression cassette containing the human skeletal α-actin (HSA) promoter. We discovered a striking phenotypic resemblance between these HSA-AR mice and mouse models of KD. This similarity is surprising given that the Q repeat in this AR transgene comprises only 22 glutamines and is expressed exclusively in skeletal muscle fibers and not in motoneurons.

Results

HSA Promoter Drives Transgene Expression Exclusively in Skeletal Muscle Fibers. We first validated our HSA expression cassette by generating HSA-LacZ (LacZ = β-galactosidase gene) reporter mice [supporting information (SI) Fig. 5*A*]. Consistent with other expression cassettes containing the HSA promoter (11, 12) these reporter mice express β-gal specifically in skeletal muscle fibers, starting at embryonic day 9.5–10.5, with no detectable expression in other tissues, including the heart, viscera, fat or spinal cord (SI Fig 5 *B* and *C*). We also created Tg mice in which a rat WT AR cDNA is driven by this same HSA expression cassette (SI Fig 6*A*), resulting in selective overexpression of AR in skeletal muscle fibers but not in other cell types within muscle (HSA-AR Tg mice; SI Fig 6*B*). As further validation of the specificity of transgene expression, we crossed our HSA-AR mice with AR mutant [testicular feminization mutation (*Tfm*)] mice. *Tfm* males express little to no AR protein (13), offering a null background for AR in which to assess AR transgene expression. As expected, Western blots of tissues from such *Tfm*/HSA-AR male progeny revealed AR in skeletal muscle, and essentially none in spinal cord, testes, or heart (SI Fig. 6*C*). Furthermore, immunostaining revealed that lumbar motoneurons of HSA-AR/*Tfm* male mice contain no AR immunoreactivity, comparable with *Tfm* male mice, but in striking contrast to the robust AR staining in motoneuronal nuclei of WT male mice (SI Fig 7). These data provide clear evidence that the HSA-AR transgene is not expressed in spinal motoneurons. Taken together, these data demonstrate that the HSA-AR transgene results in specific overexpression of AR in skeletal muscle fibers and not elsewhere. Despite prominent nuclear staining for AR in muscle fibers (SI Fig 6*B*) of HSA-AR Tg male mice, we find no clear evidence of AR-positive aggregates.

Overexpression of AR in Skeletal Muscle Fibers Causes Androgen-Dependent Motor Dysfunction and Early Death. We detected transgene expression in seven founding lines of HSA-AR Tg mice and selected two (L78 and L141) for in-depth characterization. These lines differ in transgene copy number (L78 < L141; SI Fig 6*D*) and have corresponding differences in transgene expression as indicated by muscle AR mRNA (SI Fig 6*E*) and protein (SI Fig 6*F*). Reduced

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Conflict of interest statement: D.A.M., S.M.B., and C.L.J. have filed a patent concerning the role of muscle fiber androgen receptor in neuromuscular disease.

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Abbreviations: AChR, acetylcholine receptor; AR, androgen receptor; HD, Huntington's disease; HSA, human skeletal α-actin; KD, Kennedy disease; L78, founding line 78 transgenic mice; L141, founding line 141 transgenic mice; LacZ, β-galactosidase gene; MyoD, myogenic differentiation gene; polyQ, polyglutamine repeat; Tfm, testicular feminization mutation; Tg, transgenic; EDL, extensor digitorum longus; T, testosterone.

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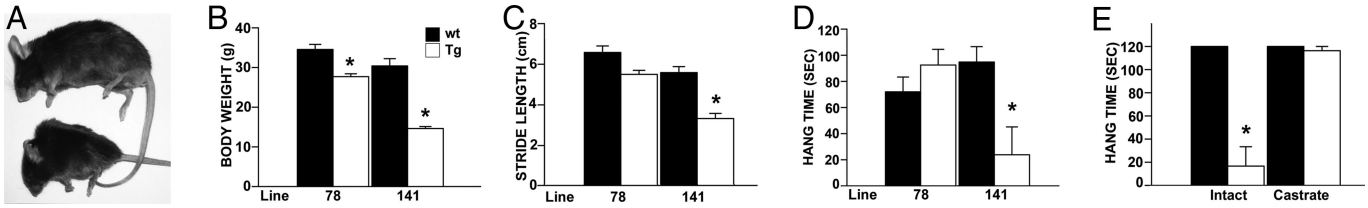

Fig. 1. Phenotype of HSA-AR male mice. (*A*) Photograph of L141 Tg male and age matched WT brother (top). L141 Tg males have reduced body weight (*A* and *B*), marked kyphosis (*A*) and motor deficits as revealed by paw print records (*C*) and the hang test (*D*). Despite reduced body weight, motor deficits are not observed in L78 Tg males, which express the AR transgene at a lower level than affected L141 Tg males (see SI Fig 6). Castrating L141 Tg males reverses deficits in hang time (*E*). Graphs represent mean ± SEM, open bars represent Tg males, and filled bars represent WT controls. *, significantly different from WT controls. WT 78 (n = 13, mean age in days: 294, range: 179–528), 78 Tg (n = 14–15, mean age: 281, range: 106–528), WT 141 (n = 12–15, mean age: 131, range: 72–218), Tg 141 (n = 6–7, mean age: 119, range: 72–179). Castrates: WT (n = 3, intact age: 80, castrate age: 90), Tg (n = 3, intact mean age: 74, range: 63–80, castrate mean age: 123, range: 90–190).

male viability at birth was observed for Tg males but not Tg females in all seven lines (survival data for L78 and L141 in SI Table 1). Treatment of pregnant dams with the anti-androgen flutamide enhances perinatal survival of Tg males (data not shown), suggesting perinatal death of Tg males depends on prenatal androgen exposure and not overexpression *per se* of the AR protein. The absence of perinatal mortality in Tg females (SI Table 1) also supports this conclusion.

Surviving Tg males of three founding lines (including L141) have a marked phenotype suggestive of motoneuron disease, including kyphosis (Fig. 1*A*), reduced body weight (Fig. 1*B*), and striking deficits in muscle strength and motor function (Fig. 1 *C* and *D*). Despite showing somewhat lower body weights, surviving Tg males of the remaining four lines (including L78) are comparable with WT brothers in motor function (Fig. 1 *C* and *D*). Given that transgene expression, at both the mRNA and protein levels, is higher in muscles of symptomatic L141 mice than in muscles of asymptomatic L78 mice, these data indicate that AR expression levels correspond to phenotypic severity. We also find that the loss of motor function is androgen-dependent in Tg males. Castration of adult L141 Tg males dramatically improves their motor function (Fig. 1*E* and SI Movie 1).

In contrast to the obvious phenotype of Tg males, Tg females from all lines appeared unaffected by expression of the AR transgene. In addition to showing normal viability perinatally, they also showed no loss of motor function as adults (untreated TG/VEH groups in Fig. 2). However, when we treated adult Tg females with exogenous testosterone (T) that approximated normal male levels of circulating T (data not shown), several aspects of the male phenotype were rapidly induced in L141 Tg females, including weight loss and motor deficits (Fig. 2). Notably, such females show significant deficits in hang time before losses in body weight, suggesting that motor deficits precede overt muscle atrophy. We learned that >9 days of T treatment is fatal to L141 Tg females. On the other hand, L78 Tg females do not become obviously atrophic with T treatment, even when the T treatment is extended to several weeks (data not shown), nor do they show any motor dysfunction (data not shown).

Skeletal Muscles of HSA-AR Tg Male Mice Show Pathology Consistent with Motoneuron Disease. These features of impaired motor function, reduced body weight and androgen dependence are akin to KD. To explore this idea further, we examined cross sections of the extensor digitorum longus (EDL) for pathology using H&E and NADH (Fig. 3*A*) stains. L141 Tg male mice showed abnormalities consistent with a KD phenotype, including both atrophic and hypertrophic fibers, internal myonuclei, fiber splitting, altered myofibrillar organization, and an overall increase in oxidative metabolism. No lymphocytic infiltrate or other histological signs of inflammation were present in the muscle. L141 Tg males also have nearly half the number of EDL fibers compared with WT controls (Fig. 3*B*), with average fiber size slightly smaller than in WT controls (mean ± SEM: WT = 1,479 ± 92.2 μm^2, Tg = 1,308 ± 92.5 μm^2) but not significantly different.

This difference in fiber number could reflect muscle fiber loss due to apoptosis, but we find no evidence of pycnosis in H&E stained muscle sections. Another possibility is that T produced by the testes prenatally interferes with myogenesis such that Tg males never achieve the normal adult number of muscle fibers. This possibility seems unlikely, however, because Tg males exposed to flutamide during late gestational myogenesis, still

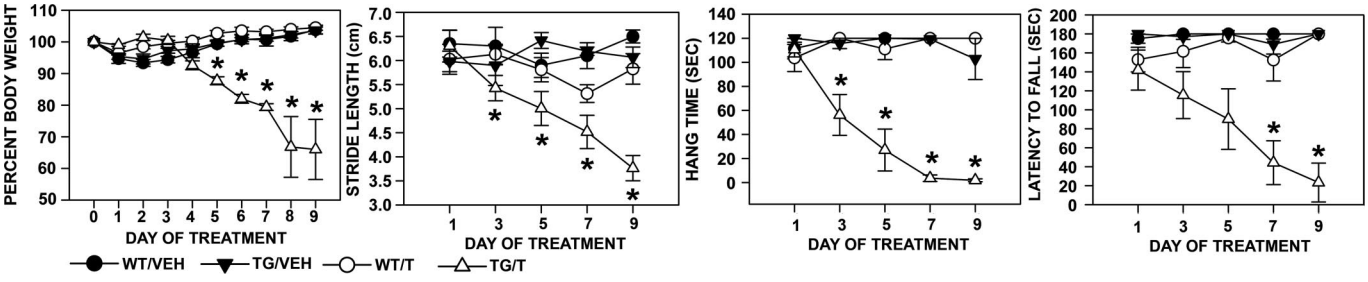

Fig. 2. T treatment of asymptomatic 141 Tg females rapidly induces disease symptoms. Percent change relative to initial body weight, stride length during gait analysis, time on the hang test (seconds), and latency to fall on a constant speed rotarod during 9 days of T or vehicle (VEH) treatment of Tg or WT females. Motor dysfunction is therefore androgen-dependent in HSA-AR Tg mice, as in polyQ AR mice. *, significantly lower than Day 0 within a treatment group. Note that only the T-treated Tg females show a significant drop on these measures over time. However, T has no effect on fiber or axon number in L141 Tg females (ANOVAs indicate no significant effects of genotype or treatment, or a significant interaction), despite its debilitating effects on motor function. T does not have these effects on body weight or motor function of Tg L78 females (data not shown), paralleling the apparently normal behavioral phenotype of L78 Tg males. Means ± SEM of n = 7 mice/group (mean age/group: 102–103 days old; overall age range: 70–114 days old).

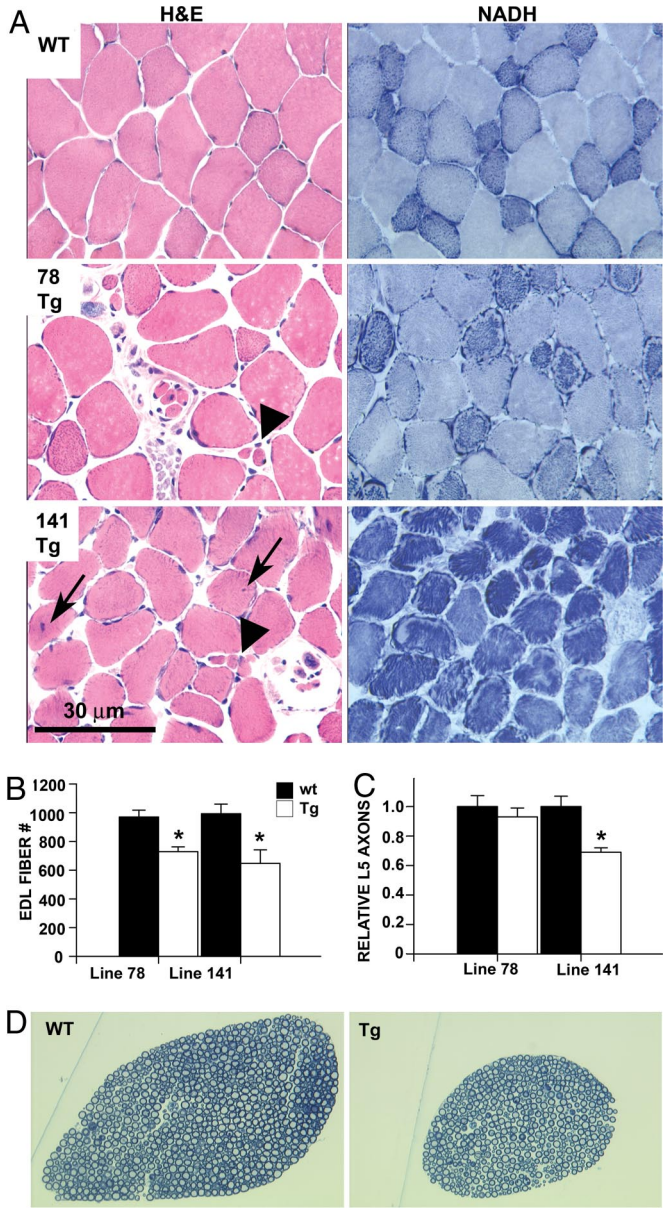

Fig. 3. Histopathology in HSA-AR Male Mice. (*A*) Photomicrographs illustrating histopathology in EDL muscle sections stained with H&E or NADH from a WT male, or L78 or L141 Tg males. Muscle pathology seen in L78 and L141 Tg males is typical of that seen in KD, including grouped atrophic fibers (arrowheads), centralized nuclei (arrows), and increased NADH staining. Muscle pathology is notably greater in the symptomatic L141 Tg males than the nonsymptomatic L78 Tg males. (*B* and *C*) Whereas the number of EDL muscle fibers (*B*) is reduced in Tg males of both lines, the number of L5 motor axons (*C*) was significantly reduced relative to WT controls only in L141 Tg males, which is the line with particularly poor motor function. Graphs represent mean ± SEM, open bars represent Tg males, and filled bars represent age-matched WT males. *, significantly different from WT males. (*D*) Toluidine blue-stained cross sections of L5 ventral roots are shown for L141 WT and Tg males. WT 78 (n = 10–13, mean age: 317 days old, range: 179–528 days old), 78 Tg (n = 13, mean age: 300 days old, range: 106–528 days old), WT 141 (n = 12–13, mean age: 129 days old, range: 72–218 days old), Tg 141 (n = 6, mean age: 116 days old, range: 72–179 days old).

show a significantly reduced number of muscle fibers in adulthood compared with their WT littermates (data not shown). More studies would be needed to clarify this issue. Surprisingly, L141 Tg females treated acutely with T in adulthood show no such deficit in muscle fibers (data not shown), despite profound losses in motor function, indicating that motor dysfunction does not depend on muscle fiber loss.

Asymptomatic L78 Tg males also show considerable pathology in the EDL (Fig. 3*A*), including significantly fewer fibers (Fig. 3*B*) compared with WT controls, even though motor function seems normal. Pathological changes in muscles from L78 Tg males suggest that either a threshold for such pathology must be reached before motor deficits are apparent or that other factors underlie the loss in function. The fact that T-treated L141 Tg females develop a severe motor deficit without any loss of fibers supports the latter possibility.

Axonopathy but Not Loss of Motoneuronal Cell Bodies in Symptomatic Tg Male Mice. Because several neuromuscular diseases, including ALS and KD are associated with a loss of motoneurons and/or motor axons at autopsy, we counted the number of motor axons in lumbar (L) ventral roots 4 and 5 and Nissl-stained motoneurons in 3 lumbar motor pools [the spinal nucleus of the bulbocavernosus (SNB), the dorsolateral nucleus (DLN), and the retrodorsolateral nucleus (RDLN)]. We found symptomatic L141 Tg males have significantly fewer L5 motor axons than age-matched WT controls (Fig. 3 *C* and *D*), although the number and size of motoneuronal somata are unaffected in these same mice (SI Table 2). Counts of the number of motor axons in L4 also indicate a significant deficit in Tg L141 males compared with their WT controls [mean ± SEM: WT = 698 ± 31.92 (n = 5) vs. Tg = 537 ± 34.82 (n = 4); $P < 0.04$]. These data suggest that HSA-AR mice exhibit axonopathy which may precede motoneuron cell death. No difference in average cross-sectional area of surviving axons (mean ± SEM: WT = 45.5 ± 4.43 μm^2; Tg = 45.2 ± 8.51 μm^2), nor in the size distribution of these axons was observed (data not shown). Notably, axon number was *un*affected in asymptomatic L78 Tg males (Fig. 3*C*), despite significant losses in muscle fiber number, suggesting that axon loss is secondary to muscle pathology. T treatment also did *not* affect the number of motor axons in L141 Tg females (data not shown), despite its profound effect on motor function. These results indicate that neither motoneuron nor muscle fiber loss are required for motor dysfunction.

Denervation-Sensitive Genes Change in the Expected Direction in Muscles of Symptomatic but Not Asymptomatic Tg Male Mice. We next examined genes that are known to be dysregulated in polyQ AR mouse models of KD and/or responsive to denervation, including VEGF, myogenin, acetylcholine receptor (AChR) alpha subunit, and myogenic differentiation (MyoD). Expression of $VEGF_{164}$ is reduced in a transgenic model of KD (8), whereas both myogenin and AChR are elevated in skeletal muscle of polyQ AR knockin mice (7). We saw similar changes in the expression of these genes, with $VEGF_{164/188}$ mRNA significantly reduced, and mRNAs for myogenin and AChR significantly increased in muscles from L141 male Tg mice relative to those from WT brothers (Fig. 4). Levels of MyoD mRNA also increased, paralleling changes after denervation (14). No such changes were observed in behaviorally asymptomatic L78 Tg males. These results are consistent with neurogenic atrophy (14, 15) and suggest that HSA-AR and other polyQ AR mouse models share a common molecular etiology. The important distinction however between our model and others is that our experimental gene is expressed only in skeletal muscles, indicating that such indices of neurogenic atrophy can result from processes that originate in muscle.

Discussion

The nature of the observed phenotype bears a striking resemblance to mouse models of KD, which also exhibit androgen-dependent motor dysfunction (4, 5, 7, 8). The loss of motor axons in HSA-AR mice is consistent with human KD patients and

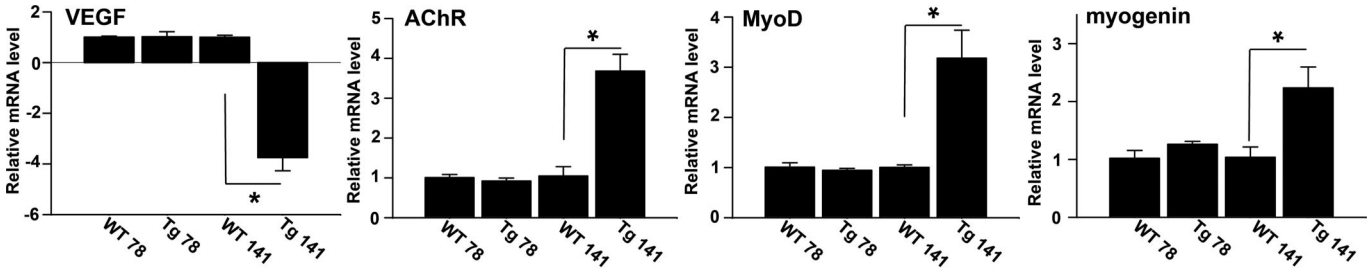

Fig. 4. Alterations in gene expression in HSA-AR Male Mice consistent with neurogenic atrophy. Quantitative RT-PCR (qPCR) estimates of mRNA abundance of VEGF isoform 164 and 188 (VEGF), AChR alpha subunit (AChR), MyoD, and myogenin in muscles from WT and Tg males of the behavioral asymptomatic L78 line and the severely affected L141 line. Down-regulation of VEGF and up-regulation of AChR alpha, MyoD, and myogenin mRNA are also observed in muscle of KD mouse models and/or after denervation. All estimates are differences in mRNA relative to WT brothers within the same line. Graphs represent mean ± SEM. *, significantly different from WT brothers. L78 mice (n = 3 WT, 3 Tg; mean age = 145 days old, range = 106–222 days old); L141 mice (n = 4 WT, 4 Tg, mean age = 115 days old, range = 72–162 days old).

many mouse models of KD (3, 4, 6, 8, 16). We also observed dysregulation of gene expression reported in several polyQ mouse models (7, 8). Whereas we did not observe AR-containing nuclear aggregates in muscle fibers or motoneurons of Tg male mice of either line, the role of aggregates in many neurodegenerative diseases, including polyQ diseases, remains controversial (17, 18). The similarities in pathology between KD and HSA-AR mice are surprising because the HSA-AR transgene is expressed exclusively in skeletal muscle fibers and contains only 22 glutamine repeats, well within the WT range (SI Fig 6*A*, verified by sequencing in all founding lines of HSA-AR Tg mice).

These results raise the intriguing question of how a protein that typically promotes anabolic responses in muscle can result in atrophy and neurodegeneration when overexpressed. Whereas an answer to this question is not yet available, molecular chaperones and the ubiquitin-proteosome pathway may be involved. A related question is whether overexpression of *any* protein in muscle fibers would be toxic. Several observations argue against this possibility. First, we find no evidence for pathology in >12 lines of HSA-LacZ mice that express β-gal in their muscle fibers (SI Fig 5), nor for mice that express Cre recombinase driven by HSA in muscle fibers (data not shown). Secondly, it is not simply expression level of AR that confers pathological properties in HSA-AR mice, as females must be treated with androgens before showing motor dysfunction. Moreover, perinatal survival of Tg males can be significantly enhanced by prenatal exposure to the anti-androgen flutamide. Finally, castration of adult Tg males largely reverses the loss of function (see SI Movie 1). Thus, in several different contexts, we find that AR becomes toxic only once activated by hormonal ligand, rather than because of overexpression *per se* of the protein.

However, it also worth noting that our model is not the first to show that overexpression of a WT protein seems capable of exerting toxicity comparable with the mutant disease version of the protein. Other examples include ataxin-1 (19, 20), α-synuclein (21), and tau (22). Remarkably, for each "disease" protein, overexpression of the WT form induces a comparable neurodegenerative phenotype as the mutant allele. For example, overexpression of WT ataxin-1 causes neurodegeneration in *Drosophila* and mouse models of spinal and cerebellar ataxia 1 (SCA1) (19, 20), suggesting that an expanded polyQ tract is not necessary for toxicity and may be only one of several ways toxicity is conferred to a protein. Similarly, in *Drosophila* models of tauopathy, overexpression of WT tau is sufficient to induce neurodegeneration (22) and gene duplication of WT α-synuclein causes Parkinson's disease similar to the mutant form of the protein (21).

Our results also reinforce the potential for myogenic contributions to KD, which has previously been proposed based on observations of AR-containing aggregates in polyQ AR mouse muscle (4). This possibility is also supported by observations that muscle AR aggregates and myopathy significantly precede motoneuron pathology in a polyQ AR knockin mouse model (7). Interestingly, R6/2 HD mice exhibit a progressive neuromuscular pathology that has been ascribed in part to primary actions of the mutant protein on skeletal muscle (23), further suggesting common pathological mechanisms in muscle that may underlie a loss of motor function in several polyQ diseases.

Our male Tg mice display behavioral, neuronal and muscular pathologies that are normally regarded as "neurogenic" in origin. The obviously atrophic appearance of animals, their loss of muscle strength coupled with losses in muscle fiber and axon number as well as the pattern of changes in gene expression in muscles is all consistent with the muscles being denervated. These changes may indeed reflect muscle denervation that is a secondary response to primary changes in the muscle, i.e., changes in the muscle which trigger axon withdrawal and loss. One possible scenario is that a decrease in muscle-derived VEGF may trigger axonopathy in our model. Because VEGF-A, which in mice is comprised mainly of $VEGF_{164}$ (24), is expressed in the sarcolemma of muscle fibers (25), such a scenario seems plausible.

Symptomatic HSA-AR male mice exhibit significant losses in L4 and L5 ventral root axons but not at the level of motoneuronal cell bodies. Similar "dying back" axonopathies are observed in many, indeed most, models of motoneuron disease, including models of ALS, spinal muscular atrophy (SMA) (26–29), and, notably, KD (3, 5, 7, 30). For example, the SOD1 (G93A), progressive motoneuropathy (pmn) and motoneuron degeneration (Mnd) mouse models all show a loss of synaptic connections before symptoms appear (27). Furthermore, in the SOD1(G93A) mouse model, degeneration is first seen at the neuromuscular junction, followed by axonal loss and eventually motoneuron loss (28). Additionally, in one model of SMA, a loss of L4 and L5 axons is seen before motoneuron loss, and motor axons are more severely affected than motoneuronal somata by the smn mutation (29). Thus, axonopathy usually precedes cell death, suggesting that motoneurons in HSA-AR male mice might eventually die as part of this process, if the mice were to survive long enough.

Mouse models of KD show similar signs of axonopathy (5, 7, 30), and indeed suggest that axonopathy, rather than cell death, may be a critical event underlying loss of motor function in this disease. A striking example is a recently described KD mouse model (5) that, despite showing profound losses in motor function, shows no evidence of motoneuronal or muscle pathology, including no loss of motoneurons. Such mice did show a change in the phosphorylation status of motoneuronal neuro-

filament proteins, suggestive of axonopathy. In short, results of our model are highly consistent with many other animal models of motoneuron disease, including models of KD, and raise the possibility that changes in muscle gene expression may be sufficient to cause both axonopathy and loss of motor function, and that neither requires motoneuronal cell death.

The relative lack of pathology in our T-treated HSA-AR Tg females despite devastating losses in function is also highly reminiscent of the KD model described above. Both models show an androgen-dependent loss in motor function in the *absence* of either muscle fiber or motoneuronal loss, suggesting that standard histopathological markers used to diagnose neurodegenerative disease may not necessarily inform us about the mechanisms underlying disease. However, a question raised by our data is whether the disease process is fundamentally the same for HSA-AR Tg males and females, given the fact that symptomatic Tg males exhibit losses in muscle fiber and axon number that symptomatic T-treated Tg females do not. Several observations suggest that the mechanisms triggering a loss of motor function may in fact be the same in the two sexes. For example, T treatment triggers a loss of motor function only for Tg females in L141 and not for Tg females in L78, just as only L141 and not L78 Tg males show deficits in motor function (Fig. 1). Muscles from both Tg males and T-treated Tg females show dysregulation of the same genes in the symptomatic line (L141, data not shown). Therefore, we suspect that the deficits in muscle fiber and axon number exhibited by Tg males but not Tg females reflect differences in the length of androgen exposure—many weeks of androgen exposure culminates in fiber and axonal loss that nine days does not. It is difficult to understand, however, why T treatment of Tg females that results in slightly lower plasma T levels than untreated adult males triggers a much more rapid loss in motor function compared with the slower progression of loss seen in Tg males. It is possible that Tg males, given a prolonged but regulated exposure to androgens, develop protective mechanisms that Tg females simply do not have time to develop.

Our findings call into question whether other disorders regarded as ''motoneuron'' diseases may actually be muscle diseases that eventually cause motoneuronal pathology. That high levels of WT AR in skeletal muscles can mimic the effects of expanded polyQ AR indicates that expanded polyQ tract is not necessary to induce an androgen-dependent motoneuronal disease and raises the possibility that similar mechanisms may also contribute to KD in humans. Moreover, because an androgen-dependent phenotype is induced by expression of an AR transgene in skeletal muscle fibers, therapeutic approaches targeting muscles and/or neuromuscular junctions may provide effective treatments for Kennedy disease.

In summary, we describe a recently discovered transgenic mouse in which muscle overexpression of AR without polyQ expansion results in neuromuscular atrophy with many key features of KD, including the following: sex-limitation, androgen-dependence, motor deficits, kyphosis, myopathy, motor axon loss, and dysregulation of genes implicated in this disease. These findings further implicate muscle AR in KD and suggest that overexpression of WT AR can recapitulate the pathological consequences of polyQ expansion of AR.

Materials and Methods

Statistical Analysis. Within founding lines, Tg Males were compared with their WT brothers or aged-matched WT males using independent groups t tests with level of significance set at $P = 0.05$ two-tailed, and n = number of animals in each group. Additional comparisons were occasionally made between L78 and L141 males using independent groups t tests with level of significance set at $P = 0.05$ as indicated. Females were analyzed using a three-way analysis of variance with treatment (T vs. blank) and genotype (WT vs. Tg) as between subjects factors, and time (day 0–day 9) as a repeated factor. Fisher's least significant difference post hoc comparisons were used to locate sources of variance.

Generation of Tg Mice. AR cDNA was subcloned from pCMV-AR (kind gift of E. Wilson, University of North Carolina, Chapel Hill, NC) into BlueScript II plasmid (pBS) and the KpnI site of the multiple cloning sequence of the resulting subclone was converted into a NotI site by using oligonucleotide linkers. LacZ was subcloned from pCMV-BGAL (Invitrogen, Carlsbad, CA). Both cDNAs were ligated into the NotI site of pBSX-HSA (kind gift of J. Chamberlain, University of Washington, Seattle, WA). HSA was originally cloned in the laboratory of L. Kedes (31). Tg animals were produced by pronuclear injection of C4 × C57BL/6J zygotes. Tg animals were identified by using PCR amplification of transgene specific regions. Founding Tg animals were mated to C57BL/6J mice, and their progeny were analyzed.

Animal Surgery and Hormone Treatment. Adult Tg and WT female siblings from L141 and L78 were ovariectomized under isoflurane anesthesia and received s.c. Silastic implants that were empty or filled with T (1.57 mm inner diameter and 3.18 mm outer diameter; effective release length of 6 mm), resulting in low physiological levels found in adult males. Behavior was measured before (day 0), and after surgery. Most animals were killed on day 9, but some T-treated Tg females were killed on day 7 depending on disease progression.

Behavioral Methods. ***Hang test.*** Mice were tested for motor function by using the hanging wire test. Mice were placed on a wire grid and turned upside down 40 cm above a counter or cage bedding, and the latency to fall up to 120 s was measured (8).

Paw print analysis. Forepaws were painted with nontoxic acrylic red paint and hindpaws painted with blue. Mice were placed at one end of a piece of paper and guided to walk along it (32). Stride length was measured from fore and hindlimbs and averaged to yield a single estimate per animal.

Rotarod. To test for muscle endurance and motor coordination, mice were tested with a Rotarod (Columbus Instruments (Columbus, OH; axle diameter 3.6 cm; speed of 16 rpm). Each animal was given three trials with 10 min in between each session, and the test was stopped after 180 s (33). Animals were trained for 1 week before first testing.

Tissue Harvesting and Processing. Under surgical anesthesia, EDL muscles were harvested, weighed, and frozen in OCT-filled cryomolds in liquid nitrogen. EDL muscles were cryostat sectioned at 10 μm and stained for Harris H&E or NADH. Quadriceps were harvested and frozen on dry ice for Western blot analysis. Other tissues were harvested and frozen on dry ice for Southern and Western blot analysis, sequencing, or quantitative real-time RT-PCR. Spinal cords for most animals were immersion fixed ≥30 days in 10% buffered formalin for later ventral root harvesting.

Quantitative Real-Time RT-PCR. ***RNA isolation and cDNA preparation.*** Total RNA was isolated from limb muscles by using TRIzol and analyzed by using gel electrophoresis and spectrophotometry. Samples were DNase I treated before reverse transcription using a $dT_{20}VN$ primer (Sigma, Oakville, ON) with SuperScript II. Resultant cDNA was diluted 1:8 for future use. Each cDNA reaction had a control reaction without reverse transcriptase (no RT control).

qPCR and analysis. Each 25-μl real-time PCR amplification used 2 μl of the cDNA or no RT control. Reactions were assembled by using SYBR Green JumpStart Taq ReadyMix (Sigma). Primers were as follows: VEGF, atcttcaagccgtcctgtgt and aatgctttctccgctctgaa;

MyoD, gacagggaggaggggtagag and tgctgtctcaaaggagcaga; myogenin, cctacagacgcccacaatct and cagggctgttttctggacat; AChR, ccacctatcgggctcagtta and tcccccaatttcatcaatgt; GAPDH ccaaggctgtaggcaaagtc and gaccacctggtcctctgtgt.

Samples were incubated at 95°C for 10 min before thermal cycling (40 cycles of: 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s) using the Mx4000 System (Stratagene, La Jolla, CA). Melting curves were determined for all PCR products. The ROX (5-carboxy-X-rhodamine, succinimidyl ester)-normalized fluorescence measurements were analyzed using the LinRegPCR program (34) to correct for efficiency of each reaction. The expression of each test gene was normalized to the level of GAPDH within each.

Morphometrical Analysis. ***Quantification of muscle number and size.*** Total fiber number and average fiber size was determined from a cross section of H&E-stained EDL from the middle of the muscle. Every fiber was counted, and the cross sectional area of individual fibers was determined stereologically by using the Stereo Investigator program (MicroBrightField, Burlington, VT). Briefly, the section was outlined at low power, and then muscle fibers were sampled by using a ×40 objective and the "fractionator" probe every 300 μm throughout the cross section. All fibers that intersected the sampling box were measured.

Quantification of axon number and size. L5 ventral roots were harvested from formalin fixed spinal cords, embedded in Poly/Bed Araldite 502, sectioned at 1 μm, and stained with Toluidine Blue by the Michigan State University Electron Microscopy Laboratory. Every myelinated axon was counted in a single cross section, and their size measured as described for fiber size, with the exceptions that a ×63 objective was used and axons were sampled every 100 μm.

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1. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH (1991) *Nature* 352:77–79.
2. Zoghbi HY, Orr HT (2000) *Annu Rev Neurosci* 23:217–247.
3. Sobue G, Hashizume Y, Mukai E, Hirayama M, Mitsuma T, Takahashi A (1989) *Brain* 112:209–232.
4. Katsuno M, Adachi H, Kume A, Li M, Nakagomi Y, Niwa H, Sang C, Kobayashi Y, Doyu M, Sobue G (2002) *Neuron* 35:843–854.
5. Chevalier-Larsen ES, O'Brien CJ, Wang H, Jenkins SC, Holder L, Lieberman AP, Merry DE (2004) *J Neurosci* 24:4778–4786.
6. McManamny P, Chy HS, Finkelstein DI, Craythorn RG, Crack PJ, Kola I, Cheema SS, Horne MK, Wreford NG, O'Bryan MK, *et al*. (2002) *Hum Mol Genet* 11:2103–2111.
7. Yu Z, Dadgar N, Albertelli M, Gruis K, Jordan C, Robins DM, Lieberman AP (2006) *J Clin Invest* 116:2663–2672.
8. Sopher BL, Thomas PS, Jr, LaFevre-Bernt MA, Holm IE, Wilke SA, Ware CB, Jin LW, Libby RT, Ellerby LM, La Spada AR (2004) *Neuron* 41:687–699.
9. Monks DA, Kopachik W, Breedlove SM, Jordan CL (2006) *Can J Physiol Pharmacol* 84:273–277.
10. Monks DA, O'Bryant EL, Jordan CL (2004) *J Comp Neurol* 473:59–72.
11. Miniou P, Tiziano D, Frugier T, Roblot N, Le Meur M, Melki J (1999) *Nucleic Acids Res* 27:e27.
12. Brennan KJ, Hardeman EC (1993) *J Biol Chem* 268:719–725.
13. Charest NJ, Zhou ZX, Lubahn DB, Olsen KL, Wilson EM, French FS (1991) *Mol Endocrinol* 5:573–581.
14. Eftimie R, Brenner HR, Buonanno A (1991) *Proc Natl Acad Sci USA* 88:1349–1353.
15. Wagatsuma A, Osawa T (2006) *Acta Physiol (Oxford)* 187:503–509.
16. Kennedy WR, Alter M, Sung JH (1968) *Neurology* 18:671–680.
17. Rusmini P, Sau D, Crippa V, Palazzolo I, Simonini F, Onesto E, Martini L, Poletti A (2007) *Neurobiol Aging* 28:1099–1111.
18. Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S (2004) *Nature* 431:805–810.
19. Tsuda H, Jafar-Nejad H, Patel AJ, Sun Y, Chen HK, Rose MF, Venken KJ, Botas J, Orr HT, Bellen HJ, Zoghbi HY (2005) *Cell* 122:633–644.
20. Fernandez-Funez P, Nino-Rosales ML, de Gouyon B, She WC, Luchak JM, Martinez P, Turiegano E, Benito J, Capovilla M, Skinner PJ, *et al*. (2000) *Nature* 408:101–106.
21. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, *et al*. (2003) *Science* 302:841.
22. Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M, Feany MB (2001) *Science* 293:711–714.
23. Ribchester RR, Thomson D, Wood NI, Hinks T, Gillingwater TH, Wishart TM, Court FA, Morton AJ (2004) *Eur J Neurosci* 20:3092–3114.
24. Ferrara N, Gerber HP, LeCouter J (2003) *Nat Med* 9:669–676.
25. Kivela R, Silvennoinen M, Touvra AM, Lehti TM, Kainulainen H, Vihko V (2006) *FASEB J* 20:1570–1572.
26. Pun S, Santos AF, Saxena S, Xu L, Caroni P (2006) *Nat Neurosci* 9:408–419.
27. Frey D, Schneider C, Xu L, Borg J, Spooren W, Caroni P (2000) *J Neurosci* 20:2534–2542.
28. Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, Khan J, Polak MA, Glass JD (2004) *Exp Neurol* 185:232–240.
29. Cifuentes-Diaz C, Nicole S, Velasco ME, Borra-Cebrian C, Panozzo C, Frugier T, Millet G, Roblot N, Joshi V, Melki J (2002) *Hum Mol Genet* 11:1439–1447.
30. Katsuno M, Adachi H, Minamiyama M, Waza M, Tokui K, Banno H, Suzuki K, Onoda Y, Tanaka F, Doyu M, Sobue G (2006) *J Neurosci* 26:12106–12117.
31. Muscat GE, Kedes L (1987) *Mol Cell Biol* 7:4089–4099.
32. Taylor MD, Vancura R, Patterson CL, Williams JM, Riekhof JT, Wright DE (2001) *J Comp Neurol* 432:244–258.
33. Barneoud P, Lolivier J, Sanger DJ, Scatton B, Moser P (1997) *NeuroReport* 8:2861–2865.
34. Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) *Neurosci Lett* 339:62–66.