

Published in final edited form as:

Neurobiol Dis. 2009 April ; 34(1): 113–120. doi:10.1016/j.nbd.2008.12.009.

Recovery of function in a myogenic mouse model of spinal bulbar muscular atrophy

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Abstract

With this paper, we deliberately challenge the prevailing neurocentric theory of the etiology of spinal bulbar muscular atrophy (SBMA). We offer data supporting an alternative view that androgen receptor (AR) acts in skeletal muscles to cause the symptoms of SBMA. While SBMA has been linked to a CAG repeat expansion in the AR gene and mutant AR is presumed to act in motoneurons to cause SBMA, we find that over-expression of wild type AR solely in skeletal muscle fibers results in the same androgen-dependent disease phenotype as when mutant AR is broadly expressed. Like other recent SBMA mouse models, transgenic (tg) females in our model exhibit a motor phenotype only when exposed to androgens, and this motor dysfunction is independent of motoneuronal or muscle fiber cell death. Muscles from symptomatic females also show denervation-like changes in gene expression comparable to a knock-in model of SBMA. Furthermore, once androgen treatment ends, tg females rapidly recover motor function and muscle gene expression, demonstrating the strict androgen-dependence of the disease phenotype in our model. Our results argue that SBMA may be caused by AR acting in muscle.

Keywords

Polyglutamine; Kennedy's Disease; Androgens; Muscle; Transgenic; Motor neuron; Denervation

Introduction

Spinal bulbar muscular atrophy (SBMA) is an adult onset, slowly progressive lower motoneuron disease characterized by proximal limb and bulbar muscle weakness and

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2008.12.009.

atrophy affecting primarily men. This disease is linked to an expansion of a CAG repeat in the androgen receptor (AR) gene, coding for an expanded polyglutamine (polyQ) repeat in the AR protein (La Spada et al., 1991). Most clinical evaluations conclude that muscle pathology represents neurogenic atrophy that is secondary to motoneuronal death (Zoghbi and Orr, 2000).

Data from our 'myogenic' mouse model challenges this neurocentric view of SBMA. We find that mice expressing an AR transgene exclusively in skeletal muscle fibers show the same male-biased, androgen-dependent loss of motor function (Monks et al., 2007) as described for other SBMA mouse models that broadly express a polyQ expanded AR (Katsuno et al., 2002; McManamny et al., 2002; Sopher et al., 2004; Yu et al., 2006). Moreover, in our model, a wild type AR with 22 glutamines mediates the androgen-induced toxicity.

Given the current view of SBMA, we were quite surprised that our transgenic (tg) mice exhibit a SBMA-like phenotype, since they neither express an expanded polyQ allele nor an AR transgene in motoneurons. Nonetheless, our tg model has the following characteristics: 1) only tg males show a loss of motor function, but 2) androgen exposure induces the disease in tg females, 3) disease symptoms in late-stage tg males are accompanied by deficits in the number of muscle fibers and motor axons, and 4) castration rescues motor function in severely diseased tg males, casting doubt on the relationship between cell loss and motor dysfunction (Monks et al., 2007). Finally, muscles of symptomatic tg males show expected changes in the expression of genes implicated in SBMA (Sopher et al., 2004; Yu et al., 2006).

That high expression of a wild type allele can induce comparable pathology as a neurodegenerative mutant allele is not without precedence, and includes synuclein implicated in Parkinson's (Singleton et al., 2003), ataxin in spinocerebellar ataxia (Fernandez-Funez et al., 2000; Tsuda et al., 2005), tau in Alzheimer's (Wittmann et al., 2001) and SOD1 in ALS (Jaarsma et al., 2000). Even AR has been reported to induce a ligand-dependent toxicity in cell models of SBMA that is independent of polyQ length (Grierson et al., 2001; Walcott and Merry, 2002), challenging our understanding of the factors regulating AR protein toxicity. Because over-expression of wt AR in muscle fibers results in a strict androgen-dependent loss of motor function mimicking a well described SBMA phenotype in mice, we hypothesize that androgen normally acts in muscles to trigger SBMA.

The current study addresses several remaining questions. Does comparable cell loss accompany the loss of function in androgen-treated tg females? Can severely compromised tg females recover motor function after the end of androgen treatment, comparable to the effect of castration in severely diseased tg males? Finally, will androgen induce the same changes in muscle gene expression in tg females as in diseased tg males, and will such changes reverse once androgen treatment ends? We now show that the precipitous loss of motor function induced by androgens in tg females is completely reversed within a week of halting hormone treatment. We further show that impairment of motor function is

independent of cell loss but is accompanied by androgen-dependent changes in muscle gene expression that also revert to normal once androgen treatment ends.

Methods

Generation and identification of transgenic mice

Transgenic (tg) mice were generated and genotyped as previously described (Monks et al., 2007). Two lines were selected for in-depth characterization: a severely affected line (L141) that expresses high levels of AR and a less severely affected line that expresses lower levels of the transgene (L78) (Monks et al., 2007). Tg animals from these two founding lines were mated to C57/BL6J mice and their progeny were analyzed. Animals were group housed, with water and food provided *ad libitum*. All animal procedures were approved and performed in compliance with the Michigan State University Institutional Animal Care and Use Committee, in accordance with the standards in the NIH Guide for the Care and Use of Laboratory animals.

Androgen treatment of tg females

Tg and wild type (wt) age-matched females (70–126 days) from L141 and L78 were ovariectomized under deep anesthesia using isoflurane, and given either a testosterone (T)-containing or blank Silastic capsule (1.57 mm i.d. and 3.18 mm o.d., effective release length of 6 mm) s.c. just caudal to the scapula. Incisions were closed by suturing the muscle wall and closing the skin with 9 mm staples. The T capsule resulted in levels of T (11.29 nmol/L +1.28 nmol/L, $n=33$) comparable to gonadally intact wt male mice levels (16.19 nmol/L +3.7 nmol/L, $n=31$). Behavior was measured 2–4 h before surgery (Day 0) and on Days 3, 5, 7, 9 after surgery. Most animals from L141 were sacrificed on day 9, but a few had to be sacrificed on Day 7, due to severe disease progression. Only females surviving to 9 days were included in statistical analyses. Some females from L78 were sacrificed after 9 days, but because L78 tg females did not express a disease phenotype at that time, we extended the treatment period for other females to 8 weeks ($n=3$ per group) to determine whether a longer exposure to T would induce disease. T levels after 8 weeks (9.5+1.2 nmol/L, $n=7$) were slightly lower than in females treated for only 10 days (see above). Behavioral data on L141 tg females has been previously reported (Monks et al., 2007), and cellular measures from these same females are reported here.

Recovery of function after T treatment

To assess whether the effects of T on motor function are reversible, a separate group of females from the symptomatic L141 line were ovariectomized and given either a T or a blank capsule as described above. On day 9, these females were re-anesthetized and capsules were removed. All behavior tests were measured on days 0 (before T treatment), 1, 3, 5, 7, 9 (during T treatment), and on days 10, 12, and 15 (1, 3 and 6 days after T removed) except for rotarod performance, cage activity, and stride length, which were measured only on Days 0, 9 and 15. Animals were sacrificed after behavioral testing on day 15, and tissues harvested.

Behavioral methods

Motor function was evaluated using the hang test, stride length, and rotarod task as previously described (Monks et al., 2007). Grip strength was also assessed using a grip strength meter (Columbus Instruments) to measure forelimb grip strength, and combined hindlimb and forelimb grip strength as in other mouse models of SBMA (Chevalier-Larsen et al., 2004; Yu et al., 2006). The grip strength meter was positioned horizontally, and mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the metal grid with their forelimbs only, or all limbs and then were pulled off. The force applied to the bar at the moment the grasp was released was recorded as the peak tension (g). The test was repeated 6 consecutive times within the same session, and the highest and lowest value from the 6 trials was dropped. The remaining four values were averaged together and recorded as the grip strength for that animal. For cage activity, four quadrants were drawn on a piece of paper and placed underneath a clear mouse cage. Each mouse was placed into the clear cage and the number of quadrants visited and the numbers of rears were recorded during 30 s.

Tissue harvesting and processing

Animals were deeply anesthetized with isoflurane. Extensor digitorum longus (EDL) muscles were placed in OCT filled cryomolds, frozen in liquid nitrogen, and stored at -80°C until sectioned on a cryostat (Leica) at $10\text{ }\mu\text{m}$. Anterior tibialis muscles were also harvested and either frozen with liquid nitrogen in OCT filled cryomolds, or in microcentrifuge tubes on dry ice for mRNA analysis. Spinal columns were placed in buffered formalin for at least 30 days before lumbar four and five (L4 and L5) ventral roots were harvested. L4 and L5 roots were embedded in Epon resin (Araldite 502: Poly/Bed 812: DDSA) and semi thin $1\text{ }\mu\text{m}$ cross sections collected and stained with toluidine blue.

Morphometric analysis

Muscles and ventral roots were quantified as previously described (Monks et al., 2007). Briefly, $10\text{ }\mu\text{m}$ EDL cross sections were stained with hematoxylin and eosin (H&E), and every fiber was counted in a single cross section from the belly of the muscle. Muscle fiber size was measured using the Stereo Investigator program (MicroBrightfield) to obtain unbiased samples. All motor axons were counted from single cross sections of L4 and 5 ventral roots. Cross sectional area of motor axons was also estimated from the same sections using the Stereo Investigator program (MicroBrightfield).

Gene expression analysis

Total RNA isolated from anterior tibialis muscles with Trizol (Invitrogen) served as a template for cDNA synthesis using the High Capacity cDNA Archive Kit from Applied Biosystems. Gene-specific primers and probes labeled with a fluorescent reporter dye and quencher were used (Applied Biosystems) for acetylcholine receptor α -subunit (AChR), myogenin and myogenic differentiation factor 1 (MyoD). TaqMan assays were performed using 5 ng aliquots of cDNA. Replicate tubes were analyzed for the expression of 18S ribosomal RNA (rRNA) using a VIC-labeled probe. Threshold cycle (Ct) values were determined by an ABI Prism 7900HT Sequence Detection System, and relative expression

levels were calculated using the standard curve method of analysis. Values are expressed relative to the control group.

Vascular endothelial growth factor isoform 164 and 188 (VEGF) mRNA was measured as previously described (Monks et al., 2007). Briefly, total RNA was isolated from limb muscles using Trizol and analyzed using gel electrophoresis and spectrophotometry. Samples were DNase I treated prior to reverse transcription using a dT₂₀ 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 reactions were then assembled using SYBR Green Jumpstart Taq ReadyMix (Sigma, Oakville, ON) and VEGF primers (atcttcaagccgctctgtgt and aatgctttctccgctctgaa) or GAPDH primers (caaggctgtaggcaaagtc and gaccacgtggtcctctgtgt). Samples were incubated at 95 °C for 10 min prior to 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-normalized fluorescence measurements analyzed using the LinRegPCR program to correct for efficiency of each reaction. The expression of VEGF was normalized to the level of GAPDH within each sample.

Statistics

Data were analyzed using Statview 5.0.1. To assess the effects of T administration on body weights and behavioral measures, 3-Way ANOVAs were performed using treatment (two levels) and genotype (2 levels) as between-subject measures, and time as a within-subject, repeated measure. To assess the effects of T on cellular measures such as muscle fiber number, axon number, fiber size, axon size, and relative gene expression, 2-way ANOVAs were performed with treatment and genotype as between-subjects factors. To assess recovery of motor functions after T removal, 2-way ANOVAs were performed with treatment (2 levels) as a between-subjects factor and time as a within-subjects factor. Significant 2-way interactions were found on all behavioral tests; therefore unpaired *t*-tests were performed at individual time points to determine when the two treatment groups differed. Unpaired *t*-tests were also performed to compare gene expression levels between the two groups. For all analyses, $p < .05$ was considered significant.

Results

Motor function rapidly recovers after the end of testosterone treatment

L141 tg females treated with testosterone (T) capsules rapidly develop a neuromuscular disease phenotype consisting of a marked loss of muscle strength (Monks et al., 2007). To determine whether the androgen-induced phenotype is reversible, we treated a cohort of L141 tg females with T for 9 days and then removed the capsules. The statistical analyses revealed significant main effects of treatment and time on all measures, and significant interactions (Supplemental Table 1). Although performance and body weight is equivalent between the two tg groups at day 0 (just before treatment begins), T induces a rapid decline in both body weight and motor performance during the 9 day treatment period (Fig.1). T-treated tg females lose 30% of their body weight during this time, and develop large deficits in motor performance. However, once the T capsules are removed, tg females regain motor

function within 3 days (Figs. 1B–D). Interestingly, we find that body weight lags behind recovery of motor function, requiring a week before body weight is fully restored after T treatment ends (Fig. 1A). Given that significant losses in body weight also lag behind significant losses in muscle strength (based on forelimb grip strength and the hang test; see Figs. 1A–D), changes in muscle strength may occur independent of changes in body weight. T-treated tg females also show marked deficits in rotarod performance, stride length and cage activity by day 9 but recovered fully on these measures by 6 days after the end of T treatment (Figs. 1E–I). Supplemental video 1 shows the females' performance on the hang test and cage activity before, during, and after the end T treatment, demonstrating the rapid decline and recovery of motor functions with and without androgens.

Testosterone treated L78 tg females do not lose motor functions

In contrast to L141 tg females, T treatment of tg females from the L78 line, in which tg males are asymptomatic, does not cause weight loss (Fig. 2A) nor muscle weakness after 9 days (Figs. 2B–D). In fact, tg females gained weight and improved performance on the hang test during this period, leading to significant effects of time on these measures (Supplemental Table 2). There were no main effects of genotype, or hormone treatment on other behavioral measures (Supplemental Table 2). No motor deficits were seen in L78 tg females even after 8 weeks of T treatment (data not shown).

No loss of muscle fibers or ventral axons despite profound losses of motor function in L141 tg female mice

Because L141 T-treated tg females show severely compromised motor function, we asked whether losses in motor axons or muscle fibers accompany the loss of motor function in this line as seen for symptomatic tg males of this same line (Monks et al., 2007). Unexpectedly, T did not affect the number of EDL muscle fibers (Fig. 3A), nor their size (Fig. 3B) despite its devastating effect on motor function in L141 T-treated tg females. Similarly, neither the number nor size of motor axons in L4 and L5 ventral roots were significantly affected by T in L141 tg females (Fig. 3C–E). There were no significant main effects of either genotype or hormone treatment on any of these measures (Supplemental Table 3). Cellular measures were not examined in L78 mice, given that no losses in muscle fibers or axons were seen in symptomatic L141 tg females. These results indicate that neither motoneuron nor muscle fiber losses are required for deficits in motor function, suggesting cell dysfunction rather than loss underlies the motor deficits triggered by T in tg females.

Both symptomatic L141 and asymptomatic L78 tg females show histopathology in skeletal muscles

EDL muscles were analyzed for pathological markers of neuromuscular disease. H&E staining reveals that L141 tg females treated with T show both small angular and rounded fibers suggestive of neurogenic atrophy and occasional fibers with centralized nuclei (Fig. 4A). However, the frequency of fibers containing centralized nuclei is low in both T- and control-treated L141 tg muscles and not significantly different ($0.39\% \pm 0.18$ vs. $0.51\% \pm 0.13$, respectively), suggesting that this pathological marker is unrelated to expression of the disease motor phenotype. While pathology in muscles of symptomatic L141 tg females is qualitatively similar to that seen in L141 tg males (Monks et al., 2007), it was considerably

less marked. For example, NADH staining suggests only mild increases in oxidative metabolism in T-treated L141 females (Fig. 4B). Surprisingly, muscles from L78 tg females that remain asymptomatic after 8 weeks of T treatment also showed pathology consisting of occasional centralized nuclei and altered myofilament organization (Figs. 4C, D). Some muscle fibers exhibit a central core of dark NADH staining, suggesting an area rich in mitochondria and largely devoid of myofilaments (Fig. 4D). This is in contrast to the relatively uniform distribution of mitochondria and myofilaments in normal, unperturbed skeletal muscle fibers, and is distinct from central core myopathies, ring fibers, or target fibers (Mills, 2007). However, given that this 'donut' pattern of staining is most prominent in muscles of T-treated L78 tg mice that exhibit normal motor function, such pathology is apparently unrelated to muscle strength or motor performance.

Changes in muscle gene expression indicative of denervation in L141 but not L78 tg females

We next examined the expression of three genes that are upregulated in L141 symptomatic tg males (Monks et al., 2007), myogenin, acetylcholine receptor α -subunit (AChR), and myogenic differentiation factor 1 (myoD), using reverse transcriptase quantitative-PCR (qPCR). There were significant main effects on AChR and myogenin gene expression (Supplemental Table 4). Muscles of symptomatic T-treated L141 tg females show a significant upregulation of AChR (Fig. 5A, $p < .0025$) and myogenin (Fig. 5B, $p < .0033$) after 9 days of T treatment compared to the other three groups (T and blank-treated wts and blank-treated tgs). There was a similar upregulation of myoD expression, although this response fell short of significance (Fig. 5C, $p = .09$), comparable to that seen in a knock-in (KI) polyglutamine mouse model of SBMA (Yu et al., 2006). Muscle gene expression in tg females without T were comparable to wt control females, showing no effect on expression of these genes. Importantly, no such changes were observed in behaviorally asymptomatic L78 tg females treated with T for 9 days (Figs. 5E–G, Supplemental Table 4). Because expression of these genes is similarly increased in denervated muscles (Klocke et al., 1994; Kostrominova et al., 2005), these data suggest that muscle denervation may underlie the loss of motor function in T-treated L141 tg females.

We also examined gene expression of vascular endothelial growth factor (VEGF), which is downregulated in muscles of symptomatic L141 tg males (Monks et al., 2007). VEGF mRNA was expressed at lower levels in T-treated tg L141 females (Fig. 5D), although this decrease was not statistically significant compared to blank-treated tg females ($p = .07$). In asymptomatic L78 females, T did not affect VEGF expression levels (Fig. 5H).

Recovery of gene expression after testosterone treatment ends

qPCR revealed that the genes upregulated in T-treated tg females were restored to normal levels 6 days after the end of T treatment. AChR, myogenin and myoD mRNA were the same in muscles of blank-treated tg females as in muscles of tg females that had formerly received T (Figs. 5I–K). Surprisingly, there was a significant reduction in the level of VEGF mRNA in females after T treatment (Fig. 5L, $p < .0096$), suggesting that L141 tg females may indeed suffer a loss of VEGF during T treatment and that the loss persists after the recovery of function.

Discussion

As in other mouse models of SBMA (Katsuno et al., 2002), the disease phenotype can be induced by androgens in our tg females. Unlike these other models, however, androgen induces the motor phenotype in our model by activating wt AR in muscle fibers. We also find that expression of the disease phenotype in tg females depends on the level of transgene expression, with tg females from the line (L141), which expresses more AR, showing an androgen-dependent disease phenotype that tg females from the line (L78), which expresses less AR, did not. This pattern parallels the difference in phenotype expression of tg males from these same two lines (Monks et al., 2007).

L141 tg females over-expressing wild type AR in muscle fibers are asymptomatic, yet rapidly lose motor function when provided with male-typical levels of T. Moreover, androgen exposure much longer than 9 or 10 days is fatal to tg females. This remarkable toxicity cannot be attributed to toxic levels of androgens, since the T implants result in circulating T levels that are slightly less than normal adult male levels. Furthermore, after 9 days of T treatment which brings L141 females close to death, full recovery from behavioral symptoms can be achieved just 3 days after T cessation. This rapid recovery seems inconsistent with a loss of motoneurons or muscle fibers, and indeed we find no significant losses after 9 days of T treatment. While symptomatic tg males have fewer muscle fibers and motor axons, our findings in tg females call into question whether cell loss plays any role in the behavioral deficits seen in our model of SBMA, and suggest that cell dysfunction may critically mediate the loss of motor function. Because recovery of function is also possible in our model, this too suggests that dysfunction rather than cellular loss underlies the deficits in motor function.

Data from other mouse models of SBMA also suggest the same conclusions that cell dysfunction rather than cell death triggers the loss of motor function in SBMA (Katsuno et al., 2002; Chevalier-Larsen et al., 2004). In AR-122Q mice described by Chevalier-Larsen et al. (2004), substantial motor deficits are present in males but no motoneuron loss or muscle pathology was observed (Chevalier-Larsen et al., 2004). Also, the AR-97Q mice described by Katsuno et al. (2002) develop signs of neurogenic muscle atrophy without motoneuron loss. These findings also support the idea that the neurological phenotype associated with SBMA may result from neuronal dysfunction rather than neuronal loss. Importantly, data from our model suggest this neuronal dysfunction can be triggered by signals that originate in the muscle.

qPCR indicates that transcripts of myogenin, and acetylcholine receptor α -subunit (AChR), which are elevated following denervation (Klocke et al., 1994; Kostrominova et al., 2005), are also increased in symptomatic L141 tg females given T, comparable to levels seen in a KI model of SBMA (Yu et al., 2006). The upregulation of these genes in symptomatic L141 tg females, and not in androgen-treated asymptomatic L78 tg nor wt females, indicates that this response to T may somehow be related to motor dysfunction. Elevation of these specific genes suggests that muscle fibers may be structurally and/or functionally denervated, and that muscle denervation may contribute to the loss of motor function in our model. What is surprising about our model however is that this putative denervation of muscle fibers is

triggered by events that originate in the muscles, since the transgene is expressed only there. This is counter to the predominant way of thinking, and implies that mutant AR need not act in motoneurons to get the same outcome, i.e., muscle denervation (Jordan and Lieberman, 2008). That expression of the same genes are increased in a knock-in model of SBMA suggests that mutant AR may act in muscles to elicit these changes (Yu et al., 2006). Skeletal muscles affected by Huntington disease, another polyQ expansion disorder, do not show a similar response (Strand et al., 2005), suggesting that this increased gene expression is specific to motoneuron diseases such as SBMA, and spinal muscular atrophy (Sedehizade et al., 1997).

Fiber type grouping was not evident in tg muscles stained with NADH as one might have expected if fibers were denervated and then re-innervated by collateral sprouts. Thus, it may be that synaptic failure rather than loss is involved. If dysfunctional motor terminals still occupy the junction, this might preclude sprouts from re-innervating such junctions. It is also possible that early perturbations in axonal transport without motoneuronal loss could trigger synaptic dysfunction and produce the denervation-like profile that we see in diseased muscles. Supporting this view, we also find perturbations in axonal transport in our model (Kemp et al., 2008).

The upregulation of AChR and myogenin could also reflect regenerative processes in the muscle that with the current methods used we failed to detect. MyoD family members (including myogenin) and AChR are typically upregulated in muscle satellite cells during development and regeneration (Duclert et al., 1991; Shi and Garry, 2006). Future studies directly examining neuromuscular synapses may help answer whether the increases in gene expression reflect regeneration of damaged muscle fibers, denervation or both.

Although VEGF mRNA levels were not significantly different in our L141 females during T treatment, they were significantly decreased in muscles of L141 females 6 days after T treatment when such females had fully recovered motor function. The lack of a significant decrease in VEGF levels in symptomatic females is inconsistent with the significant decrease in VEGF mRNA levels in muscles of affected L141 tg males (Monks et al., 2007). This discrepancy suggests that either we failed to detect a real decrease in VEGF mRNA during T treatment or that VEGF expression responds more slowly to androgens than expression of AChR and myogenin. Whatever the case may be, given that the level of VEGF mRNA is down after motor function has recovered suggests that changes in VEGF may be a response rather than a cause of disease.

Tg muscles contain fibers with the pathological feature of centralized nuclei. However, we find that such fibers are no more frequent in T-treated L141 mice with poor motor function than in control-treated tg females that have normal motor function. This finding suggests that centralized nuclei are unrelated to the loss of motor function in our model. Also unrelated to the loss of motor function is the alteration in the myofibrillar network revealed by NADH staining, since we see more of this type of muscle pathology after androgen treatment in asymptomatic L78 tg females than in symptomatic L141 tg females. Thus, such commonly noted histological signs are also likely to be epiphenomena rather than causes of motor dysfunction.

Our mice directly challenge the assumption that only an expanded polyQ tract can induce the disease symptoms of SBMA. We show that a wt AR in skeletal muscles can mimic the effects of expanded polyQ AR, suggesting that the expanded polyQ tract is not necessary to induce an androgen-dependent motoneuronal disease and may be only one of several ways toxicity is conferred to an AR protein. However, we are not the first to demonstrate that over-expression of the wt allele can induce symptoms similar to those induced by a pathological mutant protein. For example, over-expression of wt ataxin-1 causes neurodegeneration in *Drosophila* and mouse models of SCA1 (Fernandez-Funez et al., 2000; Tsuda et al., 2005). Similarly, in *Drosophila* models of tauopathy, over-expression of wt tau is sufficient to induce neurodegeneration (Wittmann et al., 2001) and gene duplication of wt α -synuclein causes Parkinson's disease similar to that induced by the mutant form of the protein (Singleton et al., 2003). Further, in cell culture models using either neuroblastoma or PC12 cells, ligand binding induces cell death in both wt and expanded polyQ AR (Grierson et al., 2001; Walcott and Merry, 2002). Interestingly, some individuals diagnosed with SBMA show the motor phenotype and associated gynecomastia but lack the expanded CAG allele of AR (Mariotti et al., 2000). Such cases raise the possibility that dysregulation of AR expression may also cause SBMA.

Other mouse models of SBMA that display a disease phenotype rely on either universal promoters, or the endogenous promoter, all of which express the allele in both motoneurons and muscle. Two SBMA models have been made using neuron-specific promoters to drive expression of the full length human polyQ allele of the AR, but neither resulted in a disease phenotype (Bingham et al., 1995; Merry et al., 1996). Thus it is possible that the motor dysfunction in the other mouse models is also a result of AR activity in the muscles alone.

While our mice do not express the expanded polyQ allele of the AR, they recapitulate many key features of SBMA, as seen in both humans and in other mouse models of this disease, and suggest that there is a common etiological pathway involved. One likely candidate is changes in muscle which trigger synaptic dysfunction, inducing a denervation-like atrophy. Our tg mice offer a unique opportunity to study early disease mechanisms of known origin that precede motor dysfunction, allowing insight into the sequence of events which trigger disease. Cellular and molecular changes seen in late-stage SBMA patients may result from, rather than cause motor dysfunction. Additionally, these mice lend themselves to testing therapeutics, as the phenotype develops quickly and is fully reversible. Our studies suggest that muscle is an important site of disease pathogenesis in SBMA, and raises the possibility that interfering with androgen action in muscles will ameliorate motor dysfunction in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

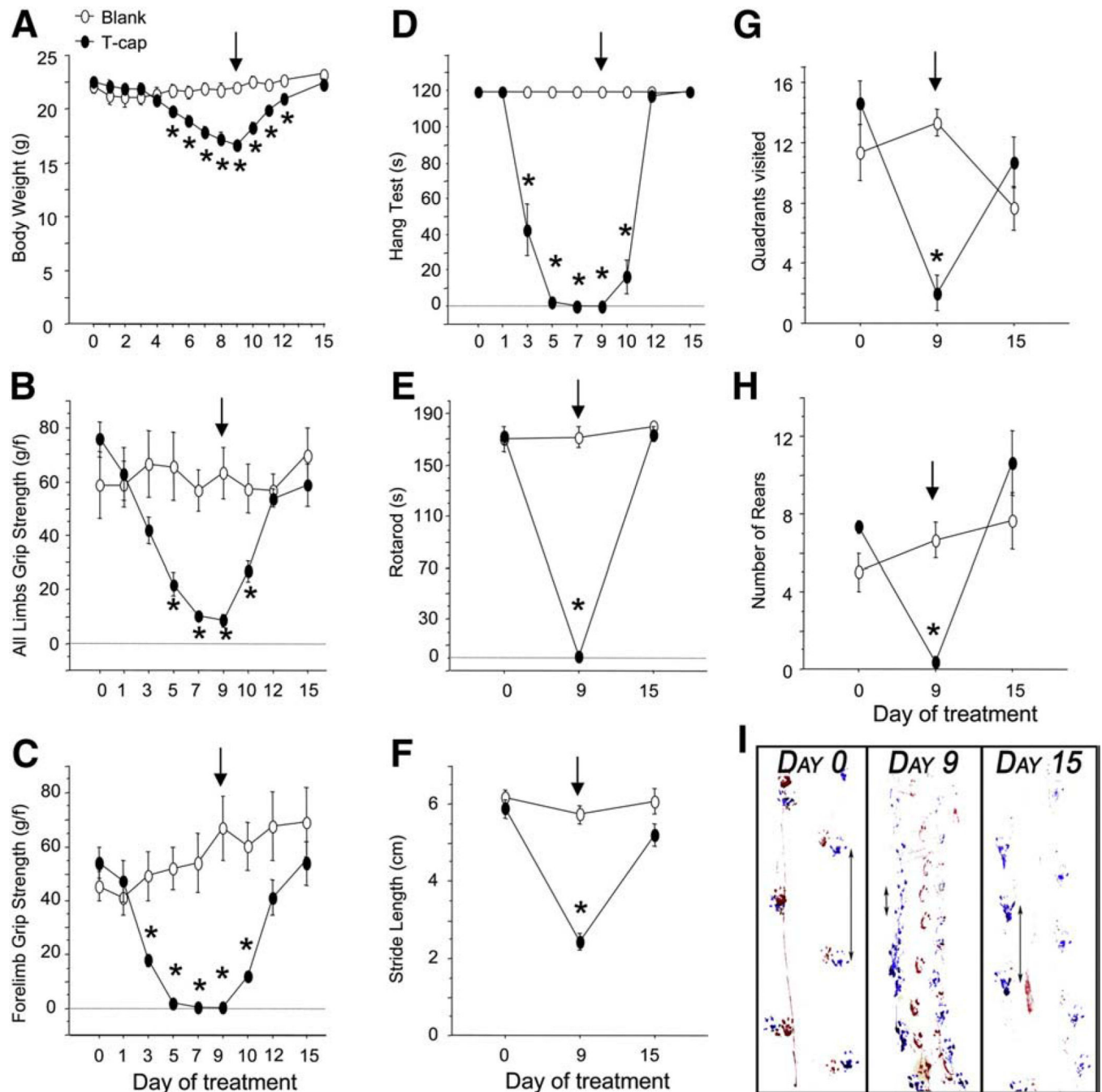
This work was supported by National Institute of Health grants NS045195 (CLJ), NS51257 (DAM) and NS055746 (APL). Additional operating support provided by the MSU Foundation (CLJ). Trainee support was provided by a

predoctoral fellowship NS54517 from NIH (JAJ). We wish to thank David Jensen, Cindy Knaff, Heather Malinowski, Diane Redenius and Sandra Troxell for considerable technical support.

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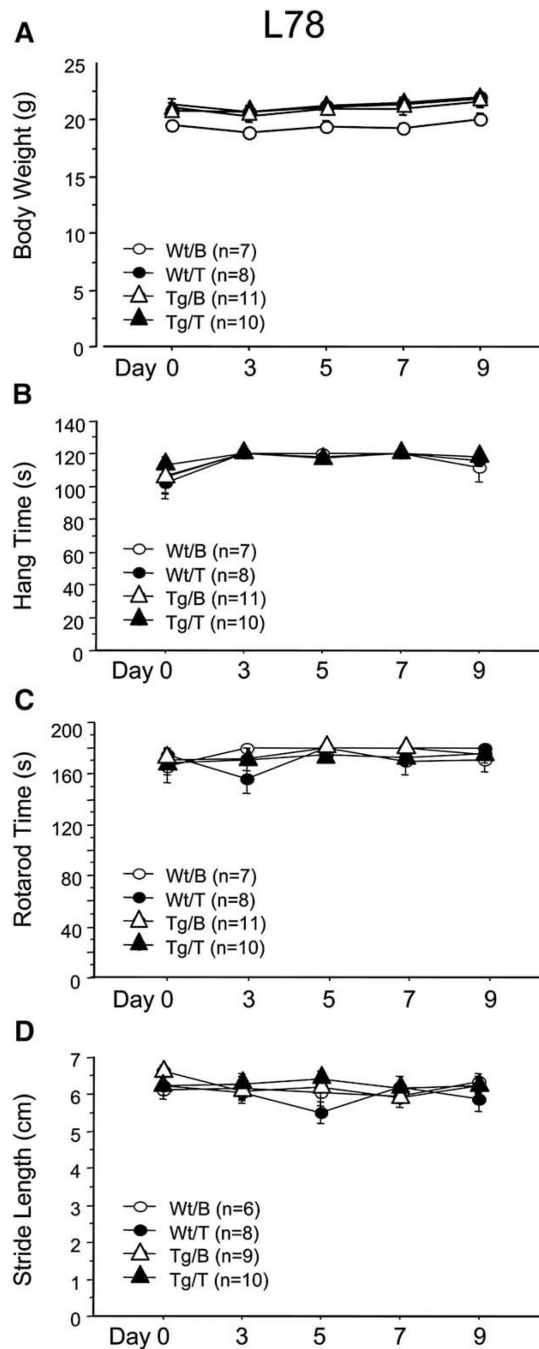
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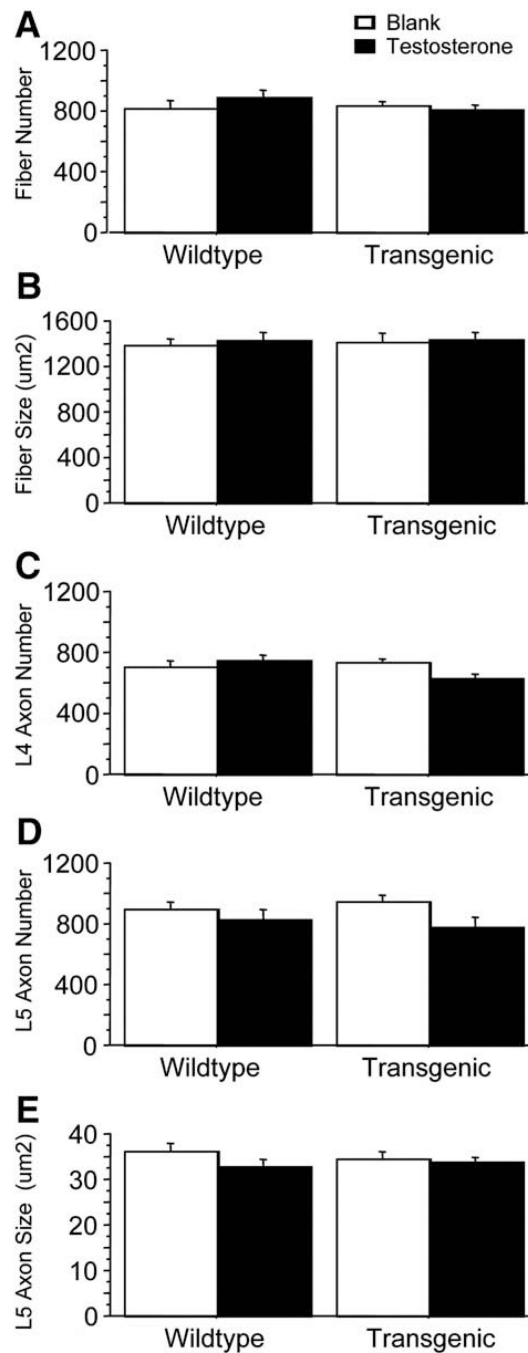
**Fig. 1.**

Transgenic (tg) females fully recover motor function after testosterone (T) treatment ends. (A) L141 tg females treated with T capsules show a rapid decline in body weight compared with females given blank (B) capsules, but completely recover their body weight after T capsules are removed on day 9 (arrow). (B–I) Strikingly, tg females also completely recover motor function after T treatment ends. Note that grip strength (B, C), hang test (D), rotarod (E), stride length (F, I) and cage activity (G, H) all recover to pretreatment levels within 6

days after the end of T treatment. *Significantly different from blank-treated tg females ($p < 0.05$).

**Fig. 2.**

T does not affect motor function in tg females of the asymptomatic L78 line. (A–D) In contrast to the devastating effect of T in L141 tg females (see Fig. 1), T has no effect on either body weight or motor performance of L78 tg females. (wt=wild type; tg=transgenic; T=testosterone capsules; B=blank capsules).

**Fig. 3.**

L141 tg females treated with T for 9 days show no evidence of cell loss despite severe motor dysfunction (see Fig. 1). L141 tg females treated with T show no reduction in the number (A) or size (B) of EDL muscle fibers, nor in the number of axons in L4 (C) or L5 (D) ventral roots, suggesting that cellular dysfunction rather than cell loss may underlie motor deficits in L141 T-treated tg females.

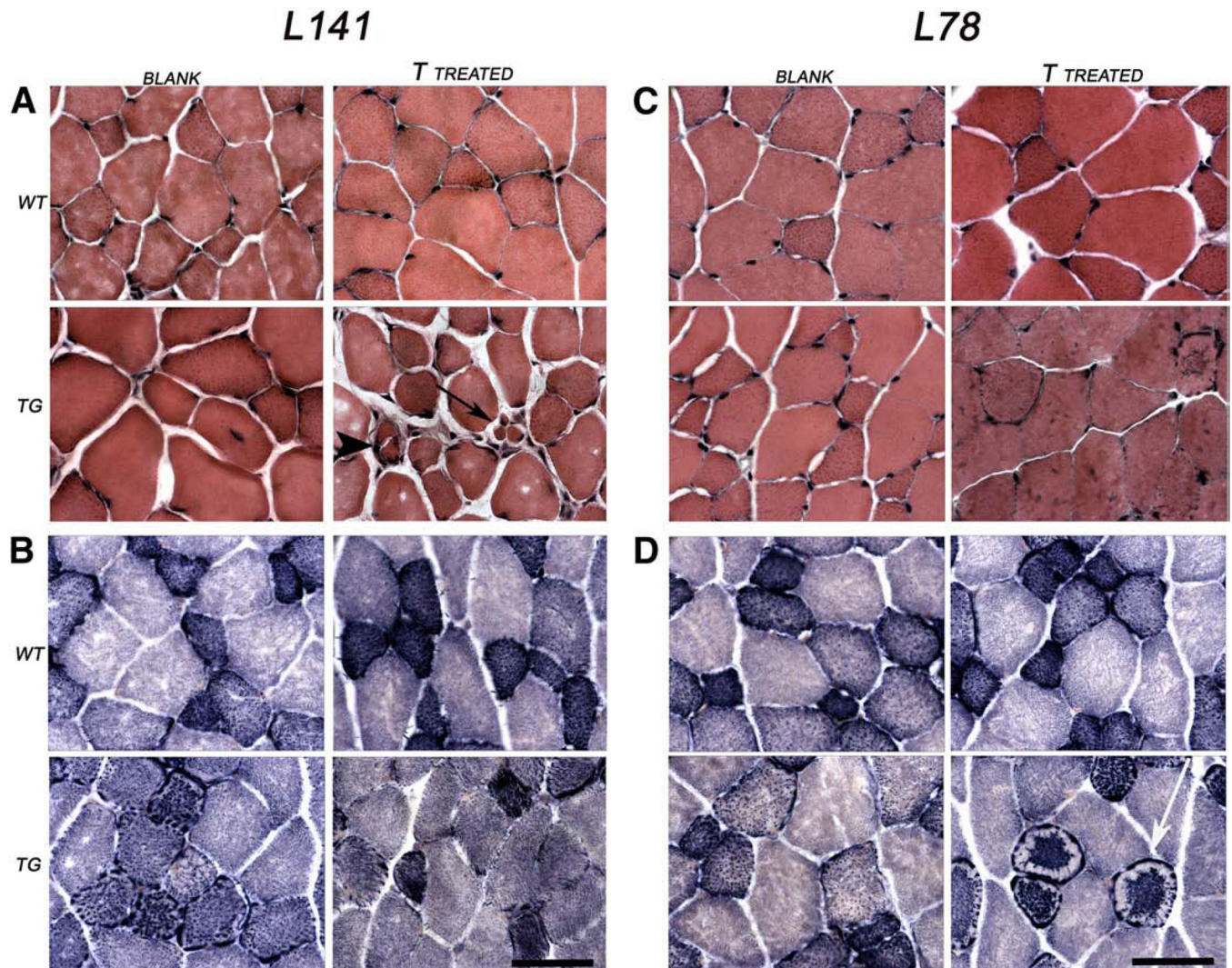


Fig. 4. Extensor digitorum longus (EDL) muscle cross sections contain pathology even in tg mice that show no motor deficits. (A) H&E stained muscles of L141 tg females treated with T (TG/T) contain small angular (arrow head), and rounded fibers (arrow). (B) Fibers appear darker in tg females treated with T in NADH stained sections, suggesting an increase in oxidative metabolism in diseased muscle. (C) H&E staining of muscles from L78 females reveals little pathology after 8 weeks of T treatment. (D) Unexpectedly, NADH staining of alternate muscle sections from these same L78 females reveals altered arrangement of myofilaments, with dark staining in the center of the fiber suggesting an area largely devoid of myofilaments (arrow). Nonetheless, this pathology does not correlate with any detected behavioral deficits. Scale bar=50 μ m.

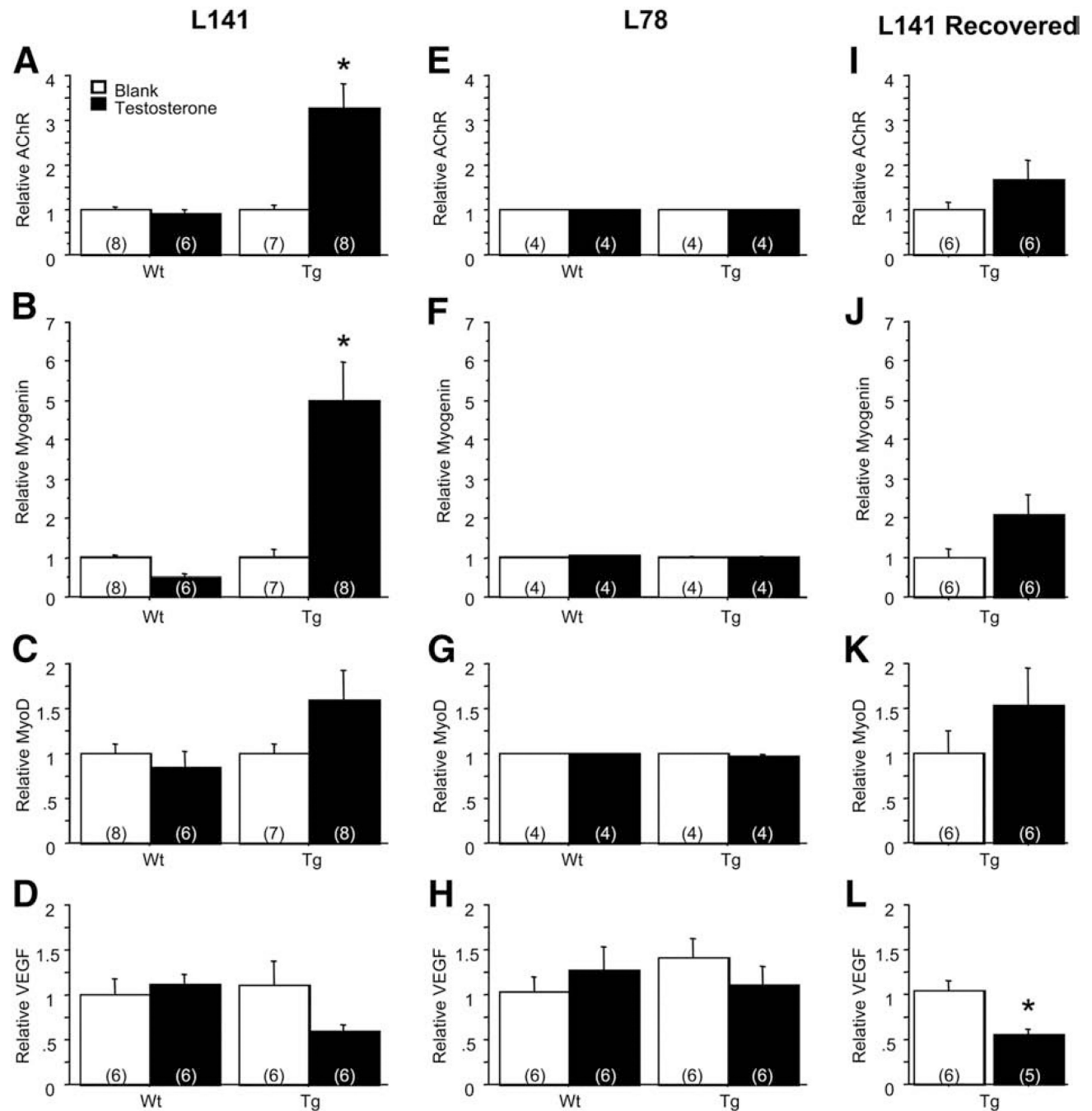


Fig. 5. qPCR reveals denervation-like changes in relative gene expression in muscles of diseased mice. (A–C) Skeletal muscle from T-treated tg L141 females show a significant upregulation of acetylcholine receptor alpha (AChR, $p < .0025$) and myogenin ($p < .003$) compared to either wt or blank-treated tg females, consistent with changes seen when muscles are denervated. Myogenic differentiation factor 1 (MyoD) mRNA levels also increased, but not significantly ($p = .09$). Importantly, neither T treatment nor expression of the AR transgene alone increase gene expression of these three genes. (D) Skeletal muscles

from T-treated L141 tg female mice also showed lower expression of vascular endothelial growth factor (VEGF), but it did not reach statistical significance ($p=.07$). (E–H) T-treated L78 tg females do not show a similar upregulation of these genes in muscle, indicating a selective dysregulation in animals showing a loss of motor function. Values are expressed relative to control (blank) treated wt females in A–H. We also found that mRNA levels for AChR (I), and myogenin (J) return to baseline levels, comparable to that of blank-treated tg females, by 6 days after T treatment ends, correlating with the recovery of motor function. However, VEGF (L) mRNA was significantly downregulated after testosterone was removed ($p=.0096$). Values are expressed relative to blank-treated tg females in I–L.