Anti-androgen flutamide protects male mice from androgen-dependent toxicity in three models of spinal bulbar muscular atrophy

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Spinal and bulbar muscular atrophy (SBMA) is a late onset, progressive neurodegenerative disease linked to a polyglutamine (polyQ) expansion in the androgen receptor (AR). Men affected by SBMA show marked muscle weakness and atrophy, typically emerging mid-life. Given the androgen-dependent nature of this disease, one might expect AR antagonists to have therapeutic value for treating SBMA. However, current work from animal models suggests otherwise, raising questions about whether polyQ-expanded AR exerts androgen-dependent toxicity through mechanisms distinct from normal AR function. In this study, we asked whether the nonsteroidal AR antagonist flutamide, delivered via a time-release pellet, could reverse or prevent androgen-dependent AR toxicity in three different mouse models of SBMA: the AR97Q transgenic (Tg) model, a knock-in (KI) model, and a myogenic Tg model. We find that flutamide protects mice from androgen-dependent AR toxicity in all three SBMA models, preventing or reversing motor dysfunction in the Tg models and significantly extending lifespan in KI males. Given that flutamide effectively protects against androgen-dependent disease in three different mouse models of SBMA, our data are proof-of-principle that AR antagonists have therapeutic potential for treating SBMA in humans, and support the notion that toxicity caused by polyQ-expanded AR utilizes at least some of the same mechanisms as normal AR before diverging to produce disease and muscle atrophy.
N-terminal domain (3). Finally, Tg males in the ‘myogenic’ model overexpress a wild-type (Wt) rat AR allele exclusively in skeletal muscle fibers (6). Castration spares or rescues androgen-dependent loss of motor function in the two Tg models and prevents androgen-dependent early death in the KI model. We tested flutamide in these three genetically distinct SBMA mouse models, reasoning that if flutamide was efficacious in three different models, such results would be compelling evidence that flutamide may have therapeutic value for human SBMA. Currently, there are no effective treatments for SBMA. We find that flutamide protects against androgen-dependent AR toxicity in all three models. These results, along with recent reports showing beneficial effects of another AR antagonist in a cell model of SBMA (14), suggest that AR antagonists, either alone, or in combination with agents that reduce testosterone production, may be an effective treatment strategy for men with SBMA.

Materials and Methods

Mice in the AR97Q and the myogenic models were maintained on a C57Bl/J6 background and mice in the KI model were maintained on a 129S1/SVLMJ background. All mice were weaned 21 days after birth, genotyped using previously described PCR (3–4, 6) and group housed with food and water provided ad libitum. Animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and in compliance with the National Institute of Health’s guidelines for the care and use of experimental animals.

Time-release flutamide pellets (Innovative Research of America, Sarasota, FL, 100 mg flutamide/pellet, 21 day release, 5 mg dose/d) were given to juvenile (28 days old) or adult (≥ 90 days old) males. At the start of flutamide treatment, juvenile AR97Q and adult KI males were asymptomatic whereas adult myogenic males were already significantly impaired. Thus, we asked whether flutamide could prevent the expression of disease for AR97Q and KI males and could reverse disease for myogenic males. Such pellets are designed to continuously release flutamide over a three week treatment period. We estimate that such pellets result in serum levels of ~30 μg/ml of flutamide (15). In our initial pilot experiments we used ‘blank’ pellets supplied by the same company and found that the effects of flutamide were not attributable to nonspecific effects of the pellet per se. However, we also found that the integrity of blank pellets was not maintained in situ, unlike the flutamide pellets, making it difficult to replace them every three weeks, as required for the flutamide pellets. For this reason, sham surgery alone served as our control; control animals were exposed to the same surgical procedures as flutamide-treated animals but did not receive a blank pellet. A battery of motor tests was used to assess disease progression as previously described (3, 5–6, 16) including the hang test, grip strength test, activity in an open field and rotarod. Body weight was also monitored throughout. At the end of flutamide treatment, blood and tissue was harvested from some mice as indicated below (5, 17).

AR97Q Model

Four-week-old asymptomatic AR97Q Tg males and age-matched Wt controls were anesthetized using isoflurane and received either flutamide pellets or nothing (n = 6–8 mice/group). Flutamide pellets were implanted subcutaneously (s.c.) on the back, near the hip, and replaced every 21 days. Animals also received ketoprofen (5 mg/kg s.c.) before recovering from anesthesia. Sham controls were subjected to the same surgical procedures as mice who received flutamide pellets. Baseline motor function was established on the day of, but prior to, surgery at 4 weeks of age (designated as treatment day 0), and continued on treatment days 3 and 7, and weekly thereafter until 63 days (13 weeks of age).

Assessing the effect of flutamide on circulating T levels of gonadally intact males. Flutamide time-release pellets were implanted in 60-day-old gonadally intact Wt male mice (N = 12) and blood was collected transcardially a week later to assess the effect of flutamide on serum T levels in Wt males. We also determined the effect of four weeks of flutamide exposure in gonadally intact Tg males starting at 28 days of age, spanning the time when exposure to androgens triggers motor dysfunction (4). Flutamide pellets were replaced with new pellets after three weeks in AR97Q Tg males with control Tg mice subjected to the same procedures but not given flutamide pellets.

Assessing the effects of flutamide on disease progression in castrated AR97Q Tg males. At four weeks of age, presymptomatic AR97Q Tg males and age-matched Wt controls were castrated under isoflurane anesthesia, and given subcutaneous (s.c.) Silastic implants containing crystalline T (1.57 mm i.d. and 3.18 mm o.d.; 6 mm effective release length). Such implants are designed to release a constant amount of T over time (18) and result in comparable levels of T as found in adult male mice (5). Half of the AR97Q Tg males and Wt controls also received a 100 mg time-release flutamide pellet s.c. (n = 11 mice/group). Care was taken to place T implants and flutamide pellets in separate subcutaneous pockets dorsolaterally to ensure maximal contact of each with the surrounding tissue. Ketoprofen (5 mg/kg s.c.) was also given before recovery. To maintain optimal release, flutamide pellets and/or T implants were replaced every 21 days until AR97Q T-treated Tg controls (no flutamide) showed end-stage disease symptoms (namely, inability to perform the hang test, difficulty righting, obvious decreases in cage activity, and/or matted, unshaven fur), at which point mice were sacrificed for tissue harvesting. Because control Tg males reached end-stage disease at markedly different ages (42–84 days), mice were sacrificed in cohorts of four, age-matched between the four conditions. Thus, for each T-treated diseased AR97Q male sacrificed, an age-matched experimental Tg male (T + flutamide) and age-matched Wt controls (T + flutamide, or T without flutamide) were sacrificed as a cohort, all having received the same length treatment. Motor function and body weight was evaluated prior to treatment on day 0, and on days 3, 7, and weekly thereafter until diseased 97Q males (T but no flutamide) showed end-stage symptoms.

Assessing the effects of flutamide on soluble and aggregated AR based on Westerns and filter trap assays. A separate group of AR97Q Tg and Wt animals at 28 days of age were...
castrated and implanted with T capsules, with half in each group also receiving flutamide as described above (n = 4 per group). When control Tg males reached end-stage (described above), they and their corresponding flutamide-treated, age-matched Tg and Wt controls were deeply anesthetized with isoflurane and the left gastrocnemius muscle and lumbar spinal cord were harvested, flash frozen in liquid nitrogen, and stored at −80°C until tissue analysis was performed. Blood was collected transcardially for measuring circulating T titers.

For Western blot analysis, muscle and cord tissue was minced and homogenized on ice in radioimmunoprecipitation assay (RIPA) buffer containing phosphatase and proteinase inhibitors. Lysates were centrifuged at 4°C for 15 minutes at 15,000 g and protein concentration was determined by protein assay (Biorad). Protein samples containing 70 μg of protein were mixed with 6X SDS sample buffer and boiled for 5 minutes at 100°C, followed by electrophoresis through 7% SDS-polyacrylamide gels and transfer to nitrocellulose membranes using a semidy transfer apparatus. After blocking in 5% nonfat milk for one hour, membranes were incubated in primary antibodies against AR (1:5000, Santa Cruz, sc-816), Hsp90 (1:5000, Santa Cruz, sc-7947), and GAPDH (1:40,000, abcam, ab8245). Immunoreactive proteins were detected by chemiluminescence. For filter trap analysis, lysates were diluted in RIPA buffer to 40 μg per sample and vacuumed through a slot-blot apparatus (Hybri-Slot Manifold, Bethesda Research Laboratories) onto 0.22 μm-pore cellulose acetate membranes preermined in 20% methanol and PBS. Membranes were then washed in PBS and probed for AR as described for Western blots. Densitometry was used to estimate the amount of monomeric (soluble) and aggregate AR in immunoblots and normalized to protein loading controls hsp90 in spinal cord and GAPDH in muscle. Estimates of AR (based on immunoblots and filter trap assays) were also normalized relative to Wt controls (no flutamide).

Knock-in (KI) Model

Beginning at 90 days of age, asymptomatic gonadally intact KI males and age-matched Wt control males received either time-release flutamide pellets or sham surgery (n = 9–10 mice/group) as described above. No T treatment was given since gonads were not removed from KI males. Flutamide pellets were replaced every 21 days until mice became moribund or died (or up to 300 days of age). Motor function was assessed prior to flutamide treatment at 90 days of age (designated as treatment day 0) and then assessed on treatment days 2, 4, 6, 8, 14 and weekly thereafter. Rotarod testing was not conducted.

Myogenic Model

To prevent perinatal death of myogenic Tg males, flutamide was administered prenatally via daily s.c. injections (5 mg flutamide in 100 μl of propylene glycol) to the dams from gestational day 15 to birth on day 21. While prenatal flutamide rescues Tg males from perinatal death, it does not prevent the later emergence of androgen-dependent disease (6, 19). Adult (122–139 days) gonadally intact Tg males and their age-matched Wt male controls were anesthetized and received flutamide pellets or sham surgery (17–19 animals per group), as described above for AR97Q mice, with treatment for this model lasting 38–42 days. Baseline motor behavior and body weight was collected 2 days prior to surgery, on Day 0 (just before surgery), days 1 and 3 of treatment, and then weekly thereafter up to 6 weeks (until day 42). Rotarod testing was not conducted for males in the myogenic model.

Statistical Methods

Results were analyzed using SPSS Statistics software. Control (no flutamide) AR97Q Tg males developed end-stage disease at markedly different times, resulting in different Ns at different time points during the treatment period, precluding the use of a repeated-measures analysis of variance (ANOVA). Hence, we ran independent t-tests to probe for significant differences between flutamide-treated and control-treated AR97Q Tg males. Tests were run only at select time points, to limit the chances of a Type I error, based on the means for the two Tg groups having nonoverlapping standard error bars. Nonetheless, we used a Bonferroni correction, the most stringent correction, to adjust the alpha level when more than a single t test was run on the same data set. A two-way ANOVA was used to evaluate the effects of genotype, treatment, and their interaction on the amount of monomeric protein in the Western blots while independent t-tests were used to assess significant differences in aggregate AR in Westerns and filter trap assays. Potential effects of flutamide in the myogenic model were assessed using a 2 × 2 × 10 (Genotype × Treatment × Day) three-way ANOVA with repeated measures on the third factor. Significant main effects and interactions were followed up with post hoc comparisons using a Bonferroni correction. For some variables, the data did not meet homogeneity of variance assumptions. For these measures, nonparametric statistics were also run to confirm significance found in the ANOVA. Statistical values presented are from the ANOVA but only those also found to be significant using nonparametric tests in those cases where homogeneity was violated. Values reported in the Results section are means ± standard error of the mean (SEM) based on n = the number of animals per group. Significant effects of flutamide on survival in the KI model were determined using the log rank test.

Results

AR97Q model

Flutamide only transiently prevents disease progression in gonadally intact Tg males. Expression of the SBMA disease phenotype in this model is highly androgen-dependent (4). Unlike the potent protective effects of prepuberal castration, flutamide treatment of prepuberal AR97Q males only transiently protected motor function based on the hang test (Figure 1), with significantly longer hang times than control-treated Tgs at 28, 35 and 42 days of treatment (P = .019, 0.005, 0.014, respectively; Figure 1a). However, once the Bonferroni correction was applied (alpha set at 0.017), significance of this effect at 28 days dropped below threshold. Moreover, by 49 days of treatment, flutamide exerted no beneficial effect on motor function. Surprisingly, flutamide had no significant effect on any other measure, including body weight (Figure 1b), grip strength (Figure 1c, d), number of rears in the open
field, stride length, and rotarod performance (data not shown).

**Flutamide increases circulating testosterone levels in gonadally intact males.** Given the rather mild beneficial effects of flutamide on motor function in AR97Q males during puberty, we examined the possibility that flutamide might drive circulating T levels up. As predicted (20–22), one week of flutamide exposure led to a significant five-fold increase in circulating T levels in adult gonadally intact Wt male mice (Table 1, \( P = .002 \)). Of greater significance, four weeks of flutamide treatment starting at P28 also induced a significant three-fold increase in circulating T levels in AR97Q Tg males (Table 1, \( P = .021 \)).

**Flutamide fully protects motor function in AR97Q Males when testosterone levels are controlled.** While the effect of flutamide on circulating T levels offers one possible explanation for why it might not have protected motor function in AR97Q Tg males, another possibility is that mutant AR disrupts motor function by a novel pathway that is inaccessible to flutamide (11). To directly test this possibility, we averted the confounding effects of flutamide on circulating T levels by testing flutamide in juvenile AR97Q Tg males that were castrated and given T. The T treatment alone triggered a progressive loss in motor function characteristic of gonadally intact Tg males, whereas flutamide treatment of such castrated T-treated males fully protected their motor function. Although flutamide had only subtle protective effects on body weight (Figure 2a), flutamide maintained the motor function of Tg males at Wt levels. Specifically, flutamide prevented the sharp decline in hang times exhibited by Tg males who received only T (Figure 2b), resulting in significantly longer hang times at 28 days of flutamide- compared to control-treated Tgs (\( P = .001 \), Bonferroni adjusted \( \alpha = 0.017 \)). Flutamide treatment also fully protected grip strength in AR97Q Tg males (Figure 2c, d), with force measures in flutamide-treated Tg males equivalent to Wt controls and significantly higher than control treated Tgs starting at 21 days of treatment (\( P = .001 \) and 0.0015 for forepaw and all paw, respectively; Bonferroni adjusted \( \alpha = 0.017 \)) with the one exception of forepaw grip at 28 days. Flutamide also fully maintained rotarod performance in AR97Q Tg males (Figure 2e), preventing the drop in performance exhibited by Tg controls at 35 days. Flutamide also prevented the marked decline in rearing behavior (Figure 2f), resulting in significantly more rears in flutamide-treated than control Tgs at both 28 and 35 days of treatment (\( P = .0115 \) and 0.0025, respectively; corrected \( \alpha = 0.025 \)). Other measures of motor activity in the open field (eg, total time spent moving) showed a similar sparing effect of flutamide (data not shown). Flutamide also apparently extended life span in this model since only control Tg males reached a moribund state, whereas those given flutamide remained healthy and active, with normal motor function. Importantly, circulating T levels did not differ between flutamide-treated and control-treated Tgs (Table 1) though they were somewhat higher in the Tg males compared to Wt males, likely due to differences in body size. We have consistently found that the same size T implant results in higher T levels in smaller animals (23–24). Regardless, flutamide potently protected the motor function of AR97Q males from the androgen-dependent toxic action of a polyQ-expanded AR.

**Flutamide affects soluble and aggregated AR in the AR97Q model.** We asked whether flutamide treatment affected the amount of monomeric or aggregated AR in T-treated castrated males, since decreases in either
might underlie the protective effect of flutamide in SBMA pathogenesis. We found that flutamide significantly reduces monomeric AR in the lumbar spinal cord of both Wt and Tg males (Figure 3a, c, \(P < 1 \times 10^{-2}\) compared to respective Wt and Tg controls). In the gastrocnemius muscle, however, flutamide did not significantly reduce monomeric AR in either Wt males (\(P = 1 \times 10^{-6}\) compared to control-treated Wts) or AR97Q Tg males (\(P = 2 \times 10^{-2}\), compared to control-treated Tgs; Figure 3b, e).

Because there was little or no evidence of AR aggregates in Wt tissue, we limited quantitative assessment of aggregate AR to Tg samples. Assessing the amount of high molecular weight AR in the stack of the Western blots indicates that flutamide modestly reduces the amount of aggregated 97Q-AR in gastrocnemius muscle but not in the spinal cords of Tg mice (Figure 3d, f; flutamide-treated vs. control-treated Tg muscle: \(P = 1 \times 10^{-2}\)). However, data based on filter trap assays suggest that flutamide has no effect on the abundance of AR aggregates in spinal cord (Figure 3g, h, \(P = 1 \times 10^{-2}\)) or muscle (Figure 3i, j, \(P = 3 \times 10^{-2}\)).

We also found that flutamide partially prevented a decrease in gastrocnemius weight caused by disease (flutamide-treated Tgs: 0.096 ± 0.016 g vs control-treated Tgs: 0.060 ± 0.003 g; \(P = 2 \times 10^{-2}\)) but had no effect on muscle weight in Wt males (flutamide-treated Wts: 0.144 ± 0.011 g vs control-treated Wts: 0.140 ± 0.002 g; \(P = 7 \times 10^{-2}\)). As expected, muscles from control-treated Wts weighed twice as much as those from diseased, control-
treated Tg males. We found no difference between treatment groups in circulating T levels (Table 1), although levels were consistently higher in this second experiment than in the first. The reason behind this difference is not clear since treatments began at the same age using the same method of T delivery for both experiments. Regardless, flutamide also fully protected the motor function of AR97Q Tg males in this second experiment (data not shown).

Knock-In (KI) Model

Flutamide treatment of KI males prolongs life-span. Treating 90-day-old gonadally intact KI males with flutamide extended their average life span compared to control-treated KI males (199.2 ± 21.7 vs 145.4 ± 15.8 days respectively, Figure 4). By 17 weeks of age, 50% of control-treated KIs had reached end-stage (died or become moribund), compared to only 20% of flutamide-treated KIs. It was a full two months later, at 26.5 weeks of age, before 50% of flutamide-treated KIs had reached end-stage. This benefit occurred despite the fact that flutamide increased circulating T levels in gonadally intact KI males (flutamide-treated KI males: 14.1 ± 1.62 nmol/l vs flutamide-treated aged-matched Wt males: 18.3 ± 4.55 nmol/l vs control-treated age-matched Wt males: 7.9 ± 4.01; mean ± SEM). Of note, untreated KI males have circulating T levels comparable to Wt males (25). That flutamide-treated KI males survived significantly longer than control-treated KIs was confirmed based on the log rank test (P = .049). KI males show only mild motor deficits that were, in our hands, not statistically significant (based on hang test, grip strength or open field; data not shown). KI males also did not show a decline in body weight over the course of disease as is characteristic of AR97Q mice (data not shown). Flutamide also did not affect any of these measures.

Myogenic Model

Flutamide treatment improves motor function of chronically impaired Tg males. We also tested the therapeutic potential of flutamide in the myogenic model. We find that flutamide significantly improved motor function of chronically diseased, gonadally intact adult Tg males (122–139 days of age). Note that treatment began when Tg males were fully symptomatic (Figure 5). Based on separate 3-way repeated measures ANOVA for each measure (hang times, grip strength, etc.), significant main effects and interactions between genotype, treatment and time were found, warranting further post hoc analyses. Separate two-way repeated measures ANOVAs were then run to determine whether flutamide significantly affected our measures within each genotype over days. Indeed, we found a significant main effect of flutamide on hang times (P = .001) and grip strength (all p values = .001) of Tg males but no effects on these measures in Wts. We also found that the effects of flutamide interacted significantly with days of treatment on all measures (all
p values = 0.001) in Tg males, warranting pair-wise comparisons between flutamide-treated and control-treated Tgs. Remarkably, flutamide significantly improved “all paw” grip strength within 24 hours (Figure 5a, \( P = 0.004 \)) with significant improvement in “all paw” grip strength emerging by 72 hours (Figure 5b, \( P = 0.001 \)). The beneficial effect of flutamide on hang times in Tg males emerged a few days later at one week of treatment with significant increases in hang times (Figure 5c, \( P = 0.015 \)). Finally, flutamide significantly increased the body weight of symptomatic Tg males starting at 14 days (Figure 5d, \( P = 0.035; \)). However, while flutamide clearly mitigated the effects of disease in myogenic Tg males, their motor function was not fully restored to normal.

**Discussion**

We examined the therapeutic potential of the AR antagonist flutamide in three genetically distinct male mouse models of SBMA: 1) the AR97Q Tg model broadly expresses a full length human AR with 97Qs, 2) the KI model expresses a targeted CAG expanded human allele in the first exon of the endogenous AR gene, and 3) the myogenic Tg model expresses Wt rat AR only in skeletal muscle fibers. Despite these differences, each model exhibits male-bias symptoms that are androgen-dependent (3–4, 6).

Given the apparent androgen-dependence of this disease, AR antagonists were expected to be therapeutic. However, previous reports indicate that the AR antagonist flutamide is neurotoxic in a fly model of SBMA (9–10), while being neither beneficial nor harmful in the AR97Q mouse model (11). Contrary to these findings, we now report that flutamide prevents androgen-dependent motor dysfunction in AR97Q males while reversing androgen-dependent motor dysfunction in myogenic males. Flutamide also greatly ameliorates androgen-dependent early death in KI males. Given that flutamide effectively protects against androgen-dependent AR toxicity in three different mouse models of SBMA, akin to its well established antagonist action on normal AR function (26–28), our data are proof-of-principle that flutamide has therapeutic potential for treating SBMA in humans.

Our results are not without caveats. While clearly showing that flutamide can antagonize the toxic action of an androgen-activated polyQ-AR, flutamide also stimulates an increase in circulating androgens in gonadally intact male mice, limiting the effectiveness of flutamide as a treatment for disease (Figure 1, Table 1). However, flutamide can potentially protect the motor function of castrated AR97Q Tg males that are treated with T to induce the disease (Figure 2. These results demonstrate that flutamide can in fact block the toxic action of a polyQ-expanded AR. While flutamide also combated androgen-dependent AR toxicity for
both KI and myogenic males (Figure 4 and 5), flutamide’s efficacy in these models was less complete, likely because KI and myogenic males were gonadally intact. Thus, the use of flutamide to treat SBMA in humans may require adjuvant treatment with other antiandrogens, such as leuprorelin, which limit T production. Results of a phase two clinical trial for leuprorelin while promising, indicate only modest beneficial effects overall on motor function (8) which could potentially be enhanced by adjuvant treatment with flutamide. Flutamide combined with leuprorelin is a standard treatment regime for advanced prostate cancer (29) and has been given to a patient with both SBMA and prostate cancer. While it was reported that the patient tolerated the treatment well for the four years observed, status of his SBMA symptoms were not reported.

There is some dispute over whether the myogenic model is a legitimate mouse model for SBMA since motor dysfunction in this model is triggered by Wt AR rather than polyQ-expanded AR (1). While this question is not readily answered, there are several compelling reasons to think that the myogenic model has clinical relevance to SBMA. First and foremost, the disease phenotype displayed by myogenic mice aligns well with that described for SBMA in humans and other mouse models of this disease (2–4, 6). Core features of the disease phenotype include a distinct male bias and androgen-dependent motor dysfunction, both of which myogenic mice show (6). Secondly, there is growing precedence linking both mutant and Wt alleles of the same gene to the same neurodegenerative disease. Notable recent examples include superoxide dismutase 1 linked to amyotrophic lateral sclerosis (ALS) (30) and α-synuclein linked to Parkinson’s disease (31). Wt AR has also been shown to exert comparable androgen-dependent toxicity as the polyQ-expanded AR in both mouse and fly models of SBMA and likely triggers toxicity through common molecular pathways (32–33). Finally, up to 17% of affected men diagnosed with SBMA show the expected cluster of symptoms [slowly progressing motor dysfunction coupled with signs of decreased androgen sensitivity (eg, gynaecomastia) and elevated serum creatine kinase (CK) levels] but nonetheless, lack the polyQ expansion in their AR gene (34–35). Thus, it is possible that dysregulation of AR expression per se causes SBMA in some individuals.

While KI males show only mild motor deficits which in this cohort of mice was not detected, we did detect the androgen-dependent early death reported for this model (3). Flutamide significantly ameliorated this early death, extending 50% survival

![Figure 4](image-url). Flutamide treatment beginning at 90 days of age significantly extends lifespan of adult knock-in (KI) males. Control-treated KI males reach 50% survival at 17 weeks of age, whereas 50% survival for flutamide-treated KI males is extended to 26.5 weeks, when only 10% of control-treated KIs remain alive. Average lifespan for flutamide-treated KIs was 199.2 ± 21.7 days compared to 145.4 ± 15.8 days for control-treated KIs (mean ± SEM, n = 10 KIs per group. Log-rank test indicates a significantly (P < .05) improved survival of SBMA KI males conferred by flutamide.

![Figure 5](image-url). Flutamide significantly improves the motor function of chronically diseased myogenic Tg males which overexpress Wt rat AR only in skeletal muscle fibers. The partial rescue of grip strength (A, B), hang times (C), and body weight (D) for diseased Tg males may reflect increased circulating testosterone caused by flutamide in gonadally intact males which could interfere with flutamide binding to AR. Plotted data represents the mean ± SEM for n = 17 – 19 animals per group. *P < .05 for flutamide-treated Tgs vs control-treated Tgs based on a 3-way ANOVA followed by pairwise comparisons.
by a full 9.5 weeks (Figure 4). Muscle dysfunction triggered by a polyQ-expanded AR likely underlies the early death of KI males (3). Striated perineal muscles, which normally express AR at a much higher level than other skeletal muscles (36–38), show a pronounced myotonic dystrophy-like phenotype, exhibiting hyperexcitability and spontaneous activity (3). This pathology causes the external urethral sphincter to be chronically contracted, preventing urination. Indeed, postmortem examination of KI males reveals engorged bladders and evidence of uremia (3). We too found engorged bladders for KI mice who became moribund. Presumably, flutamide prolongs the survival of KI males by combating the toxic action of polyQ-expanded AR in perineal muscles.

There are several possible nonexclusive mechanisms that could explain how flutamide protects motor function from androgen-induced AR toxicity. One possibility is that flutamide interferes with T’s ability to enhance AR half-life and/or to promote nuclear translocation. Both mechanisms have been implicated as critical factors in androgen-dependent AR toxicity (9, 39–40). Finding decreased levels of soluble 97Q-AR in spinal cord and muscle may mean that flutamide combats toxicity by reducing AR stability (Figure 3). However, given that these effects are rather subtle, it seems unlikely that this mechanism alone fully accounts for the protective effect of flutamide in these SBMA models. While it is also possible that flutamide improved motor function by preventing the formation of AR aggregates, supporting evidence is not strong. Flutamide decreased aggregates only in muscle and only based on high molecular bands in Westerns and not filter trap assays (Figure 3). Moreover, whether aggregates are mediators are toxicity has been widely challenged, for SBMA and other polyglutamine diseases (41–42). Since flutamide stimulates nuclear transport of both Wt and the polyQ-expanded AR (9, 43–44), the beneficial effect of flutamide on symptoms of SBMA probably does not involve blocking the ligand-induced nuclear transport of polyQ-expanded AR.

Flutamide might also modulate other ligand-activated mechanisms implicated in androgen-dependent AR toxicity, including its transcriptional activity and/or post-translational modifications. For example, recent data suggest that AR toxicity triggered by androgens requires not only nuclear localization (9, 39), but transcriptional activity (32). Therefore, flutamide may interfere with AR transcriptional activity, perhaps by blocking the binding of polyQ-AR to its target genes, as shown for Wt AR (45). Flutamide may also affect which regulatory elements polyQ-AR binds to which in turn can change the complement of recruited coregulators as demonstrated for Wt AR (46–47). Recent data identifies the AF2 domain of the polyQ-expanded AR as critically mediating toxicity in SBMA (32). This same region recruits corepressors when bound to flutamide (48). Thus, while it is possible that flutamide blocks AR binding to DNA altogether, it seems more likely that flutamide represses its transcriptional activity once bound to DNA, by favoring the recruitment of corepressors over coactivators.

The polyQ-expanded AR is also hyperacetylated compared to normal AR, both in the presence or absence of ligand (49). This hyperacetylation of polyQ-expanded AR has also been implicated in androgen-dependent AR toxicity, since overexpression of SIRT1 which deacetylates AR, reduces AR toxicity in cell models of SBMA (50). Hence, flutamide may also confer protection against disease in mice by deacetylating AR via SIRT1 (51), and/or by blocking the recruitment of secondary coactivators such as CBP and p300 which normally acetylate AR (52).

Interdomain interactions of AR, which are also androgen-dependent and contribute to receptor dimerization, ligand association, and AR stability (28, 53–54), have also been implicated in androgen-dependent AR toxicity (14). Flutamide has been shown to interfere with this interaction in Wt AR (45, 53). Thus, flutamide may be protective in SBMA models by interrupting this interdomain interaction. Whatever the molecular pathway(s) involved, the protective effect of flutamide on motor function probably does not involve rescuing motoneurons from death, since none of the three models show significant motoneuron death (4, 6, 16).

Conclusion
Finding that flutamide greatly ameliorates disease symptoms in three genetically distinct mouse models of SBMA indicates that flutamide may indeed have therapeutic value for men affected by SBMA, particularly if combined with treatments that lower androgen production, a standard treatment regime for prostate cancer (29). Comparisons across these three SBMA models likely offer valuable insight into the essential mechanisms underlying SBMA in humans, given that each express clinically relevant features of the disease, namely that males and not females develop an androgen-dependent disease phenotype of impaired motor function (7, 55–56). Understanding the mechanism(s) by which flutamide combats androgen-dependent AR toxicity will undoubtedly shed light on the mechanisms by which mutant AR causes motor dysfunction in SBMA. These data, along with other recent work in the field (14), strongly argue that AR antagonists, either alone, or in combination with agents that limit the pro-
duction of endogenous T, offer an avenue by which motor function of SBMA patients can be improved.

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