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Disease Models & Mechanisms 5, 141-145 (2012) doi:10.1242/dmm.007849

Testosterone treatment fails to accelerate disease in a transgenic mouse model of spinal and bulbar muscular atrophy

Erica S. Chevalier-Larsen[1] and Diane E. Merry[1,*]

SUMMARY

Evidence from multiple animal models demonstrates that testosterone plays a crucial role in the progression of symptoms in spinal and bulbar muscular atrophy (SBMA), a condition that results in neurodegeneration and muscle atrophy in affected men. Mice bearing a transgene encoding a human androgen receptor (AR) that contains a stretch of 112 glutamines (expanded polyglutamine tract; AR112Q mice) reproduce several aspects of the human disease. We treated transgenic male AR112Q mice with testosterone for 6 months. Surprisingly, testosterone treatment of AR112Q males did not exacerbate the disease. Although transgenic AR112Q males exhibited functional deficits when compared with non-transgenics, long-term testosterone treatment had no effect on motor function. Testosterone treatment also failed to affect cellular markers of disease, including inclusion formation (the accumulation of large nuclear aggregates of mutant AR protein) and levels of unphosphorylated neurofilament heavy chain. These data suggest that the mechanism of disease in SBMA saturates at close to endogenous hormone levels and that individuals with SBMA who take, or have taken, testosterone for its putative therapeutic properties are unlikely to suffer adverse effects.

INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA) is a slowly progressive adult onset neurodegenerative disease that affects men in mid-life. The disease causes degeneration and atrophy of the muscles of the proximal limb girdle and the bulbar muscles of the jaw (Kennedy et al., 1968), as well as the accumulation of nuclear aggregates of mutant androgen receptor (AR) protein, otherwise known as neuronal intranuclear inclusions (NIIs) (Li et al., 1998). SBMA results from a CAG expansion in the first exon of the *AR* gene, located on the proximal long arm of the X chromosome (La Spada et al., 1991). In the rare instances when female carriers manifest disease, their symptoms are mild (Ferlini et al., 1995). Initially, it was unclear whether female carriers were protected from disease symptoms owing solely to X-inactivation. However, the finding of homozygous females with very mild symptoms (Schmidt et al., 2002) and evidence from several cell and animal models of SBMA indicate that testosterone plays a crucial and causative role in disease pathogenesis. Cellular models of disease show an increase in nuclear inclusion formation upon hormone treatment (Stenoien et al., 1999; Walcott and Merry, 2002), whereas fly models demonstrate that consumption of testosterone induces disease as well as nuclear inclusion formation (Takeyama et al., 2002). Transgenic mouse models have indicated that both surgical and chemical castration of SBMA males alleviates or prevents disease progression, depending on the stage of disease at the time of intervention (Katsuno et al., 2002; Katsuno et al., 2003; Chevalier-Larsen et al., 2004). Furthermore, administration of testosterone to transgenic female SBMA mice exacerbates disease phenotype (Katsuno et al., 2002), as does giving testosterone to male SBMA mice treated with leuprorelin, a lutenizing hormone-releasing hormone (LHRH) analog (Katsuno et al., 2003).

For years, many patients with SBMA received testosterone treatment. The rationale for this treatment was complex, but a partial loss of AR function coupled with the known anabolic effects on muscle suggested that testosterone might prove beneficial (Sheffield-Moore, 2000). Despite the evidence indicating that testosterone contributes to the progression of SBMA, these patients have not reported any negative effects as a result of androgen administration (Goldenberg and Bradley, 1996; Neuschmid-Kaspar et al., 1996). However, one case study reported on the substantial decline of a patient with SBMA, with subsequent recovery upon cessation of testosterone treatment (Kinirons and Rouleau, 2008). However, the fact that this patient was taking paroxetine, a selective serotonin reuptake inhibitor (SSRI), might complicate the interpretation of this case. Paroxetine has been shown to enhance the conversion of dihydrotestosterone (DHT) to androstanediol (Griffin and Mellon, 1999), and thus there might have been relatively low relative levels of DHT within his spinal motor neurons. Treatment of this patient with testosterone might have restored his DHT levels and exacerbated disease. Whether the same would occur in SBMA patients not treated with paroxetine is unknown.

Previously, we created and characterized mice that were transgenic for the full-length expanded human *AR* gene, containing 112 CAG repeats, driven by the prion protein (PrP) promoter (AR112Q mice) (Chevalier-Larsen et al., 2004). The disease in this model progresses slowly, with AR112Q males beginning to exhibit mild motor dysfunction at 3 months of age and developing late-stage disease by 10 months of age (Chevalier-Larsen et al., 2004). Furthermore, lifespan is unaffected. In the current study, we treated

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Received 3 March 2011; Accepted 19 August 2011

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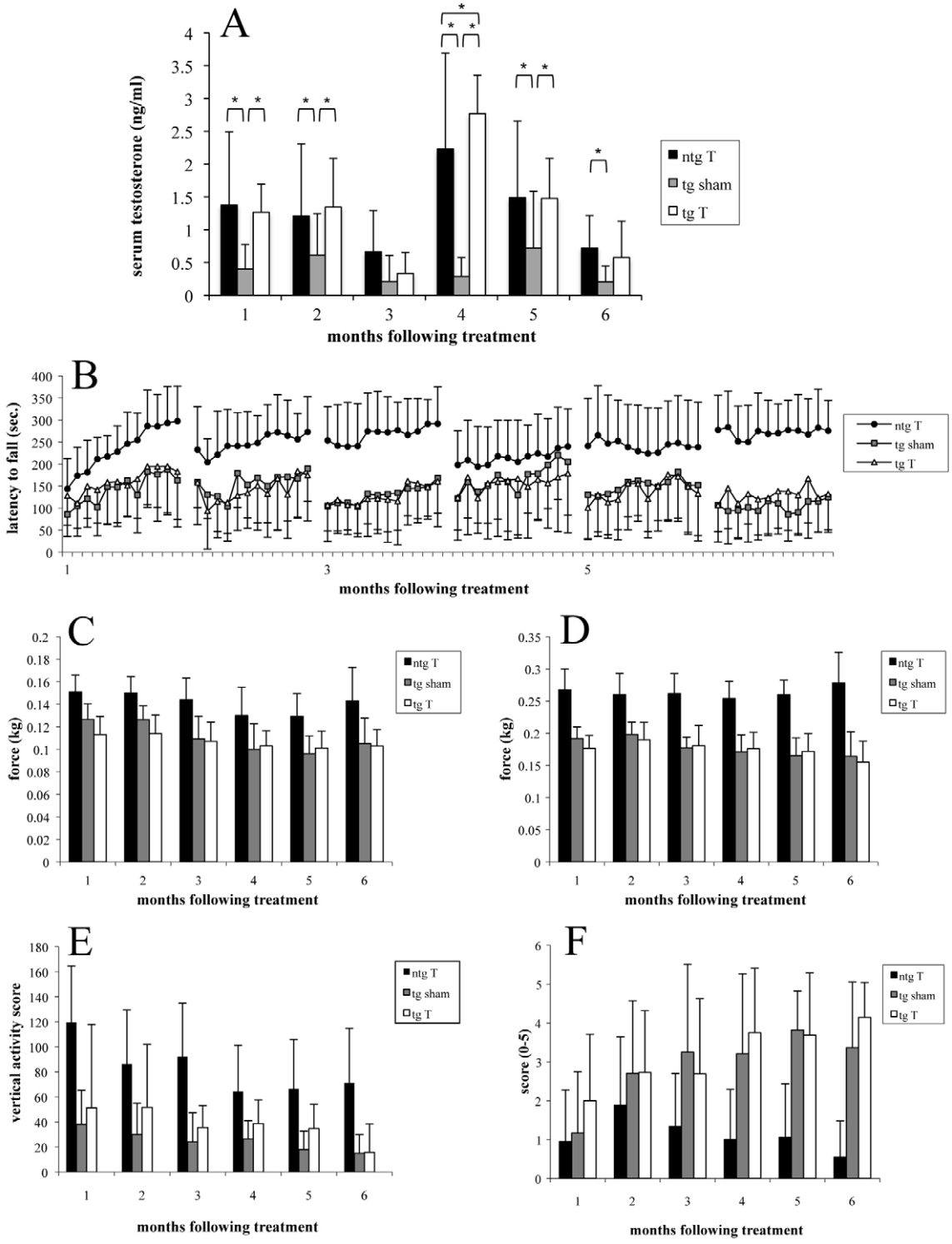

Fig. 1. Disease phenotype is unaffected by testosterone treatment. (A) Testosterone treatment resulted in elevated serum testosterone levels in transgenic (tg) T-treated (n=8) and non-transgenic (ntg) T-treated (n=10) animals when compared with tg sham-treated (n=6) mice (P<0.05), with the exception of months three and six. Although, testosterone levels dropped at the end of pellet life, testosterone levels were still significantly elevated in ntg T-treated mice in month six and there was a trend towards elevated testosterone in tg T-treated animals. (B) Ability to remain on a rotarod was impaired in AR112Q T-treated (n=10) and AR112Q sham-treated (n=12) males when compared with T-treated non-transgenics (n=14) (P<0.05), but rotarod performance was similar between T-treated AR112Q males and sham-treated AR112Q males (P>0.05). (C-E) Grip strength [using forepaws (C) or all paws (D)] and vertical activity (E) of all AR112Q males was reduced in comparison to T-treated non-transgenic males (P<0.05), but no significant decrease in any of these measures was observed upon T-treatment of AR112Q males (P>0.05). (F) Clasping behavior increased with age in all AR112Q males and was not affected by T-treatment; non-transgenic males rarely showed clasping behavior.

a cohort of these AR112Q male mice with testosterone, beginning at 2 months of age, for a period of 6 months. Behavioral and histopathological measures of disease indicate no change in severity or age of onset of disease with testosterone treatment (T-treatment). These data have important clinical implications for patients and suggest that there is a point at which the role of hormone in the pathogenic mechanism of SBMA becomes saturated.

RESULTS

Transgenic AR112Q and non-transgenic mice were implanted with timed-release pellets designed to deliver 4-6 ng/ml of testosterone for 90 days. Over the course of the experiment, implantation of testosterone pellets increased circulating testosterone an average of threefold, from 0.40±0.23 ng/ml (AR112Q sham-treated; n=10) to 1.26±0.48 ng/ml [non-transgenic (ntg) T-treated; n=8] and 1.17±0.35 ng/ml [transgenic (tg) T-treated; n=6]; this is a significant elevation of testosterone levels in treated animals (ntg T-treated and tg T-treated) over those implanted with placebo pellets (tg sham-treated) (P<0.001). Although 90-day testosterone pellets were used, we observed a decline in potency of the pellets by the end of the 90-day period (months three and six) (Fig. 1A). Implantation of new pellets at the end of 90 days restored circulating testosterone levels (Fig. 1A).

Although circulating testosterone was elevated in treated mice, motor function assays did not reveal any effect of testosterone treatment on phenotype. Motor function assays were performed monthly for 6 months; results were consistent for all 6 months of treatment. Beginning at 3 months of age (1 month following treatment), AR112Q males showed decreased rotarod performance, regardless of T-treatment, when compared with non-transgenic T-treated males (Fig. 1B). Decreased grip strength was apparent in all AR112Q animals, regardless of T-treatment, when animals gripped with either forepaws only (Fig. 1C) or all paws (Fig. 1D). As previously observed (Chevalier-Larsen et al., 2004), weakness was more pronounced when assessing all paws, implying greater weakness in the hindlimbs. Vertical activity, or rearing behavior, was also reduced in AR112Q males (Fig. 1E), although no deficits were seen in T-treated AR112Q males when compared with sham-treated AR112Q males. Clasping behavior was greater in both T-treated and sham-treated AR112Q males when compared with T-treated non-transgenic mice and increased steadily with time (Fig. 1F). T-treatment had no effect on weight or survival and no gross alterations in muscle fiber morphology were observed (data not shown).

Cellular markers of disease were also examined in spinal cord and brain tissue to determine whether T-treatment expedited cellular dysfunction that might not be detected by behavioral changes. As with the behavioral analyses, no changes were detected between the T-treated and sham-treated AR112Q males. NIIs were present in both T-treated and sham-treated males; NII size and frequency were similar between these two groups (Fig. 2A). A total of 49.6±6.5% of motor neurons from lumbar spinal cord contained NIIs in tg sham-treated animals, whereas NIIs were seen in 48.5±10.5% of motor neurons in tg T-treated mice (Fig. 2B). T-treated non-transgenic mice did not exhibit NIIs (Fig. 2A). Similarly, the level of unphosphorylated neurofilament heavy chain (NF-H) was reduced in the neuronal soma of all AR112Q animals when compared with non-transgenic mice (Fig. 2A), and the extent to which unphosphorylated NF-H immunoreactivity was reduced was similar between all AR112Q animals, regardless of T-treatment (Fig. 2C). In summary, signs of cellular pathogenesis were consistent with those observed during characterization of this disease model and were unchanged by long-term treatment with testosterone.

DISCUSSION

Although it is clear that testosterone plays a key role in SBMA pathogenesis, the mechanism leading to neurodegeneration remains unknown, although an increased understanding of the specific aspects of AR metabolism that contribute to its misfolding, aggregation and toxicity have come to light in recent years (Montie et al., 2009; Nedelsky et al., 2010; Orr et al., 2010). The data from this study suggest that, whatever the mechanism that leads to neuronal dysfunction, there is a threshold at which the effect of testosterone on this pathway plateaus. One possibility is that this

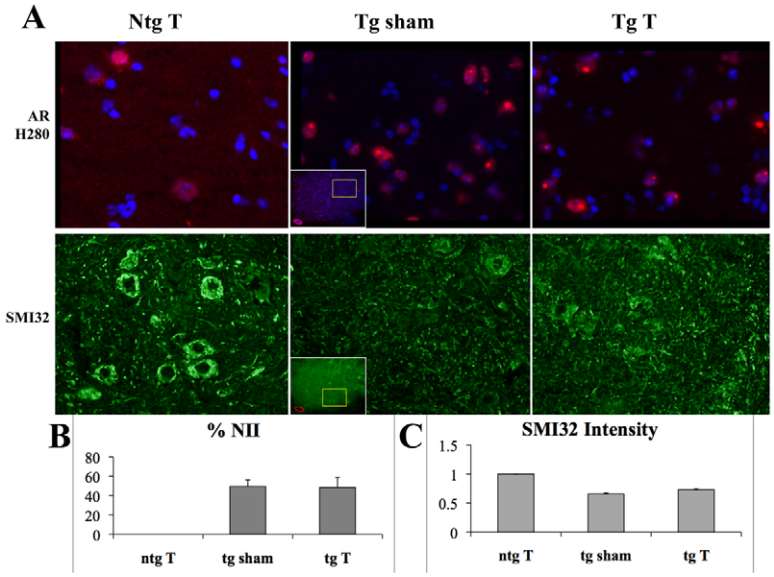

Fig. 2. Testosterone treatment does not alter cellular disease markers. NIIs were detected with AR-H280 antibody in spinal cord of all AR112Q males (A, top); no differences in NII frequency were detected upon T-treatment (B; n=2). Nuclei of non-transgenic (ntg) T-treated males show diffuse nuclear AR and no NIIs (A). Unphosphorylated NF-H, as detected by SMI32 immunostaining (A, bottom), was abundant in neuronal soma of the non-transgenic spinal cord but was reduced in AR112Q tissue. (C) Quantification of SMI32 intensity did not reveal any differences between transgenic (tg) T-treated and tg sham-treated males (n=2). Panels showing NIIs (A, top) and SMI32 (A, bottom) are not from the same fields. Insets in A represent the location within the anterior horn from which higher-magnification images were taken (yellow box). The red oval outlines the central canal, with the dorsal aspect to the left and the ventral aspect to the right.

threshold is due to saturation of AR with ligand. Our AR112Q mice overexpress human AR. It is therefore likely that testosterone would have a greater capacity to exert its pathogenic effects in AR112Q mice than in SBMA patients expressing normal levels of AR. Despite their increased capability to bind ligand, testosterone in excess of endogenous levels had little effect on phenotypic or cellular markers of disease progression.

Several previous studies have demonstrated that hormone withdrawal can ameliorate disease onset and/or progression (Katsuno et al., 2002; Katsuno et al., 2003; Chevalier-Larsen et al., 2004; Li et al., 2007). It is possible that the brief decline in elevated testosterone levels seen at the end of month three of these studies might alleviate any adverse effects of hormone treatment. This seems unlikely, however, because behavioral improvement due to androgen ablation was not observed in mice of a comparable age until 3 months following castration (Chevalier-Larsen et al., 2004) and elevated testosterone levels were restored in month four, following pellet re-implantation.

A caveat to the studies reported here is that our transgenic mice express the mutant AR primarily in the nervous system, through the use of the PrP promoter, and thus the evaluation of the mutant protein (and added testosterone) in muscle, which plays an important role in disease pathogenesis (Yu et al., 2006; Monks et al., 2007), is limited. However, expression of the PrP-promoter-driven transgene in muscle was observed, albeit at levels lower than those in neural tissue (Chevalier-Larsen et al., 2004), and nuclear inclusions in muscle were also noted (data not shown). Whether testosterone treatment would exacerbate disease through effects on muscle is unknown, although we find the possibility of different pathogenic processes in motor neurons and muscle unlikely.

Mutant AR protein might exert its toxic effects on neurons through multiple pathways, including disruption of protein folding homeostasis, interference with protein degradation, transcriptional dysregulation and/or by inhibition of axonal transport. It is possible that a threshold level of mutant AR present in the nucleus would lead to maximal disruption of neuronal function. In motor neurons, testosterone and DHT have been shown to downregulate AR expression (Vismara et al., 2009); thus, an excess of these ligands might actually moderate the prevalence of the mutant AR. It is likely that DHT, not testosterone, is the ligand responsible for neuronal pathology in SBMA, given the high motor neuron levels of the testosterone-to-DHT converting enzyme 5α-reductase (Pozzi et al., 2003). In this case, the conversion of testosterone to DHT via 5α-reductase could represent a point of saturation in the pathological pathway. This hypothesis is particularly attractive because it would suggest a mechanism for cell-type specificity in SBMA. However, manipulation of 5α-reductase activity with the inhibitor dutasteride failed to modulate disease in SBMA patients (Fernandez-Rhodes et al., 2011) or in a transgenic mouse model of SBMA (Maria Cho, Heather Montie, Mary Rosemiller, Yuhong Liu and D.E.M., unpublished results). Regardless of the molecular mechanism by which this saturation occurs, our data are encouraging in that SBMA patients who had previously taken supplemental testosterone are unlikely to have exacerbated their disease.

TRANSLATIONAL IMPACT

Clinical issue

Spinal and bulbar muscular atrophy (SBMA) is a neurodegenerative disorder that is caused by a polyglutamine expansion in the androgen receptor (AR) and affects approximately 1 in every 40,000 men. Affected men typically develop muscle weakness of the proximal limbs and bulbar muscle, tremors and fasciculations in mid-life. Muscle cramping can precede clinical symptoms. Signs of androgen insensitivity, such as gynecomastia and reduced fertility, often accompany muscle deficits. Female carriers of the disease are protected both by X-inactivation of the gene encoding the mutant AR and by lower levels of circulating AR ligands (testosterone and dihydrotestosterone). Prior to the discovery that disease pathogenesis is androgen-dependent, many individuals with SBMA received androgens therapeutically, based on the assumption at the time that a loss of AR function contributed to disease symptoms.

Results

This study examines the impact of exogenous testosterone on disease progression in a mouse model of SBMA to determine whether testosterone treatment of the patient population might in fact have exacerbated the disease. The data indicate that the addition of exogenous testosterone does not exacerbate disease progression in intact male mice that are transgenic for a mutant AR. Behavioral measures of disease in testosterone-treated transgenic males are indistinguishable from transgenic males that are sham treated. Additionally, cellular markers of disease in testosterone-treated transgenic males are comparable to those in sham-treated transgenic males.

Implications and future directions

These data suggest that it is unlikely that androgen therapy has hastened the progress of disease in SBMA patients. They also suggest that there is a threshold at which the contribution of testosterone to disease pathogenesis becomes saturated.

METHODS

Genotyping was performed as described previously (Chevalier-Larsen et al., 2004).

2-month-old male mice were anesthetized with inhaled isofluorane (3%). The area between the shoulder blades was shaved and the surgical area sterilized with alcohol. A small (1-2 cm) dorsal midline incision was made. A blunt probe was inserted into the incision to create a subcutaneous pocket. A pellet containing a 90-day-release 12.5 mg testosterone pellet or a placebo pellet (both from Innovative Research of America) was placed into the pocket and the incision was closed with a wound clip. Wound clips were removed 3 days later. Mice were re-implanted with hormone or sham pellet once during the course of this study. All animal surgeries were performed according to the guidelines of the Office of Laboratory Animal Welfare (OLAW) and the Institutional Animal Care and Use Committee (IACUC) at Thomas Jefferson University.

Blood samples were obtained monthly from each animal via retro-orbital eye bleed. Sera were assessed for circulating testosterone levels using a colorimetric EIA kit (Caymen Chemical) according to the manufacturer's instructions.

All behavioral assays were conducted monthly following pellet implantation. Testing was carried out as previously described (Chevalier-Larsen et al., 2004). An Ugo Basile (Italy) rotarod was used.

Immunofluorescence of brain and spinal cord tissue was carried out as previously described (Chevalier-Larsen et al., 2004). Antibodies used include AR H280 (1:100; Santa Cruz) and SMI32 (1:1000; Sternberger Monoclonal). Motor neurons of the lumbar spinal cord were scored as inclusion-positive whether they had

several punctate or single, large inclusions. Unphosphorylated neurofilament immunoreactivity was quantified using ImageJ software; integrated density values were acquired for regions of interest delineated around motor neuron soma of the lumbar spinal cord.

FUNDING

This work was supported by the National Institutes of Health [grant R01 NS32214] to D.E.M.

AUTHOR CONTRIBUTIONS

E.S.C.-L. and D.E.M. conceived and designed the experiments, analyzed the data, and wrote the manuscript. E.S.C.-L. performed the experiments.

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