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Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy

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Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motor neuron disease that affects males. It is caused by the expansion of a polyglutamine (polyQ) tract in androgen receptors. Female carriers are usually asymptomatic. No specific treatment has been established. Our transgenic mouse model carrying a full-length human androgen receptor with expanded polyQ has considerable gender-related motor impairment. This phenotype was abrogated by castration, which prevented nuclear translocation of mutant androgen receptors. We examined the effect of androgen-blockade drugs on our mouse model. Leuprorelin, a lutenizing hormone–releasing hormone (LHRH) agonist that reduces testosterone release from the testis, rescued motor dysfunction and nuclear accumulation of mutant androgen receptors in male transgenic mice. Moreover, leuprorelin treatment reversed the behavioral and histopathological phenotypes that were once caused by transient increases in serum testosterone. Flutamide, an androgen antagonist promoting nuclear translocation of androgen receptors, yielded no therapeutic effect. Leuprorelin thus seems to be a promising candidate for the treatment of SBMA.

SBMA, also known as Kennedy disease, is an adult-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations and bulbar involvement[1,2]. This disorder affects males; female carriers are usually asymptomatic[3,4,5]. No specific treatment for SBMA has been established.

The molecular basis of SBMA is the expansion of a trinucleotide CAG repeat encoding the polyQ tract in the first exon of the androgen receptor gene[6]. The CAG repeat within androgen receptor genes ranges in size from 5 to 33 repeats in normal subjects, but from 40 to 62 in patients with SBMA[7]. There is an inverse correlation between the CAG repeat size and the age at onset, or the disease severity adjusted by the age at examination in SBMA[8,9] as well as other polyQ diseases[10].

The cardinal pathological finding in SBMA is nuclear inclusions containing mutant and truncated androgen receptors with expanded polyQ tracts in the residual motor neurons of the brain stem and spinal cord[11] and some other visceral organs[12]. The presence of nuclear inclusions is also a pathological hallmark in most other polyQ diseases, and is considered to be relevant to pathophysiology[10]. Although the entire mechanism of polyQ-induced neuronal dysfunction and subsequent cell loss has not been clarified, nuclear localization of the mutant protein could be essential in the pathogenesis of polyQ diseases[13,14].

A transgenic mouse model was designed that expresses the full-length human androgen receptor containing 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken β-actin promoter[15]. Although no transgenic lines with 24 CAG repeats manifested any characteristic SBMA phenotypic traits, three of five lines with 97 CAG repeats (AR-97Q) showed progressive motor impairment, which was notably pronounced and accelerated in male AR-97Q mice, but was not noted or was far less severe in female AR-97Q mice. Thus, this model recapitulated not only the neurologic disorder, but also the phenotypic difference with gender that is a feature specific to SBMA. Nuclear localization of mutant androgen receptors was considerable in the male transgenic mice but not in the females, in agreement with the gender-related phenotypic expression. Castrated male AR-97Q mice showed considerable improvement in symptoms, pathological findings and nuclear localization of the mutant androgen receptors, whereas testosterone caused notable exacerbation in female AR-97Q mice, indicating that large amounts of serum testosterone were essential for the phenotypic expression of SBMA, and that testosterone deprivation conferred therapeutic effects in this disease.

Here we examined the effect of two androgen-blockade drugs used in the treatment of prostate cancer. Although flutamide had no effect on the phenotypic expression of SBMA, leuprorelin reversed both the symptomatic and histopathological phenotypes. Our results indicate that ligand-dependent nuclear translocation of mutant androgen receptors is the main source of the pathogenesis of SBMA, and that leuprorelin suppresses this translocation.

RESULTS
Leuprorelin rescues the phenotypic expression of SBMA
Leuprorelin-treated male AR-97Q mice showed considerable amelioration of symptoms, pathological findings and nuclear localization of
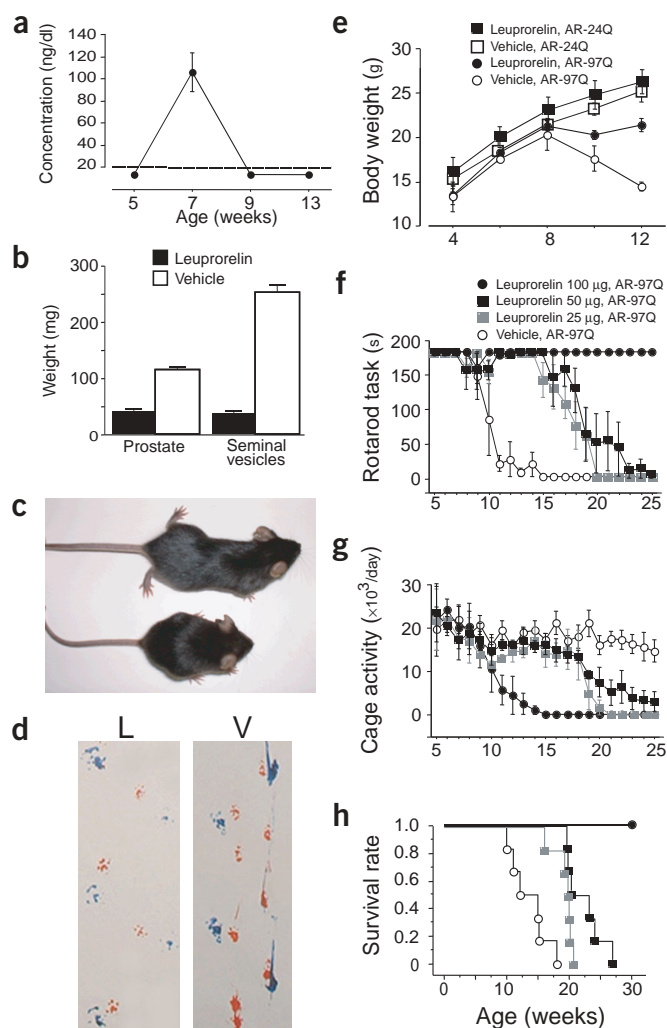
Figure 1 Effects of leuprorelin on symptoms in male AR-97Q mice.
(**a**) Serum testosterone in AR-97Q mice ($n = 6$). (**b**) Weights of prostates and seminal vesicles of 13-week-old #4-6 mice. (**c**) The leuprorelin-treated AR-97Q male mouse (top) did not show the muscular atrophy seen in the vehicle-treated male mouse (bottom; 12-week-old #4-6 mice).
(**d**) Footprints of 12-week-old leuprorelin-treated (L) and vehicle-treated (V) male AR-97Q #4-6 mice . Red, front paw; blue, hind paws. (**e**) Effect of leuprorelin on body weight in AR-24Q #5-5 and AR-97Q #7-8 mice.
(**f**–**h**) Rotarod task (**f**), cage activity (**g**) and survival rates (**h**) of AR-97Q #7-8 mice. Key applies to **f**–**h**.

the transgene protein compared with vehicle-treated AR-97Q mice. Leuprorelin initially increased serum testosterone by exerting agonist effects at the LHRH receptor, but subsequently reduced it to undetectable amounts (**Fig. 1a**). The androgen blockade was also confirmed by the decreased weights of the prostate and seminal vesicles ($P < 0.001$ for both; **Fig. 1b**). This reduction was indistinguishable from that in castrated mice (data not shown). Leuprorelin caused infertility in both male AR-97Q mice and normal littermates at a dose of 100 µg, although the mice were fertile at doses of 25 or 50 µg leuprorelin. The leuprorelin-treated AR-97Q mice showed notable amelioration of muscle atrophy and reduced body size (**Fig. 1c**). By footprint analysis, the vehicle-treated AR-97Q mice had motor weakness and dragged their hind legs, but these symptoms were substantially attenuated by leuprorelin treatment (**Fig. 1d**). Leuprorelin treatment profoundly suppressed progressive emaciation, which was evident in the vehicle-

treated mice (**Fig. 1e**). Although it is known to increase body mass in human subjects, leuprorelin did not induce significant obesity in male AR-24Q mice (**Fig. 1e**). The leuprorelin-treated male mice had significantly ($P < 0.0001$) less or almost no motor impairment, as assessed by rotarod task and cage activity (**Fig. 1f,g**). Leuprorelin also significantly ($P=0.0005$) prolonged life (**Fig. 1h**). Although the effect on fertility could be abrogated by dose reduction, the therapeutic effects of leuprorelin were insufficient at a lower dose (**Fig. 1f–h**).

By western blot analysis of total tissue homogenate or nuclear fraction, leuprorelin-treated male AR-97Q mice had many fewer mutant androgen receptors smearing from the top of the gel than did vehicle-treated male mice (**Fig. 2a,b**). This indicated that leuprorelin successfully reduced insoluble nuclear androgen receptor fragments. The leuprorelin-treated mice had much less diffuse nuclear staining and fewer nuclear inclusions detected with the 1C2 antibody to polyQ (**Fig. 2c**). Muscle histology showed considerable amelioration of neurogenic muscle atrophy, such as grouped atrophy and small angulated fibers, with leuprorelin treatment (**Fig. 2c**).

Testosterone given to mice from 13 weeks of age substantially aggravated the neurological symptoms (**Fig. 3a,b**) and pathological findings by immunohistochemistry with 1C2 (**Fig. 3c**) of leuprorelin-treated AR-97Q mice.

Leuprorelin reverses symptomatic and pathological phenotypes

Leuprorelin-treated AR-97Q mice showed a decrease in body weight and deterioration in the rotarod task at an age of 8–9 weeks (**Figs. 1e,f** and **3a**), when serum testosterone initially increased through the agonistic effect of leuprorelin (**Fig. 1a**). This change was transient and was followed by sustained amelioration along with consequent suppression of testosterone production. Footprint analysis also showed temporary exacerbation of motor impairment (**Fig. 4a**). Immunostaining of tail specimens obtained from the same mouse showed an increase in the number of the muscle fibers at 4 weeks of leuprorelin administration by nuclear 1C2 staining, although this staining was diminished by another 4 weeks of treatment. Vehicle-treated AR-97Q mice showed robust deterioration of nuclear 1C2 staining (**Fig. 4b,c**).

Flutamide does not suppress SBMA phenotype

In contrast, flutamide treatment did not ameliorate the symptoms, pathological features or nuclear localization of the mutant androgen receptors in male transgenic mice. Flutamide significantly ($P < 0.0001$) decreased the weights of the prostate and seminal vesicles (**Fig. 5a**). There was no significant difference in the androgen-blockade effects of leuprorelin and flutamide. Flutamide treatment of male AR-97Q mice did not ameliorate muscle atrophy or body size reduction (**Fig. 5b**). By footprint analysis, both flutamide-treated and vehicle-treated mice showed motor weakness and dragged their hind legs (**Fig. 5c**). Both flutamide-treated and vehicle-treated male AR-97Q mice showed progressive emaciation (**Fig. 5d**). Flutamide had no effect on the rotarod task, cage activity or life span (**Fig. 5e–g**).

Western blot analysis showed mutant androgen receptors smearing from the top of the gel in whole-tissue homogenates of both flutamide-treated and vehicle-treated mice (**Fig. 6a**). These mutant androgen receptors localized in the nuclear fraction (**Fig. 6b**). Flutamide-treated mice showed no diminution in diffuse nuclear staining or nuclear inclusions (**Fig. 6c**).

DISCUSSION

Our study shows that leuprorelin rescues symptomatic and pathological phenotypes in our transgenic mouse model of SBMA. Leuprorelin prevents testicular testosterone production by downregulating LHRH
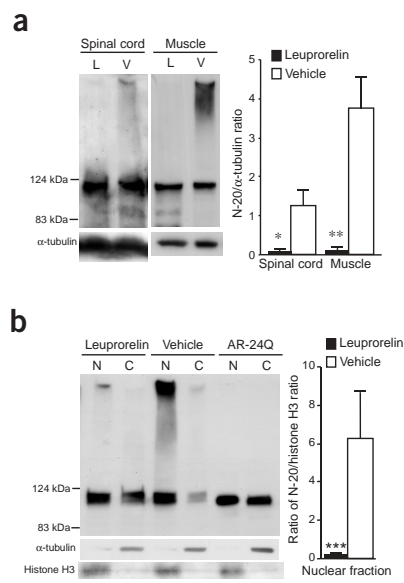
Figure 2 Effects of leuprorelin on mutant androgen receptor expression and neuropathology in male AR-97Q mice. (**a**) Western blot analysis with an antibody to androgen receptor (N-20) of total homogenates of the spinal cords and muscles of 13-week-old leuprorelin-treated (L) and vehicle-treated (V) male AR-97Q #7-8 mice. *, $P = 0.011$; **, $P = 0.015$. Left margin, molecular sizes. Bottom, α-tubulin (control blot). (**b**) Western blot analysis with N-20 of nuclear (N) and cytoplasmic (C) fractions of muscles of the male mice (13 weeks old; #7-8) given leuprorelin (L) or vehicle (V) and a transgenic mouse (13 weeks old; #5-5) with androgen receptors with 24 CAG repeats (AR-24Q). Smearing of mutant androgen receptors was present in the nuclear fraction lanes. ***, $P < 0.0001$. Left margin, molecular sizes. Bottom, accuracy of fractionation confirmed with a nuclear marker (histone H3) and a cytoplasmic marker (α-tubulin). (**c**) Immunohistochemical study with 1C2, showing diffuse nuclear staining and nuclear inclusions in the spinal anterior horns and muscles of 13-week-old male leuprorelin-treated and vehicle-treated AR-97Q #7-8 mice and H&E staining of the muscle of vehicle-treated and leuprorelin-treated male mice.

receptors in the pituitary, and has been used extensively for medical castration in the therapy of prostate cancer, based on the androgen sensitivity of the tumor[16]. Its safety and tolerability have been widely approved, although it has possible side effects, including decreased libido, impotence, hot flushes, osteoporosis and fatigue. Here, fertility was decreased in leoprorelin-treated mice, and this effect was nullified by dose reduction. Leuprorelin decreases plasma testosterone by 95% or more[17]. Blockade of testosterone production was apparent in our transgenic mouse model and, furthermore, testosterone exacerbated phenotypic expression in leuprorelin-treated male transgenic mice. These findings indicated that suppression of testosterone was responsible for the therapeutic effect of leuprorelin. Ligand-dependent nuclear translocation of androgen receptor may be involved in the pathogenesis of SBMA[15]; here, leuprorelin seemed to prevent mutant androgen receptor translocation and suppressed its nuclear accumulation and subsequent neuronal dysfunction. Indeed, both western blot analysis and immunostaining with 1C2 antibody showed that leuprorelin substantially reduced nuclear accumulation of mutant androgen receptors. Leuprorelin could be a promising therapeutic agent for SBMA, given its minimal invasiveness and established safety. In clinical trials, however, the patient's desire for fertility should be taken into account, and the appropriate clinical dose should be carefully determined with reference to our dose-response study.

As leuprorelin initially acts as an LHRH agonist, serum testosterone temporarily increased after 2 weeks of treatment in our transgenic mice. As expected from this initial testosterone surge, nuclear staining with antibody to polyQ and motor dysfunction of the mice deteriorated in the early stage of leuprorelin therapy. Nevertheless, serial observations of tail specimens showed that nuclear accumulation of mutant androgen receptors was transient and was actually reversed by sustained leuprorelin treatment. Behavioral tests also demonstrated

immediate recovery of motor function after initial deterioration, and long-term stabilization of neurologic function was achieved by leuprorelin treatment. The reversibility of polyQ pathogenesis has also been shown by turning off gene expression in an inducible mouse model of Huntington disease[18]. Our results, however, indicated that preventing nuclear translocation of mutant androgen receptors was

Figure 3 Effects of testosterone in leuprorelin-treated male AR-97Q mice. (**a**,**b**) Rotarod task (**a**) and cage activity (**b**) of male AR-97Q #4-6 mice treated with leuprorelin ($n = 6$) or leuprorelin plus testosterone ($n = 6$; time of testosterone administration indicated by bar in graph). (**c**) Immunostaining with 1C2, showing diffuse nuclear staining and nuclear inclusions in the spinal anterior horns and muscles of 17-week-old leuprorelin-treated and testosterone-treated AR-97Q male #4-6 mice.
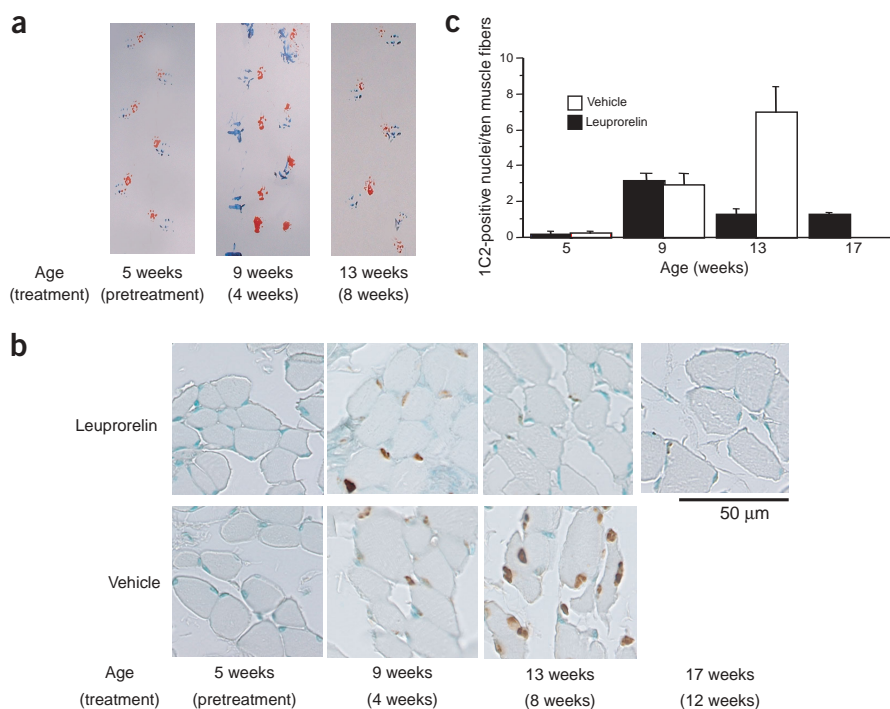
a

Age
(treatment) | 5 weeks
(pretreatment) | 9 weeks
(4 weeks) | 13 weeks
(8 weeks)

c
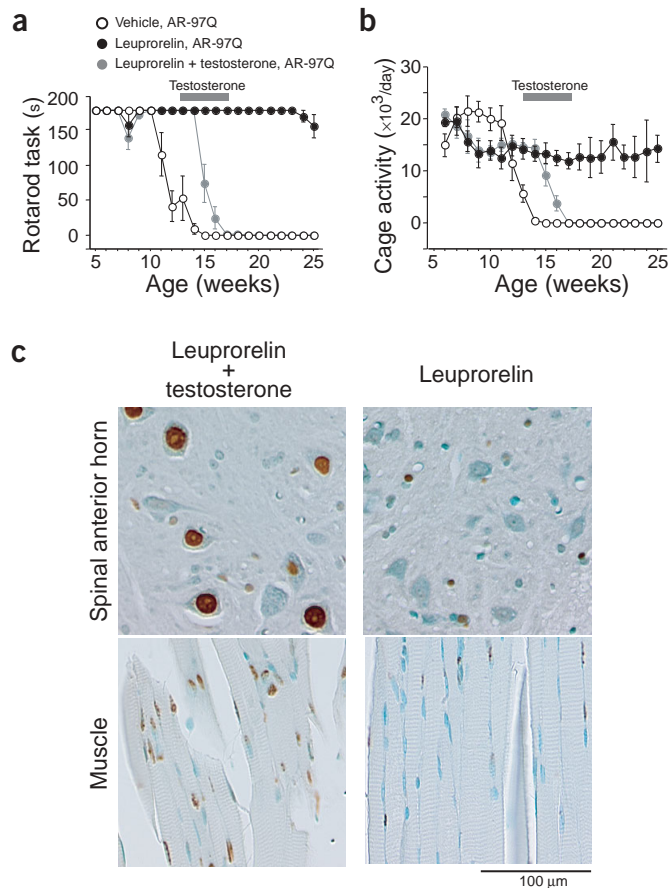
Figure 4 Reversal of symptoms and pathological findings with leuprorelin treatment. (**a**) Serial footprints of a leuprorelin-treated male AR-97Q #2-6 mouse. (**b**) 1C2 nuclear staining of tail muscles. Left to right, serial sections of same #4-6 mice treated with leuprorelin (upper panels) or vehicle (lower panels) (**c**) Quantification of 1C2 nuclear staining of the tail muscle ($n = 5$ for each group).

b

Leuprorelin

Vehicle

50 µm

Age
(treatment) | 5 weeks
(pretreatment) | 9 weeks
(4 weeks) | 13 weeks
(8 weeks) | 17 weeks
(12 weeks)

enough to reverse both symptomatic and pathological phenotypes in our transgenic mice. As the pathophysiology of AR-97Q mice is neuronal dysfunction without neuronal cell loss[15], our results indicated that polyQ pathogenesis was reversible at least in its dysfunctional stage. We need to determine the early dysfunctional period in human polyQ diseases.

In contrast to the notable success of leuprorelin therapy, flutamide produced no beneficial effects despite its sufficient antiandrogen effects. Flutamide, the first androgen antagonist discovered, has very specific affinity for androgen receptors, and competes with testosterone for binding to the receptor. It has been used to treat prostate cancer, usually in association with an LHRH agonist, to block the action of adrenal testosterone[17,19]. Although flutamide suppresses the androgen-dependent transactivation, it does not reduce plasma testosterone. Furthermore, flutamide does not inhibit, but may even facilitate, the nuclear translocation of androgen receptors[20,21]. Flutamide also promoted nuclear translocation of mutant androgen receptors containing expanded polyQ in cell and *Drosophila* models of SBMA[22,23]. This may be the reason flutamide produced no therapeutic effect in our transgenic mouse model of SBMA. Flutamide is not likely to be a therapeutic agent for SBMA.

As in other polyQ diseases, a toxic gain of mutant androgen receptor function has been considered the main cause of the pathogenesis of SBMA[24]. Although the expansion of polyQ tract inhibits the transcriptional activities of androgen receptors and promotes androgen receptor degradation[25], motor impairment has not been noted in patients with severe testicular feminization lacking androgen receptor
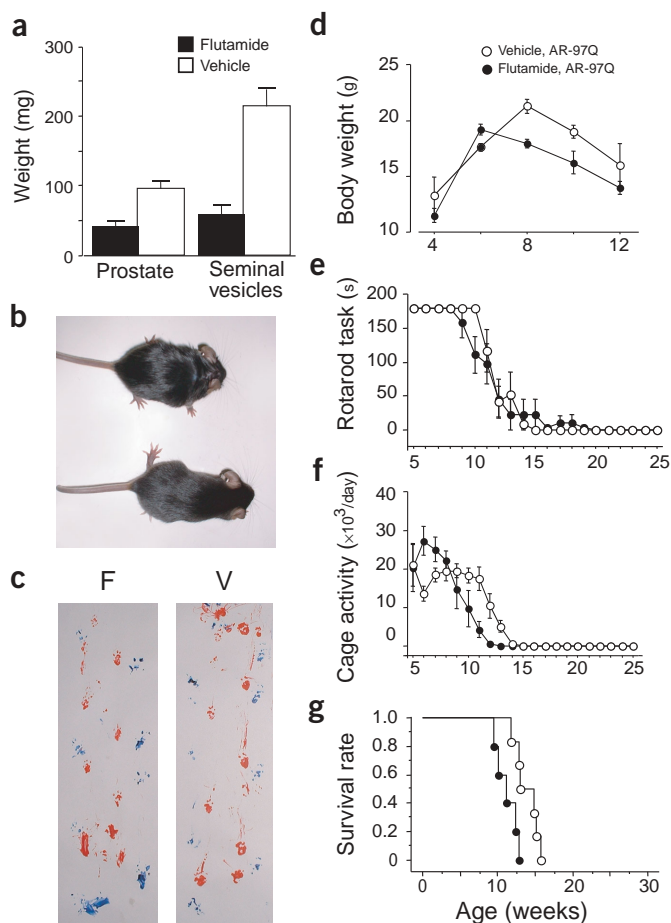
function[26]. Thus, the neurologic impairment in SBMA cannot be attributed to the loss of androgen receptor function[27]. SBMA has been considered an X-linked disease, whereas other polyQ diseases show autosomal dominant inheritance. If the toxic gain of mutant androgen receptor function is the main pathogenic process in SBMA, symptoms should be manifested in female patients, as in other polyQ diseases. Female patients, however, rarely have clinically characteristic phenotypes , even if they are homozygous[3–5]. Thus, SBMA symptoms are manifested only in the presence of large amounts of serum testosterone, as in male patients. This hypothesis is supported by the finding that female transgenic mice showed subtle phenotypic expression that was amplified by testosterone administration[15]. Taken together, the ligand effect,

Figure 5 Effects of flutamide on the symptoms of male AR-97Q mice. (**a**) Weights of prostates and seminal vesicles of 12-week-old #4-6 mice. (**b**) Flutamide-treated (top) and vehicle-treated male mice (11-week-old #4-6 mice). (**c**) Footprints of 11-week-old flutamide-treated (F) and vehicle-treated (bottom) male AR-97Q #4-6 mice . Red, front paws; blue, hind paws. (**d–g**) Body weights, rotarod tasks, cage activity and survival rates of flutamide-treated ($n = 6$) and vehicle-treated ($n = 6$) male AR-97Q #4-6 mice.
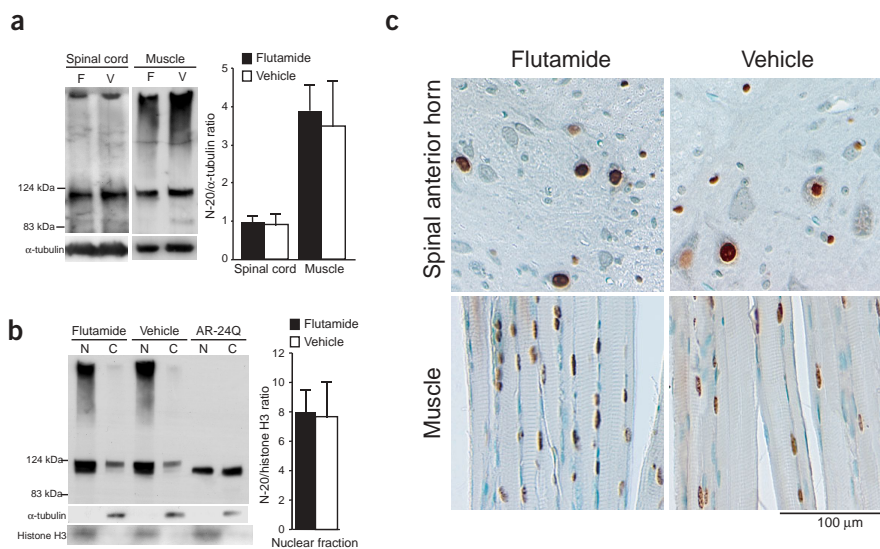
a

b

c

F   V

d

e

f

g

Figure 6 Effects of flutamide on mutant androgen receptor expression and neuropathology in male AR-97Q mice. (**a**) Western blot analysis with an antibody to androgen receptor (N-20) of total homogenates from the spinal cords and muscles of 13-week-old flutamide-treated (F) and vehicle-treated (V) male AR-97Q #7-8 mice . Left margin, molecular sizes. Bottom, α-tubulin (control blot). (**b**) Western blot analysis with N-20 of nuclear (N) and cytoplasmic (C) fractions from the muscles of 13-week-old #7-8 male mice given flutamide (F) or vehicle (V), and a 13-week-old transgenic mouse with androgen receptors with 24 CAG repeats (AR-24Q; #5-5). Left margin, molecular sizes. Bottom, accuracy of fractionation confirmed with a nuclear marker (histone H3) and a cytoplasmic marker (α-tubulin). (**c**) Immunostaining with 1C2, showing diffuse nuclear staining and nuclear inclusions in the spinal anterior horns and muscles of flutamide-treated and vehicle-treated mice (12-week-old #4-6 mice).

rather than protein expression of mutant androgen receptors is important in SBMA pathogenesis and provides a theoretical basis for the treatment effects.

Although there have been no notably effective therapeutic approaches to polyQ diseases, some promising results have been reported using transgenic animal models. Molecular chaperones, which renature misfolded mutant proteins, have exerted beneficial effects in cell and animal models of polyQ diseases. Heat-shock protein (HSP) 70 and HSP40 yielded preventive effects in a SBMA cell model[28]. Overexpression of HSP70 had preventive effects in our transgenic mouse model of SMBA[29] as well as in SCA1 cell and transgenic mouse models[30,31]. Increasing the expression of or enhancing the function of molecular chaperones could also be a potential therapy for SBMA. Alternatively, histone deacetylase inhibitor suppresses polyQ toxicity in cell and *Drosophila* models[32,33], but its effect is not sufficient in a mouse model of Huntington disease[34]. An ideal treatment for polyQ diseases could be a combination of these and other therapeutic strategies. Our study has indicated that hormonal therapy with LHRH agonist, such as leuprorelin, could be central to SBMA therapy.

METHODS

Generation and maintenance of transgenic mice. We generated AR-24Q and AR-97Q mice as described before[15]. We subcloned a full-length human androgen receptor fragment containing 24 or 97 CAG repeats[35] into a pCAGGS vector[36] digested with *Hin*dIII, microinjected the result into fertilized eggs of BDF1 mice and obtained five founders with AR-97Q. We maintained these mouse lines by back-crossing with C57Bl/6J. We examined all the symptomatic lines (#2-6, #4-6, #7-8). All animal experiments were approved by the Animal Care Committee of Nagoya University Graduate School of Medicine.

Neurological and behavioral testing. We analyzed the rotarod task in mice using an Economex Rotarod (Colombus Instruments) and measured cage activity with the AB system (Neuroscience, Tokyo, Japan) as described before[15,37].

Hormonal intervention and serum testosterone assay. We injected leuprorelin acetate (provided by Takeda Pharmaceutical) subcutaneously at a dose of 25, 50 or 100 μg per mouse every 2 weeks from 5 weeks of age. We administered leuprorelin at a dose of 100 μg unless otherwise indicated. We injected control AR-97Q male mice with a vehicle containing D-mannitol, carmellose sodium and polysolvate 80. We gave leuprorelin-treated AR-97Q mice either leuprorelin only or leuprorelin plus 20 μg testosterone enanthate dissolved in sesame oil (injected subcutaneously) weekly from the age of 13 weeks. We dis-

solved flutamide (Sigma-Aldrich) in 10% ethanol and 90% sesame oil, and gave it at a dose of 1.8 mg per mouse once every second day[38]. We injected the control AR-97Q male mice with vehicle. We assayed serum testosterone with the Coat-A-Count Total Testosterone radioimmunoassay (Diagnostic Products Corporation).

Protein expression analysis. We exsanguinated mice anesthetized by ketamine-xylazine, and 'snap-froze' their tissues with powdered CO_2 in acetone. We homogenized the tissues (2,500g for 15 min at 4 °C) in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS and 1 mM 2-mercaptoethanol with 1 mM PMSF and 6 μg/ml aprotinine. We loaded each lane of a 5–20% SDS-PAGE gel with 160 μg protein for nerve tissue and 80 μg protein for muscle (both from the supernatant fraction). This was transferred to Hybond-P membranes (Amersham Pharmacia Biotech) in a transfer buffer of 25 mM Tris, 192 mM glycine, 0.1% SDS and 10% methanol. After immuno-probing with N-20, a rabbit antibody to the androgen receptor (1:1,000 dilution; Santa Cruz Biotechnology), we did secondary antibody probing and detection with the ECL Plus kit (Amersham Pharmacia Biotech). We quantified the signal intensity of the bands smearing from the top of the gel using the NIH Image program (NIH Image version 1.62). The quantitative data of three independent western blots were expressed as mean ± s.d. We reprobed membranes with antibody to α-tubulin (1:5,000 dilution; Santa Cruz Biotechnology). We extracted nuclear and cytoplasmic fractions with NE-PER nuclear and cytoplasmic extraction reagents (Pierce). We reprobed membranes of fractionated western blot with antibody to α-tubulin (1:5,000 dilution and antibody to histone H3 (1:400 dilution; Upstate Biotechnology).

Immunohistochemistry and muscle histology. We perfused 20 ml of a 4% paraformaldehyde fixative in phosphate buffer (pH 7.4) through the left cardiac ventricles of mice deeply anesthetized with ketamine-xylazine, postfixed the tissues overnight in 10% phosphate-buffered formalin, and processed them for paraffin embedding. Then we deparaffinized tissue sections 4 μm in thickness, dehydrated them with alcohol, treated them with formic acid for 5 min at room temperature and stained them with 1C2 (1:10,000 dilution; Chemicon), as described before[15,37,39]. After formalin fixation, we washed tail specimens with 70% ethanol and decalcified them with 7% formic acid and 70% ethanol for 7 d followed by paraffin embedding. To assess 1C2-positive cells in muscle, we calculated the number of 1C2-positive cells in more than 500 fibers in the entire area and expressed the results as the number per 100 muscle fibers. We air-dried cryostat sections (6 μm in thickness) of gastrocnemius muscles and stained them with H&E.

Statistical analysis. We analyzed data using the unpaired *t*-test and considered *P* values ≤0.05 to be statistically significant.

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