neurons was assessed on consecutive sections (Fig. 3A, B). *DCTN1* and *EGR3* downregulations were both marked and independent of the degree of cytoplasmic pNF-H accumulation (Fig. 3B), implying that these genes were widely downregulated even in motor neurons with little or no pNF-H accumulation. By contrast, levels of *ACATN*, *DR5*, and CCNC ranged from just above the control levels to much higher levels and were well correlated with the degree of cytoplasmic pNF-H accumulation ($r = 0.48–0.60$, $p < 0.001$ to 0.0001, Fig. 3B).

These observations indicate that downregulation of *DCTN1* and *EGR3* occurs before the appearance of the neurodegeneration marker pNF-H and thus is a relatively early event in the neurodegeneration process. In contrast, the changes in *ACATN* and *DR5* expression were milder than those in *DCTN1* and *EGR3*, but proportional to pNF-H accumulation, suggesting that their upregulation is a relatively late event, occurring after the appearance of the neurodegeneration marker pNF-H.

Gene Expression Changes Occur Before Appearance of Motoneuronal Cytoplasmic Accumulation of Ubiquitylated Proteins: *DCTN1*, *EGR3*, *ACATN* and *DR5* Are Changed Even in the Motor Neurons Without Ubiquitylated Protein Accumulation

The correlation of gene expression with cytoplasmic accumulation of ubiquitylated protein in individual motor neurons was assessed on consecutive sections (Fig. 4A, B). In patients with ALS, *DCTN1* and *EGR3* were markedly downregulated, and *ACATN* and *DR5* were upregulated in motor neurons both with and without cytoplasmic accumulation of ubiquitylated proteins (Fig. 4B). However, the degree of downregulation or upregulation was significantly greater in the motor neurons with ubiquitylated protein accumulation compared with those without (Fig. 4B), suggesting that cytoplasmic accumulation of ubiquitylated proteins may be partially correlated to expression changes of *DCTN1*, *EGR3*, and *ACATN*. However, because the expression of these genes changed markedly even in the motor neurons without ubiquitylated proteins, it would imply that cytoplasmic ubiquitylated protein accumulation is a rather late event in the process of motor neuron degeneration.

We further examined the correlation between these 4 gene expression levels and subgroups of motor neurons with 3 different types of ubiquitylated protein accumulation (i.e. dot-like accumulations, skein-like accumulations, and large round inclusions of ubiquitylated proteins), and found that the expression levels of all 4 genes were changed before all types of ubiquitylated protein accumulation (data not shown).

DISCUSSION

We demonstrated that *DCTN1*, *EGR3*, *ACATN*, *CCNC*, and *DR5* are differentially expressed in the residual motor neurons in sporadic ALS. Furthermore, the expression levels of these genes are differentially correlated with the levels of pathologic markers, the numbers of residual motor neurons, and the degrees of cytoplasmic accumulation of pNF-H and ubiquitylated protein, which are considered to reflect degeneration processes of motor neurons in sporadic ALS (5, 16–18, 22). These 5 genes were selected from those showing the most marked and specific altered expression levels among 4,845 genes that we had previously assessed in

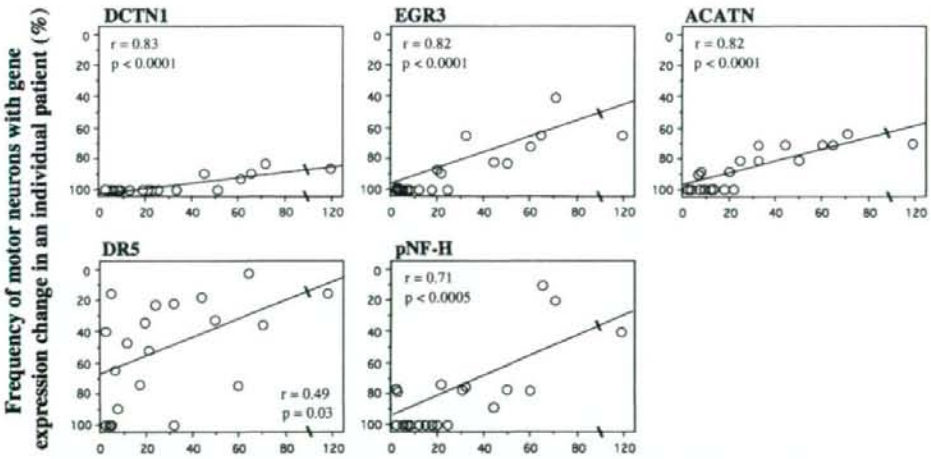

FIGURE 2. Frequency of motor neurons with gene expression change correlates with the extent of motor neuron loss. The correlation analyses between frequencies of motor neurons with gene expression changes (relative to controls) for *DCTN1*, *EGR3*, *ACATN*, *DR5*, and pNF-H and numbers of residual motor neurons were performed in the 20 patients with amyotrophic lateral sclerosis. The mean number of motor neuron patients in controls was 195 ± 17 (± SD) (range 176–225)/10 sections (n = 8). The values are inversely ordered on the y axis.

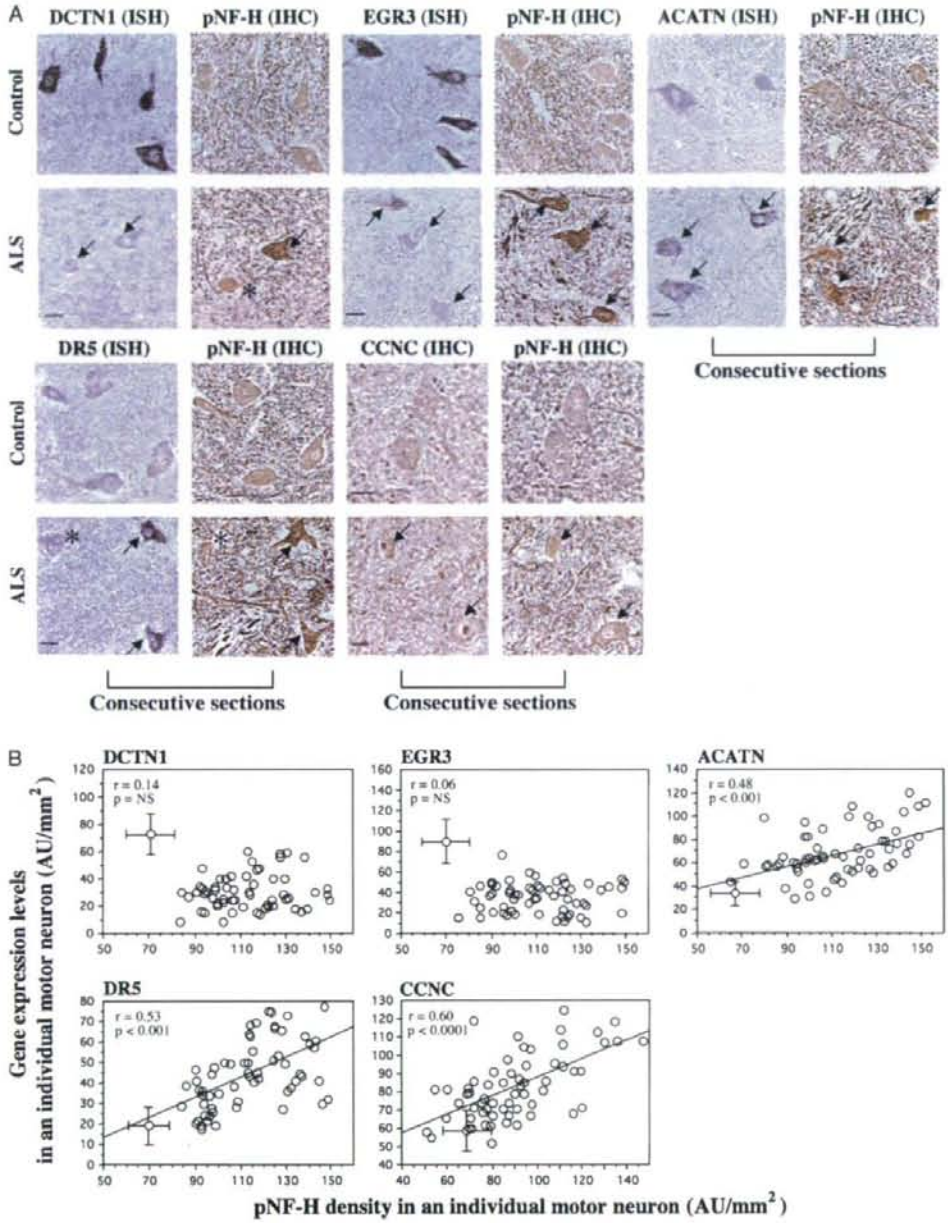

FIGURE 3. Gene expression and cytoplasmic phosphorylated neurofilament H (pNF-H) accumulation. **(A)** Gene expressions and pNF-H accumulation in identical motor neurons. Representative in situ hybridization (ISH) for *DCTN1*, *EGR3*, *ACATN*, and *DR5*, and immunohistochemistry (IHC) for CCNC are shown compared with pNF-H staining (IHC) on consecutive spinal cord sections from patients with amyotrophic lateral sclerosis (ALS) and control patients. The accumulation of cytoplasmic pNF-H was prominent in ALS motor neurons. Arrows denote motor neurons with gene expression or protein accumulation changes compared with control patients, and asterisks denote those with unchanged levels. Scale bars = 25 μm. **(B)** Expression levels of genes were compared with the level of pNF-H accumulation in individual motor neurons. Expression levels of *ACATN*, *DR5*, and CCNC were correlated with the accumulation of pNF-H, whereas those of *DCTN1* and *EGR3* were not. Consecutive transverse spinal cord sections were assessed from 8 representative patients with ALS. The control values for gene and protein expression levels and the accumulation of pNF-H are shown as means ± SD for 8 control cases. AU, arbitrary absorbance units; NS, not significant.

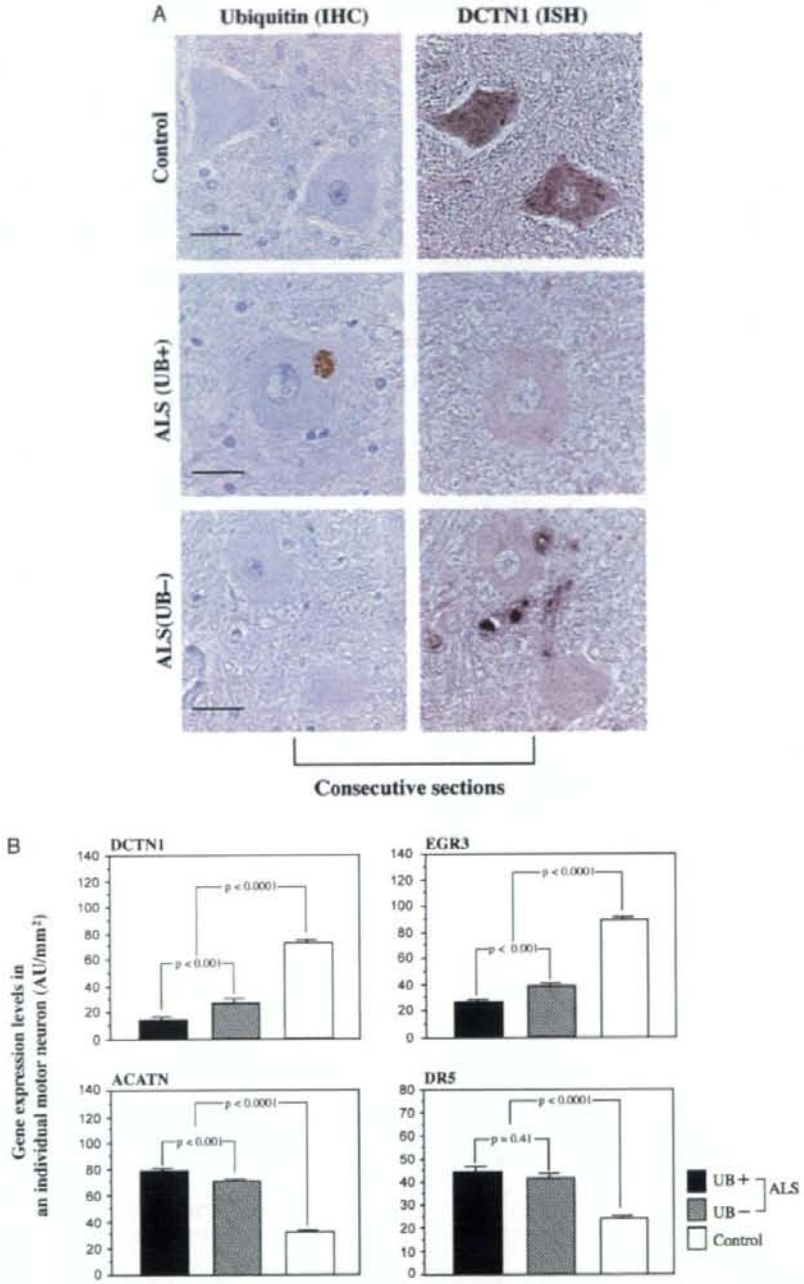

FIGURE 4. Gene expression and cytoplasmic accumulation of ubiquitylated proteins. **(A)** Representative *DCTN1* in situ hybridization (ISH) and ubiquitin (UB) immunohistochemistry (IHC) in identical motor neurons of consecutive sections are shown in patients with amyotrophic lateral sclerosis (ALS) and control patients. Scale bars = 25 μm. **(B)** Expression levels of genes were compared among the motor neurons that were either positive (n = 56) or negative (n = 175) for ubiquitylated proteins in 8 representative patients with ALS and those (n = 209) in 8 control patients. Expression levels of *DCTN1*, *EGR3*, and *ACATN* were significantly different in ubiquitin-positive compared to ubiquitin-negative neurons in ALS, whereas that of *DR5* was not. The values are shown as means ± SE. AU, arbitrary absorbance units.

a differential gene expression profile in isolated, laser-captured motor neurons from patients with sporadic ALS (15). Thus, we consider these gene expression levels to reflect most significantly the molecular events of neurodegeneration processes in motor neurons.

We selected 3 pathologic features as markers for neurodegeneration: the residual population of spinal motor neurons, cytoplasmic accumulation of pNF-H, and cytoplasmic accumulation of ubiquitylated proteins. Cytoplasmic accumulation of pNF-H has been demonstrated to occur in ALS motor neurons, even when they have normal morphologic appearances, and is thought to be a consequence of impaired axonal transport (5, 23–25). Hence, it is considered to be a histologic marker indicating neuronal degeneration and dysfunction before neuronal death (5, 16, 17). Hence, the accumulation of pNF-H is a rather early event in the motor neuron degeneration process. The presence of ubiquitylated proteins in the motor neuron cytoplasm has also been identified as a histopathologic marker of motor neuron degeneration (18). Ubiquitylated inclusions are thought to be aggregated, modified, and misfolded proteins that are ubiquitylated by motor neuron ubiquitin ligase (9). Although ubiquitylated, round inclusions are considered to occur in rather advanced stages of degeneration, it is not known whether dot-like and skein-like small faint ubiquitylated accumulations occur in the early stages of neurodegeneration.

The striking observation was that *DCTN1* expression was the most widely and most strongly downregulated among the genes examined in the residual motor neuron population, and was also severely downregulated even in the patients with large populations of motor neurons and in the motor neurons without pNF-H accumulations. The dramatic change in *DCTN1* in ALS seems to be specific for motor neurons because *DCTN1* expression was preserved in neurons in the dorsal nucleus of Clarke and the intermediolateral nucleus in the spinal cord, Purkinje cells of the cerebellum, and cortical neurons in the occipital cortex in patients with ALS. These observations suggest that *DCTN1* downregulation is the specific molecular event that occurs before the appearance of these pathologic markers, and is, therefore, a rather early event in the molecular sequences of neurodegeneration, at least among the events related to the genes examined. *DCTN1* codes for a protein that is a component of the retrograde transport protein complex with dynein (26, 27) and has been identified as a causative gene for human lower motor neuron disease (28, 29). Furthermore, it has been suggested that polymorphic amino acid substitution is a modifying factor accelerating pathogenesis and progression of sporadic ALS (30). A mouse model overexpressing dynamitin, which eventually results in late-onset progressive motor neuron degenerative disease, demonstrates the involvement of the dynactin-dynein complex (23). Two dominant point mutations in dynein cause progressive motor neuron degeneration in mice (31). These findings suggest that retrograde axonal transport involving the dynactin-dynein complex is strongly associated with motor neuron dysfunction and eventual motor neuron degeneration (32). By taking into account these findings, our present results strongly suggest that the downregulation of *DCTN1* in motor neurons may play a significant role in this process and may lead to the subsequent sequences of motor neuron degeneration in sporadic ALS. This hypothesis should be tested by further study on another cohort of patients with ALS and by in vitro and in vivo experiments.

Another interesting observation was that *ACATN*, *DR5*, and the CCNC protein were upregulated in subpopulations of residual motor neurons and that their upregulation was well correlated to the accumulation of pNF-H and the degree of motor neuron loss. ACATN functions as a cofactor for acetylation of gangliosides and has been demonstrated to suppress proapoptotic activity of GD3 ganglioside (33–36). In the *Drosophila* model, knockout of *ACATN* leads to a lethal phenotype owing to brain damage (Y. Hirabayashi, personal communication, 2007). DR5 is another cell death-related receptor as a member of the tumor necrosis factor (TNF) receptor family (TNFR10b) (37). CCNC is a cell cycle regulator protein and increases in *CCNC* expression are associated with its nuclear translocation, as was also demonstrated in this study (38). The aberrant activation of cell cycle regulators has been proposed as a pathway inducing motor neuron death in ALS (39, 40). Moreover, upregulated *DR5* was colocalized in motor neurons with CCNC nuclear translocation and also in those with downregulated TNFR-associated factor 6 (TRAF6) in our study (data not shown). The downregulated TRAF6, which is associated with nuclear factor-κB activation for cell survival, may not be able to sequester the overexpressed DR5 signaling, leading to a pathway of cell death (41). Taken together, expressions of these genes are involved in the cell death-related pathway. Upregulation of these genes occurs in subpopulations of motor neurons in parallel to or after the emergence of histopathologic markers such as pHF-H accumulation and motor neuron loss, suggesting that they occur in a relatively late phase of neurodegeneration, especially compared with *DCTN1* downregulation. The observation that active motor neuron degeneration processes for cell death that are probably mediated via cell death-related gene expression, such as *ACATN*, *DR5* and CCNC upregulation, occur in subpopulations of the remaining motor neurons with sustained *DCTN1* downregulation is consistent with our previous results that motor neurons in the remaining motor neuron pool randomly enter into the active degeneration process even up to the terminal stage in sporadic ALS (42).

The appearance of ubiquitylated protein accumulations or ubiquitylated inclusions is one of the hallmarks of motor neuron degeneration in sporadic ALS (18). In this study, however, the expression levels of *DCTN1*, *EGR3*, *ACATN*, and *DR5* were significantly altered before the appearance of ubiquitylated protein accumulations. Because the morphologic features of cytoplasmic ubiquitylated protein accumulations vary considerably, ranging from fine dot-like or skein-like accumulations to large inclusions, the simple assessment of ubiquitin-positive or negative materials may not be sufficient to identify neurodegeneration. However, even when we assessed ubiquitylated accumulation in a more precise manner, the expressions of these 4 genes were markedly altered independent of the appearance

of ubiquitylated protein accumulations. These findings suggest that appearance of ubiquitylated protein accumulation is a later pathologic event, occurring after the expressions of a number of genes are already altered. Alternatively, we may speculate that ubiquitylated protein accumulation is a secondary consequence of the series of molecular events accompanied by the alterations of a wide-range of gene expressions.

The present study also demonstrates that microarray analyses on laser-captured motor neurons followed by histopathologic analyses on tissues from large numbers of patients can provide significant information about molecular events in motor neuron degeneration and dysfunction in patients with sporadic ALS. The most serious problem in developing effective therapy for sporadic ALS is the lack of animal or cell models that properly reflect the motor neuron degeneration processes of sporadic ALS or even certain aspects of them. This is not a longitudinal and chronologic analysis of degeneration process in identical motor neurons, and it is not clear whether the changes seen in the present study represent the primary causes or secondary effects in the disease process because of the inherent problem of studying human disease using autopsy materials. However, we may be able to speculate that these results of human studies reflect the molecular sequence of motor neuron degeneration of ALS. Our present approach would provide an avenue for developing new molecular-targeted therapies for sporadic ALS by creating animal or cell models mimicking the molecular events seen in human patients.

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Review Article

Therapeutic strategies for spinal and bulbar muscular atrophy (SBMA)

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CONTENTS

Abstract

Spinal and bulbar muscular atrophy (SBMA) is an adult-onset neurodegenerative disease characterized by slowly progressive weakness, atrophy of bulbar, facial and limb muscles, and mild androgen insensitivity. The cause of this disease is expansion of a trinucleotide CAG repeat which encodes the polyglutamine tract within the first exon of the androgen receptor (AR) gene. SBMA occurs exclusively in adult males, whereas both heterozygous and homozygous females are usually asymptomatic. Lower motor neurons in the anterior horn of the spinal cord and those in the brainstem motor nuclei are predominantly affected in SBMA, and other neuronal and non-neuronal tissues are also widely involved to a lesser extent. SBMA is considered an intractable disease, but several therapeutic approaches have been developed based on new insight into the pathogenesis. There are several lines of evidence indicating that testosterone, the ligand for ARs, plays a crucial role in the pathogenesis of neurodegeneration in SBMA, leading to clinical trials of androgen deprivation therapies. Moreover, animal studies have revealed other key molecules in the pathogenesis of SBMA, such as heat shock proteins (HSPs), transcriptional co-activators and axon motors, suggesting additional therapeutic targets.

Nomenclature

Spinal and bulbar muscular atrophy (SBMA) is also known as Kennedy's disease, named after William R. Kennedy, whose study on 11 patients from 2 families depicted the clinical and pathological features of this disorder (1). Alternative names for this disease include bulbospinal neuronopathy and bulbospinal muscular atrophy.

Clinical features

SBMA chiefly affects adult males. The prevalence of this disease is estimated to be 1-2 per 100,000, although a considerable number of patients may be misdiagnosed as having other neuromuscular diseases, including amyotrophic lateral sclerosis (ALS) (2, 3). Patients of various ethnic backgrounds have been reported throughout the world.

The major symptoms of SBMA are weakness, atrophy and fasciculations of bulbar, facial and limb muscles, which are attributable to degeneration of lower motor neurons in the spinal cord and brainstem (4, 5). Subclinical dysfunction of upper motor neurons has been suggested by electrophysiology and magnetic resonance spectroscopy, although histopathological evidence is not sufficient (6, 7). In extremities, involvement is usually predominant in the proximal musculature, and is occasionally asymmetric. The onset of weakness is usually between 30 and 60 years of age, but is often preceded by nonspecific symptoms such as postural tremor and muscle cramps. Typically, affected individuals require a wheelchair 15-20 years after the onset of weakness (8-10). Although fasciculations in the extremities are rarely present at rest, they are easily recognized when patients hold their arms horizontally or bend their legs while lying on their backs. These contraction fasciculations are especially noticeable in the face, neck and tongue, and are especially conspicuous in the early stage of the disease. Fatigue after exercise may also be present. Bilateral facial and masseter muscle weakness, poor uvula and

soft palatal movements, and atrophy of the tongue with fasciculations are often encountered. Speech has a nasal quality in most cases due to reduced velopharyngeal closure. Some patients experience laryngospasm, a sudden sensation of dyspnea, although the clinical implication of this symptom is unclear (11). Advanced cases often develop dysphagia, eventually resulting in aspiration or choking. Muscle tone is usually hypotonic and no pyramidal signs are detected. The deep tendon reflex is diminished or absent, with no pathological reflex. Sensory involvement is largely restricted to a sense of vibration, which is affected distally in the legs. Cerebellar symptoms, dysautonomia and cognitive impairment are absent. Patients occasionally demonstrate signs of androgen insensitivity, such as gynecomastia, testicular atrophy, dyserection and decreased fertility, some of which are detected before the onset of motor symptoms. Abdominal obesity is common, whereas male pattern baldness is rare in patients with SBMA.

Electromyogram shows neurogenic abnormalities, and distal motor latencies are often prolonged in nerve conduction studies. Both sensory nerve action potentials and sensory evoked potentials are reduced or absent (12). Endocrinological examinations frequently reveal partial androgen resistance with elevated serum testosterone levels (13). Serum creatine kinase levels are elevated in the majority of patients, and hyperlipidemia, liver dysfunction and glucose intolerance are also detected in some patients. Profound facial fasciculations, bulbar signs, gynecomastia and sensory disturbances are the main clinical features distinguishing SBMA from other motor neuron diseases, although genetic analysis is indispensable for diagnosis. Female cases are usually asymptomatic, but some express subclinical phenotypes including high-amplitude motor unit potentials on electromyography (14).

The progression of SBMA is usually slow, but life-threatening respiratory tract infection often occurs in the advanced stage of the disease, resulting in early death in some patients. The cardinal cause of death is aspiration pneumonia (8). No specific therapy for SBMA has been established. Testosterone has been used in some patients, although it has no effect on the progression of SBMA.

Genetics

The cause of SBMA is expansion of a trinucleotide CAG repeat which encodes the polyglutamine tract in the first exon of the androgen receptor (AR) gene (15). The CAG repeat within the AR ranges in size from 9 to 36 in normal subjects, but from 38 to 62 in SBMA patients (16). Expanded polyglutamine tracts have been found to cause several neurodegenerative diseases, including SBMA, Huntington's disease, several forms of spinocerebellar ataxia and dentatorubral-pallidoluysian atrophy (DRPLA) (17). In these disorders, known as polyglutamine diseases, the CAG repeat has a strong tendency to further expand, accelerating the disease onset in successive generations (18). As documented in other polyglutamine diseases, the CAG repeat size correlates well with the age of onset in SBMA, but does not appear to dictate the rate of disease progression (8, 19).

The AR, the causative protein of SBMA, is a 110-kDa nuclear receptor which belongs to the steroid/thyroid hormone receptor family (20). The AR mediates the effects of the androgens testosterone and dihydrotestosterone through binding to an androgen response element (ARE) in the target gene to regulate its expression. The AR is essential for major androgen effects, including normal male sexual differentiation and pubertal sexual development, although an AR-independent nongenomic function of androgen has been reported. The AR is expressed not only in primary and secondary sexual organs, but also in nonreproductive organs, including the kidney, skeletal muscle, adrenal gland, skin and nervous system, suggesting a far-reaching influence on a variety of mammalian tissues. In the CNS, the AR expression level is relatively high in spinal and brainstem motor neurons, the same cells which are vulnerable in SBMA. The AR gene is located on chromosome Xq11-12. This 90-kb DNA contains 8 exons coding for the functional domains specific to the nuclear receptor family. The first exon codes for the *N*-terminal transactivating domain. Exons 2 and 3 code for the DNA-binding domain, whereas exons 4 through 8 code for the ligand-binding domain. The *N*-terminal transactivating domain, in which a CAG trinucleotide repeat locates, possesses a major transactivation function maintained by interaction with general transcriptional co-activators such as CREB-binding protein (CBP), transcription initiation factor TAFII130 and steroid receptor co-activator-1 (SRC-1). The CAG repeat beginning at codon 58 in the first exon of AR encodes the polyglutamine tract. The length of this repeat is highly variable because of the slippage of DNA polymerase upon DNA replication. Whereas abnormal elongation causes SBMA, a shorter CAG repeat is likely to increase the risk of prostate cancer (21). Transcriptional co-activators also possess glutamine-rich regions modulating protein-protein interactions with the *N*-terminal transactivating domain of AR.

Histopathology

Histopathological studies have provided important information on the pathogenesis of polyglutamine-mediated neurodegeneration. The fundamental histopathological finding in SBMA is loss of lower motor neurons in the anterior horn of the spinal cord, as well as in the brainstem motor nuclei, except for the third, fourth and sixth cranial nerves (4). The number of nerve fibers is reduced in the ventral spinal nerve root, reflecting motor neuronopathy. Sensory neurons in the dorsal root ganglia are less severely affected, and large myelinated fibers demonstrate a distally accentuated sensory axonopathy in the peripheral nervous system. Neurons in the Onufrowicz nuclei, intermediolateral columns and Clarke's columns of the spinal cord are generally well preserved. Muscle histopathology includes both neurogenic

and myogenic findings: there are groups of atrophic fibers with a number of small angular fibers, fiber type grouping and clamps of pyknotic nuclei, as well as variability in fiber size, hypertrophic fibers, scattered basophilic regenerating fibers and central nuclei.

A pathological hallmark of polyglutamine diseases is the presence of nuclear inclusions (NIs). In SBMA, NIs containing the pathogenic AR are found in the residual motor neurons in the brainstem and spinal cord, as well as in non-neuronal tissues, including the prostate, testes and skin (22). These inclusions are detectable using antibodies recognizing a small portion of the *N*-terminus of the AR protein, but not by those against the *C*-terminus of the protein. This observation implies that the *C*-terminus of the AR is truncated or masked upon formation of NI. A full-length AR protein with an expanded polyglutamine tract is cleaved by caspase-3, releasing a polyglutamine-containing toxic fragment, and the susceptibility to cleavage is polyglutamine repeat length-dependent (23). Thus, proteolytic cleavage is likely to enhance the toxicity of the pathogenic AR protein. Electron microscopic immunohistochemistry shows dense aggregates of AR-positive granular material without limiting membrane, both in neural and non-neural inclusions, in contrast to other polyglutamine diseases where NIs take the form of filamentous structures. Although NIs are a disease-specific histopathological finding, their role in pathogenesis has been heavily debated. Several studies have suggested that NIs may indicate a cellular response coping with the toxicity of abnormal polyglutamine protein (24). Instead, the diffuse nuclear accumulation of the mutant protein has been considered essential for inducing neurodegeneration in polyglutamine diseases, including SBMA.

An immunohistochemical study on autopsied SBMA patients using an anti-polyglutamine antibody demonstrated that diffuse nuclear accumulation of the pathogenic AR is more frequently observed than NIs in the anterior horn of the spinal cord (25) Intriguingly, the frequency of diffuse nuclear accumulation of the pathogenic AR in spinal motor neurons strongly correlates with the length of the CAG repeat in the AR gene. No such correlation has been found between NI occurrence and the CAG repeat length. Similar findings have also been reported in other polyglutamine diseases. Taken together, it appears that the pathogenic AR containing an elongated polyglutamine tract principally accumulates within the nuclei of motor neurons in a diffusible form, leading to neuronal dysfunction and eventual cell death. In support of this hypothesis, neuronal dysfunction is halted by genetic modulation preventing nuclear import of the pathogenic polyglutamine-containing protein in cellular and animal models of polyglutamine diseases (17).

Since the human AR is widely expressed in various organs, nuclear accumulation of the pathogenic AR protein is detected not only in the CNS, but also in non-neuronal tissues such as scrotal skin. The degree of pathogenic AR accumulation in scrotal skin epithelial cells tends to be correlated with that in the spinal motor neurons in autopsy specimens, and it is well correlated with CAG repeat length and inversely correlated with the motor functional scale (26). These findings indicate that scrotal skin biopsy with anti-polyglutamine immunostaining is a good biomarker for monitoring SBMA pathogenic processes. Since SBMA is a slowly progressive disorder, appropriate biomarkers would help improve the power and cost-effectiveness of longitudinal clinical treatment trials.

Molecular pathogenesis

Aggregation of mutant AR

In order to develop therapies for neurodegenerative diseases, it is essential to understand the molecular pathogenesis causing neuronal dysfunction and loss. Several targets of intervention have emerged from basic research on polyglutamine diseases, providing candidate therapeutic agents for SBMA.

The expanded polyglutamine tract in AR has been implicated in the pathogenesis of SBMA in two different, but not mutually exclusive, ways: 1) loss of normal AR function induces neuronal degeneration; and 2) the pathogenic AR acquires toxic properties, damaging motor neurons. Since the AR possesses trophic effects on neuronal cells, one can assume that loss of AR function may play a role in the pathogenesis of SBMA. Expansion of the polyglutamine tract mildly suppresses the transcriptional activities of the AR, probably because it disrupts the interaction between the *N*-terminal transactivating domain of AR and transcriptional co-activators (20). Although this loss of function of AR may contribute to androgen insensitivity in SBMA, the pivotal cause of neurodegeneration in SBMA is believed to be a gain of toxic function of the pathogenic AR due to expansion of the polyglutamine tract. This hypothesis is supported by the observation that motor impairment has never been observed in severe testicular feminization patients lacking AR function or in AR knockout mice. Moreover, a transgenic mouse model carrying an elongated CAG repeat driven by a human AR promoter demonstrated motor impairment, suggesting that the expanded polyglutamine tract is sufficient to induce the pathogenic process of SBMA (27).

Aggregation of abnormal protein has been considered to be central to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, ALS and prion disease. An expanded polyglutamine stretch alters the conformation of causative proteins, resulting in aggregation of the proteins. It is now widely accepted that aggregation of these abnormal proteins in neurons is the primary event in the pathogenesis of polyglutamine diseases. The rate-limiting step of aggregation has been proposed to be the formation of an oligomeric nucleus, which may occur after a repeat length-dependent conformational change of the polyglutamine monomer from a random coil to a parallel, helical β-sheet (28). Several experimental observations indicate that the formation of toxic oligomers, or intermediates, of abnormal polyglutamine-containing protein instigates a

series of cellular events which lead to neurodegeneration (29). This is also the case in a mouse model of SBMA, in which soluble oligomers are detectable prior to the onset of neuromuscular symptoms (30). Additionally, it has also been suggested that the toxicity of pathogenic AR is intensified by post-transcriptional modifications, including transglutamination and caspase-mediated proteolytic cleavage (23, 31, 32). On the other hand, Akt-induced phosphorylation of AR blocks ligand binding and thereby mitigates toxicity in cultured motor neurons (33).

Testosterone-dependent neurodegeneration in SBMA

SBMA is unique among polyglutamine diseases in that the pathogenic protein, AR, has a specific ligand, testosterone, which alters the subcellular localization of the protein by favoring its nuclear uptake. The AR is normally confined to a multiheteromeric inactive complex in the cell cytoplasm, and translocates into the nucleus in a ligand-dependent manner. This ligand-dependent intracellular trafficking of AR appears to play an important role in the pathogenesis of SBMA. The phenotypic difference with gender, which is a specific feature of SBMA, has been recapitulated in a transgenic mouse model of SBMA expressing the full-length human AR containing 97 CAGs under the control of a cytomegalovirus (CMV) enhancer and a chicken β-actin promoter (AR-97Q) (34). Affected AR-97Q mice demonstrate small body size, short life span, progressive muscle atrophy and weakness, as well as reduced cage activity, all of which are markedly pronounced and accelerated in male AR-97Q mice, but either not observed or far less severe in female AR-97Q mice. The onset of motor impairment is detected by the rotarod task at 8-9 weeks of age in the male AR-97Q mice, but at 16 weeks or more in females. Diffuse nuclear staining and less frequent NIs detected by 1C2 are demonstrated in the neurons of the spinal cord, brainstem and dorsal root ganglia, as well as in non-neuronal tissues such as heart, skeletal muscle and pancreas. Male AR-97Q mice show markedly more abundant diffuse nuclear staining and NIs than females, in agreement with the symptomatic difference according to gender. Despite the profound gender difference in pathogenic AR protein expression, there is no significant difference in the expression of the transgene mRNA between male and female AR-97Q mice, indicating that the testosterone level plays an important role in the sexual difference of phenotypes, especially in the post-transcriptional stage of the pathogenic AR.

The role of androgen has been further exemplified by hormonal interventions. Castrated male AR-97Q mice show profound improvement of symptoms, histopathological findings and nuclear localization of the pathogenic AR compared with sham-operated male AR-97Q mice. In contrast, subcutaneous injection of testosterone causes significant aggravation of symptoms, histopathological features and nuclear localization of the pathogenic AR in female AR-97Q mice (34). Since the nuclear translocation of AR is ligand-dependent, testosterone appears to show toxic effects in female AR-97Q mice by accelerating nuclear translocation of the pathogenic AR. This view is supported by the clinical observation that testosterone administration exacerbated neuromuscular symptoms of a patient with SBMA (35). The nuclear accumulation of pathogenic AR protein with an expanded polyglutamine tract is likely essential for neuronal cell dysfunction and degeneration in the majority of polyglutamine diseases. It thus appears logical that reducing testosterone levels would improve phenotypic expression by preventing nuclear localization of the pathogenic AR. In support of this hypothesis, ligand-dependent neurodegenaration has also been revealed in other animal models of SBMA (36, 37). It should be noted that testosterone deprivation by castration reverses motor dysfunction in a transgenic mouse model of SBMA showing fairly slow progression (37).

Transcriptional dysregulation

Disruption of transcriptional machinery has also been hypothesized to underlie the pathogenesis of polyglutamine diseases (38). Gene expression analysis indicates that transcriptional disruption is an early change in the pathogenesis of mouse models of polyglutamine diseases. Transcriptional co-activators such as CBP are sequestrated into the polyglutamine-containing NIs through protein-protein interactions in mouse models and patients with polyglutamine diseases (39). Alternatively, the interaction between transcriptional co-activators and soluble pathogenic protein has also been demonstrated in animal models of polyglutamine diseases, as well as in *post mortem* tissues of patients (40). The expression of genes regulated through CBP-mediated transcription is decreased in mouse models of polyglutamine diseases (38). CBP functions as a histone acetyltransferase (HAT), regulating gene transcription and chromatin structure. It has been indicated that the HAT activity of CBP is suppressed in cellular models of polyglutamine diseases. Taken together, transcriptional dysregulation due to a decrease in histone acetylation is likely to underlie the pathogenesis of neurodegeneration in polyglutamine diseases. This hypothesis is exemplified by our observation that acetylation of nuclear histone H3 is significantly diminished in the spinal cord of SBMA mice (41). Additionally, dysfunction of CBP results in decreased expression of vascular endothelial growth factor (VEGF) in another mouse model of SBMA, indicating that transcriptional alteration is a trigger for neurodegeneration in this disease (42)

Disruption of axonal transport

Motor neurons possess an extremely long axons along which molecular motors transport essential components, such as organelles, vesicles, cytoskeletons and signal molecules. This implies that axonal trafficking plays a fundamental role in the maintenance of the normal function of motor neurons. Obstruction of axonal transport has attracted attention as a cause of neuronal dysfunction in

a variety of neurodegenerative diseases, including SBMA (43, 44). A mutation in the genes for proteins regulating axonal trafficking, dynein and dynactin 1, has been shown to cause motor neuron degeneration in both humans and rodents (45, 46).

In a mouse model of SBMA, neurofilaments and synaptophysin accumulate at the distal motor axons. A similar intramuscular accumulation of neurofilaments has been detected in the skeletal muscle of SBMA patients. Fluoro-gold labeling and sciatic nerve ligation have demonstrated an impaired retrograde axonal transport in transgenic SBMA mice (47). The mRNA level of dynactin 1 is significantly reduced in SBMA mice, resulting from pathogenic AR-induced transcriptional dysregulation. These pathological events are reversed by castration, which prevents nuclear accumulation of pathogenic AR. Overexpression of dynactin 1 mitigates the neuronal toxicity of the pathogenic AR in a cell culture model of SBMA. These observations indicate that polyglutamine-dependent transcriptional dysregulation of dynactin 1 plays a crucial role in reversible neuronal dysfunction in the early stage of SBMA. Pathogenic AR containing an expanded polyglutamine has also been demonstrated to activate c-Jun *N*-terminal kinase (JNK), leading to inhibition of kinesin-1 microtubule-binding activity and eventual disruption of anterograde axonal transport (48). It is noteworthy that JNK inhibitors reverse the suppression of neurite outgrowth by pathogenic AR in cultured cells.

Therapeutic strategies

Testosterone deprivation therapy

Leuprorelin is a potent luteinizing hormone-releasing hormone (LHRH) analogue that suppresses the release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This drug has been used for a variety of sex hormone-dependent diseases, including prostate cancer, endometriosis and prepuberty. The primary pharmacological target of leuprorelin is the anterior pituitary. Through its agonist effect on LHRH-releasing cells, it initially promotes the release of gonadotropins, resulting in a transient increase in the serum level of testosterone or estrogens. After this surge, the continued use of this drug induces desensitization of the pituitary by reducing LHRH receptor binding sites and/or uncoupling of receptors from intracellular processes. Within about 2-4 weeks of leuprorelin administration, serum testosterone levels decrease to the extent achieved by surgical castration. The effects are maintained during treatment, suggesting that continuous administration of leuprorelin is required for its clinical use. This drug has thus been provided as a sustained-release depot taking the form of polymer microspheres. On the other hand, flutamide, the first androgen antagonist discovered, has highly specific affinity for AR and competes with testosterone for binding to the receptor. It has been used for the treatment of prostate cancer, usually in association with an LHRH analogue, in order to block the action of adrenal testosterone. Although flutamide suppresses androgen-dependent transactivation, it does not reduce plasma levels of testosterone.

Leuprorelin successfully inhibits nuclear accumulation of the pathogenic AR, resulting in marked improvement in neuromuscular phenotypes in male AR-97Q mice (Fig. 1) (49). Leuprorelin initially increases serum testosterone levels by activating the LHRH receptor, but subsequently reduces levels to undetectable levels. Androgen-blocking effects were also confirmed by reduced weights of the prostate and seminal vesicles. Leuprorelin-treated AR-97Q mice show a longer life span, larger body size and better motor performance compared with vehicle-treated mice. Leuprorelin appears to improve neuronal dysfunction by preventing ligand-dependent nuclear translocation of the pathogenic AR in the same way as castration.

Given its minimal invasiveness and established safety, leuprorelin appears to be a promising therapeutic agent for SBMA. In a preliminary open trial, 6-month treatment with leuprorelin significantly diminished nuclear accumulation of pathogenic AR in the scrotal skin of patients, suggesting that androgen deprivation intervenes in the pathogenic process of human SBMA, as demonstrated in animal studies (26). A multicenter trial of leuprorelin acetate is currently under way to verify the clinical benefits of androgen deprivation for SBMA patients.

In contrast, the AR antagonist flutamide does not ameliorate symptoms, pathological features or nuclear localization of the pathogenic AR in male AR-97Q mice, although there is no significant difference in the androgen-blocking effects between flutamide and leuprorelin. Flutamide does not inhibit, and may even facilitate, the nuclear translocation of AR. Consistent with the mouse study, this AR antagonist also promotes nuclear translocation of the pathogenic AR containing an expanded polyglutamine in cellular and fly models of SBMA (36, 50). Therefore, flutamide is not likely to be a useful therapeutic agent for SBMA.

Castrated or leuprorelin-treated AR-97Q mice show phenotypes similar to those seen in female AR-97Q mice, implying that motor impairment of SBMA patients can be reduced to the level in females. SBMA is considered an X-linked disease, whereas other polyglutamine diseases show autosomal dominant inheritance. In fact, female SBMA patients hardly manifest clinical phenotypes, although they possess a similar number of CAG repeats in the disease allele of the AR gene as their siblings with SBMA (14, 51). Reduction in the mutant AR expression due to X inactivation may prevent females from manifesting the disease, but hormonal intervention studies using mouse and fly models clearly suggest that low levels of testosterone prevent nuclear accumulation of pathogenic AR protein, resulting in a lack of neurological phenotypes in females. This view is strongly supported by the observation that manifestation of symptoms is minimal even in homozygous SBMA females (52). Therefore, it seems inappropriate to regard SBMA as an X-recessive inherit-

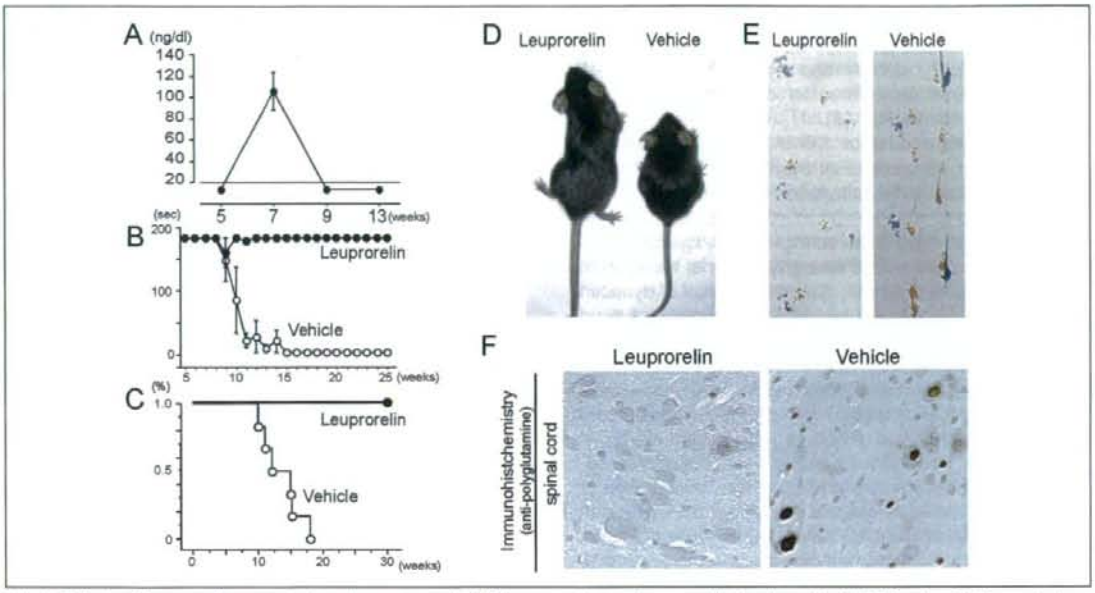

Fig. 1. Effects of leuprorelin on mutant androgen receptor (AR) expression and neuropathology in male AR-97Q mice. **A**. Serum testosterone levels in AR-97Q mice. Leuprorelin initially increased serum testosterone levels but subsequently reduced them to undetectable levels. **B and C**. Rotarod task (**B**) and survival rate (**C**) of AR-97Q mice. Leuprorelin markedly improved motor function of the mice at the dose administered. **D**. Leuprorelin prevented muscle atrophy in AR-97Q mice. **E.** Walking pattern and strides were also improved by androgen deprivation. **F**. Immunohistochemistry using 1C2 showed marked differences in diffuse nuclear staining and nuclear inclusions between leuprorelin-treated and vehicle-treated AR-97Q male mice in the spinal anterior horn.

ed disease, but rather its neurological phenotype is likely to depend on testosterone concentration.

AR co-regulators, such as ARA70, are alternative therapeutic targets because they control the function and cellular distribution of AR. It has been shown that 5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one (ASC-J9) dissociates AR and ARA70, resulting in suppression of pathogenic AR aggregation, as well as amelioration of neuromuscular symptoms in a mouse model of SBMA, without severely depleting serum levels of testosterone. The treatment was also effective even after the onset of muscle weakness in mice (53).

Manipulation of heat shock proteins

Many components of the ubiquitin-proteasome pathway and molecular chaperones are known to co-localize with polyglutamine-containing NIs, implying that failure of cellular defense mechanisms underlies neurodegeneration in polyglutamine diseases. Heat shock proteins (HSPs), stress-inducible molecular chaperones, are another key to elucidation of the pathogenesis of SBMA. HSPs are classified into different families according to molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (54). These HSPs are either constitutively expressed or inducibly synthesized after cellular stress. HSPs play a crucial role in maintaining correct folding, assembly and intracellular transport of proteins. For example, HSP70 and HSP90, essential components of the AR-chaperone complex in the cell cytoplasm, regulate the function, nuclear translocation and degradation of the AR (55). Under toxic conditions, HSP synthesis is rapidly upregulated and non-native proteins are refolded as a consequence. Therefore, HSPs have attracted a great deal of attention as cytoprotective agents for conditions such as ischemia and malignancy.

Several studies suggest that polyglutamine elongation interferes with the protective cellular response against cytotoxic stress (28). Truncated ARs with an expanded polyglutamine tract delay the induction of HSP70 after heat shock (56). The threshold of HSP induction is known to be relatively high in spinal motor neurons (57). Expression levels of HSPs are decreased in brain lesions in an animal model of Huntington's disease and in the SBMA mouse (58, 59). Therefore, impairment of the HSP induction capability is implicated in the pathogenesis of motor neuron degeneration in SBMA.

Not only are HSPs implicated in the pathogenesis of neurodegeneration, they are also potent suppressors of polyglutamine toxicity. There is increasing evidence that HSPs abrogate polyglutamine-mediated cytotoxicity by refolding and solubilizing the pathogenic proteins (28, 29). HSP70 cooperates with HSP40 in functioning as a molecular chaperone. These HSPs are proposed to prevent the initial conformation conversion of abnormal polyglutamine-containing protein from a random coil to a

β-sheet, leading to attenuation of toxic oligomer formation (28). Overexpression of HSP70, together with HSP40, inhibits toxic accumulation of abnormal polyglutamine-containing protein and suppresses cell death in a variety of cellular models of polyglutamine diseases, including SBMA (60). HSP70 has also been shown to facilitate proteasomal degradation of abnormal AR protein in a cell culture model of SBMA (61).

The favorable effects of HSP70 have been verified in studies using mouse models of polyglutamine diseases. Overexpression of the inducible form of human HSP70 markedly ameliorated symptomatic and histopathological phenotypes in our transgenic mouse model of SBMA (62). These beneficial effects are dependent on HSP70 gene dose and correlate with the reduction in the amount of nuclear-localized AR protein. It should be noted that the amount of the soluble form of pathogenic AR was also significantly decreased by HSP70 overexpression, suggesting that degradation of pathogenic AR may have been accelerated by overexpression of this molecular chaperone. Overexpression of CHIP, or *C*-terminus of Hsc70 (heat shock cognate protein 70)-interacting protein, has also been shown to prevent nuclear accumulation of pathogenic AR and thereby ameliorate motor symptoms in a transgenic mouse model of SBMA (63).

Favorable effects obtained by genetic modulation of HSP suggest that pharmacological induction of molecular chaperones might be a promising approach to SBMA and other polyglutamine diseases. Geranylgeranylacetone (GGA), an acyclic isoprenoid compound with a retinoid skeleton, has been shown to strongly induce HSP expression in various tissues (64). Oral administration of GGA upregulates the levels of HSP70, HSP90 and HSP105 via activation of heat shock factor 1 (Hsf1) in the CNS and inhibits nuclear accumulation of the pathogenic AR protein, resulting in amelioration of polyglutamine-dependent neuromuscular phenotypes of SBMA transgenic mice (59). Given its extremely low toxicity, this compound has been used as an oral antiulcer drug. Although a high dose appears to be needed for clinical effects, GGA appears to be a safe and promising therapeutic candidate for polyglutamine-mediated neurodegenerative diseases, including SBMA.

Inhibition of HSP90 has also been demonstrated to arrest neurodegeneration in the SBMA mouse (65). HSP90 functions in a multichaperone complex, assisting proper folding, stabilization and assembly of so-called client proteins, including various oncoproteins and the AR (66). The HSP90-client protein complex is stabilized when it is associated with p23, a co-chaperone interacting with HSP90. Treatment with 17-allylaminogeldanamycin (17-AAG), a potent HSP90 inhibitor, dissociated p23 from the HSP90-AR complex, and thus facilitated proteasomal degradation of pathogenic AR in cellular and mouse models of SBMA. 17-AAG thereby inhibits nuclear accumulation of this protein, leading to marked amelioration of motor phenotypes in the SBMA mouse model, without detectable toxicity. Of interest is the finding that the pathogenic AR is preferentially targeted to proteasomal degradation in the presence of 17-AAG compared with wild-type AR. Given a high association between p23 and the AR containing an expanded polyglutamine, it appears logical that pathogenic AR is more dependent on HSP90 to maintain folding and function than wild-type AR, and thus is particularly susceptible to HSP90 inhibition. 17-AAG is also capable of inducing HSP70 in cellular and mouse models of SBMA. Thus, 17-AAG, which is now undergoing clinical trials for a wide range of malignancies, may be a good candidate for the treatment of SBMA (67, 68).

Restoration of transcriptional activity

The histone acetylation level is determined by an interplay between histone acetyltransferase and histone deacetylase (HDAC). The recruitment of HDAC to target genes represses transcription, leading to aberrant cellular function. Since suppression of HDAC activity results in augmentation of histone acetylation and subsequent restoration of gene transcription, HDAC inhibitors have been considered to be of therapeutic benefit in polyglutamine diseases (40, 69, 70). Butyrate was the first HDAC inhibitor to be discovered, and the related compound, phenylbutyrate, has been successfully employed in experimental cancer therapy. Oral administration of sodium butyrate ameliorates symptomatic and histopathological phenotypes of our mouse model of SBMA through upregulation of histone acetylation in nervous tissues (41). This compound has also been shown to alleviate neurodegeneration in a mouse model of DRPLA (71). Although sodium butyrate is likely to be a promising treatment for SBMA, this compound yielded beneficial effects only within a narrow therapeutic range of doses in the mouse model. Careful dose determination is therefore necessary when using HDAC inhibitors for the treatment of polyglutamine diseases.

It should be borne in mind that there are several HDACs with different biological properties (72). The HDACs have been classified into three classes: class I (HDAC1, HDAC2, HDAC3 and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) and class III (SIRT members). In particular, HDAC6 induces compensatory autophagy under conditions of ubiquitin-proteasome system impairment in a fly model of SBMA (73). Furthermore, the expression of HDAC6 was sufficient to rescue degeneration associated with ubiquitin-proteasome system dysfunction *in vivo* in an autophagy-dependent manner. Additionally, activation of class III HDACs by resveratrol has been shown to alleviate polyglutamine toxicity in a *Caenorhabditis elegans* model of Huntington's disease (74). These studies suggest the need for the development of selective HDAC inhibitors which mitigate polyglutamine toxicity without deleterious effects on normal cellular function.

Potential agents not fully characterized in animal models

Several compounds have been shown to suppress polyglutamine cytotoxicity in cellular models (75). These

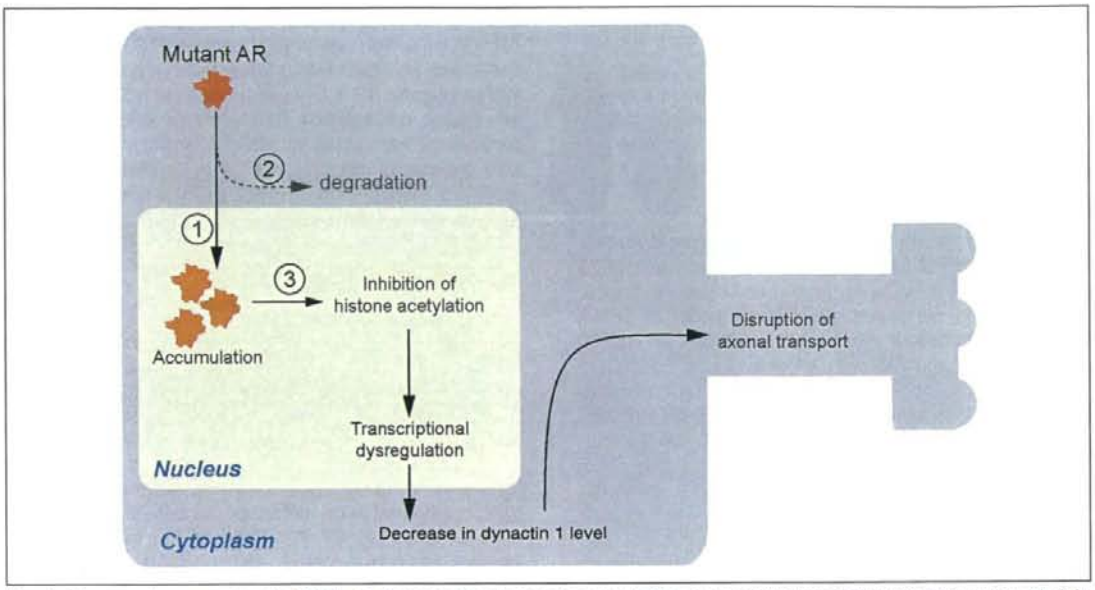

Fig. 2. Therapeutic approaches to SBMA. Ligand-dependent nuclear accumulation of mutant AR has been construed as the culprit in causing neurodegeneration in SBMA. Several therapeutic strategies have emerged from animal studies, including: 1) inhibitors of accumulation (*e.g.*, leuprorelin, ASC-J9, geranylgeranylacetone [GGA]); 2) facilitators of proteasomal degradation (*e.g.*, 17-allylaminogeldanamycin [17-AAG]); and 3) activators of transcription (*e.g.*, histone deacetylase [HDAC] inhibitors). Downstream events such as disrupted axonal transport may also be therapeutic targets.

agents are potentially applicable for SBMA, although they have not been thoroughly characterized in animal models. In addition to pharmacological approaches, RNA interference, peptide inhibitors, cell transplantation and trophic factor supplementation are potential strategies if safety and delivery problems are solved (76, 77).

Clinical perspectives

Analysis of cellular and animal models has provided rational therapeutic approaches to SBMA (Fig. 2). We may need to combine these strategies, because each agent has potential adverse effects when used over the long term (78). Given that various therapeutic strategies for SBMA have emerged thanks to the use of animal models mimicking human diseases, it is of utmost importance to pursue intensive clinical studies to verify the results from animal studies. When we apply candidate agents to patients, it should be taken into account that the majority of therapeutics emerging from animal studies are disease-modifying therapies, but not agents for symptomatic relief. Given that SBMA is a slowly progressive disease, long-term clinical trials are likely to be necessary to verify clinical benefits of disease-modifying therapies by targeting clinical endpoints such as occurrence of aspiration pneumonia or becoming wheelchair-bound. Suitable surrogate endpoints, which reflect the pathogenesis and severity of SBMA, are thus needed to assess the therapeutic efficacy in drug trials. To this end, appropriate biomarkers should be identified and validated. We may need to combine several parameters to appropriately use biomarkers in the development of therapeutics (26, 79). Quantitative analysis of natural history, including genetic, biological and anthropological data, is also necessary for long-term evaluation of therapeutic agents for SBMA.

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Neurobiology of Disease

CHIP Overexpression Reduces Mutant Androgen Receptor Protein and Ameliorates Phenotypes of the Spinal and Bulbar Muscular Atrophy Transgenic Mouse Model

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Spinal and bulbar muscular atrophy (SBMA) is an inherited motor neuron disease caused by the expansion of a polyglutamine tract within the androgen receptor (AR). The pathologic features of SBMA are motor neuron loss in the spinal cord and brainstem and diffuse nuclear accumulation and nuclear inclusions of the mutant AR in the residual motor neurons and certain visceral organs. Many components of the ubiquitin-proteasome and molecular chaperones are also sequestered in the inclusions, suggesting that they may be actively engaged in an attempt to degrade or refold the mutant AR. C terminus of Hsc70 (heat shock cognate protein 70)-interacting protein (CHIP), a U-box type E3 ubiquitin ligase, has been shown to interact with heat shock protein 90 (Hsp90) or Hsp70 and ubiquitylates unfolded proteins trapped by molecular chaperones and degrades them. Here, we demonstrate that transient overexpression of CHIP in a neuronal cell model reduces the monomeric mutant AR more effectively than it does the wild type, suggesting that the mutant AR is more sensitive to CHIP than is the wild type. High expression of CHIP in an SBMA transgenic mouse model also ameliorated motor symptoms and inhibited neuronal nuclear accumulation of the mutant AR. When CHIP was overexpressed in transgenic SBMA mice, mutant AR was also preferentially degraded over wild-type AR. These findings suggest that CHIP overexpression ameliorates SBMA phenotypes in mice by reducing nuclear-localized mutant AR via enhanced mutant AR degradation. Thus, CHIP overexpression would provide a potential therapeutic avenue for SBMA.

Key words: CHIP; polyglutamine; SBMA; transgenic mice; protein degradation; androgen receptor

Introduction

Polyglutamine (polyQ) diseases are inherited neurodegenerative disorders caused by the expansion of trinucleotide CAG repeats in the causative genes (Gatchel and Zoghbi, 2005). To date, nine polyQ diseases have been identified (Di Prospero and Fischbeck, 2005). One of these is spinal and bulbar muscular atrophy (SBMA), characterized by premature muscular exhaustion, progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles (Kennedy et al., 1968; Sobue et al., 1993; Sperfeld et al., 2002; Atsuta et al., 2006). In SBMA, a polymorphic CAG repeat with 14–32 CAGs expands to 40–62 CAGs in the first exon of the androgen receptor (*AR*) gene (La Spada et al., 1991; Tanaka et al., 1996). CAG repeat size is inversely correlated with the age at onset and positively correlated with disease severity in SBMA (Doyu et al., 1992; Igarashi et al., 1992; La Spada et al., 1992). The histopathologic hallmarks of SBMA are lower motor neuronal loss (Sobue et al., 1989), diffuse nuclear accumulation, and nuclear inclusions (NIs) of expanded polyQ mutant AR in the residual motor neurons in brainstem and spinal cord as well as in some other visceral organs (Li et al., 1998a,b; Adachi et al., 2005). Such NIs are common pathological features in polyQ diseases and also colocalize with many components of the ubiquitin-proteasome and molecular chaperones (Adachi et al., 2001; Schmidt et al., 2002; Ross and Poirier, 2004), raising the possibility that the ubiquitin-proteasome system and molecular chaperones may actively attempt to degrade or refold components of the inclusions (Stenoien et al., 1999; Ross and Pickart, 2004). Furthermore, these proteasomes and chaperones should also facilitate refolding or proteolysis of toxic misfolded proteins (McClellan et al., 2005) and may play a role in protecting neuronal cells against the toxic properties of expanded polyQ (Cummings et al., 1998; Kobayashi et al., 2000).

C terminus of heat shock cognate protein 70 (Hsc70)-interacting protein (CHIP) has three tetratricopeptide repeat (TPR) domains that interact with the molecular chaperones heat shock protein 70 (Hsp70) and Hsp90 (Ballinger et al., 1999; Connell et al., 2001) and a U-box domain that interacts with the proteasome, conferring CHIP with E3 ubiquitin ligase activity

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(Hatakeyama et al., 2001; Jiang et al., 2001). Wild-type AR is one of the CHIP substrates (Cardozo et al., 2003; He et al., 2004). CHIP also interacts with misfolded proteins trapped by molecular chaperones and degrades them, thus acting as a "quality control" E3 (Cyr et al., 2002; Murata et al., 2003). In fact, CHIP suppressed inclusion formation and cellular toxicity in cell, zebrafish, and *Drosophila* polyQ disease models (Jana et al., 2005; Miller et al., 2005; Al-Ramahi et al., 2006).

In this study, we examine whether CHIP exerts therapeutic effects on a cultured cell model and a transgenic mouse model expressing the mutant AR to explore a potential strategy for SBMA therapy. We report that CHIP markedly ameliorated motor and pathological phenotypes and that this amelioration was correlated with the reduction of monomeric mutant AR and mutant AR protein complexes in the SBMA models.

Materials and Methods

Cell culture. SH-SY5Y cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with plasmids encoding ARs containing normal (24 CAGs) or expanded (65 CAGs) polyQ repeats (Waza et al., 2005). Stable clones expressing these normal and mutant ARs were established by selection with the antibiotic G418 (0.4 mg/ml final concentration). The androgen receptor is not expressed in untransfected SH-SY5Y cells. All cell cultures were propagated in the absence of androgen. In Western blots from these cultures, we detected a band of monomeric mutant AR in the separating gel but could hardly detect the high-molecular-weight mutant AR protein complex, which was retained in the stacking gel. Therefore, this cultured cell model is better suited for estimating the change in monomeric mutant AR expression. There was no difference in viability between cells expressing the wild-type and mutant ARs in the absence of androgen using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

DNA transfection. Plasmid pcDNA3-CHIP, encoding FLAG-tagged human CHIP, was kindly provided by Dr. Keiji Tanaka (Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (Murata et al., 2001). AR stable cells were plated in six-well dishes in 2 ml of DMEM/F-12 containing 10% charcoal-stripped fetal bovine serum with penicillin and streptomycin, and each dish was transfected with 4 μg of the vector containing CHIP or mock (negative control) using Lipofectamine 2000 according to the manufacturer's instructions. Transfection efficiency was 60–70%. The cells were cultured for 48 h at 37°C under 5% CO_2.

Transgene construction. Full-length human CHIP cDNA was generated from total RNA extracted from SH-SY5Y cells by reverse transcription-PCR. Full-length human CHIP was constructed by subcloning CHIP inserts derived from the full-length human CHIP cDNA into the pcDNA3.1-myc-his mammalian expression vector (Invitrogen) using PCR. Then, the myc-tagged CHIP fragments were subcloned into the pCAGGS vector (Niwa et al., 1991). All constructs were confirmed by DNA sequence analysis. The final plasmids were digested to remove the transgene.

Generation and maintenance of Tg mice and genotyping. We generated CHIP overexpression mice by microinjection of the transgene into BDF1 fertilized eggs and obtained four founders. BDF1 homozygous CHIP transgenic females were mated with BDF1/B6 male mice expressing full-length human AR with 24 (AR-24Q mice, 5-5 line) or 97-polyQ tracts (AR-97Q mice, 7-8 line), producing a mixed BDF1 and B6 genetic background. First-generation AR-24Q/CHIP$^{(tg/-)}$ or AR-97Q/CHIP$^{(tg/-)}$ mice were mated with either CHIP$^{(tg/-)}$ or CHIP$^{(tg/tg)}$ mice to produce all AR or AR/CHIP double-transgenic mice for each analysis. We screened mouse tail DNA by PCR for the presence of the transgene using the primers 5′-CATCTCAGAAGAGGATCTGTG-3′ and 5′-GGTCGAGGGATCTTCATAAG-3′.

Neurological and behavioral assessment of SBMA model mice. The AR-24Q and AR-97Q mice were generated and maintained as described previously (Katsuno et al., 2002). All animal experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and under the approval of the Nagoya University Animal Experiment Committee. The AR-97Q male mice showed progressive muscular atrophy and weakness as well as diffuse nuclear staining and NIs of the mutant AR. These phenotypes were very pronounced in male transgenic mice, similar to the situation in SBMA patients. The mouse rotarod task (Economex Rotarod; Ugo Basile, Comerio, Italy) was performed on a weekly basis, and cage activity was measured weekly with the AB system (Neuroscience, Tokyo, Japan) as described previously (Katsuno et al., 2002; Minamiyama et al., 2004). Spontaneous motor activity was monitored for 24 h periods; all spontaneous movements, both vertical and horizontal, including locomotion, rearing, and head movements, were counted and automatically totaled.

Immunohistochemistry and histopathology. Mice were deeply anesthetized with ketamine–xylazine and transcardially perfused with 20 ml of 4% paraformaldehyde fixative in phosphate buffer, pH 7.4. Spinal cord and skeletal muscle tissues were removed, postfixed overnight in 10% phosphate-buffered formalin, and processed for paraffin embedding. Sections (6 μm thick) of the above tissues were deparaffinized, dehydrated with alcohol, and treated in formic acid for 5 min at room temperature. For the immunohistochemical studies, the paraffin sections were preheated in a microwave oven for 10 min. The sections were blocked with normal animal serum (1:20) and incubated with mouse anti-expanded polyQ antibody (1:10,000; 1C2; Millipore, Billerica, MA), anti-CHIP antibody (1:1000; Medical and Biological Laboratories, Nagoya, Japan), and mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Roche Diagnostics, Mannheim, Germany). Primary antibodies were probed with a biotinylated anti-species-specific IgG (Vector Laboratories, Burlingame, CA), and the immune complexes were visualized using streptavidin-horseradish peroxidase (Dako, Glostrup, Denmark) and 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) as a substrate. Sections were counterstained with Mayer's hematoxylin. Paraffin-embedded sections (6 μm thick) of the gastrocnemius muscles were air dried and stained with hematoxylin and eosin. For double-immunofluorescence staining of the spinal cord, sections were blocked with 5% normal goat serum and then sequentially incubated with anti-CHIP antibody (1:1000; Medical and Biological Laboratories) and 1C2 antibody (1:10,000; Millipore) at 4°C overnight. The sections were then incubated with Alexa 488-conjugated goat anti-chicken IgG (1:1000; Invitrogen) and Alexa 568-conjugated goat anti-mouse IgG (1:1300; Invitrogen) for 8 h at 4°C. The stained sections were examined and photographed with a confocal laser-scanning microscope (LSM 5 PASCAL; Carl Zeiss MicroImaging, Tokyo, Japan).

Patients. Tissue from nine patients with clinicopathologically and genetically confirmed SBMA (51–84 years of age; mean, 64.3 years), and three non-neurological controls (51–76 years of age; mean, 64.0 years) were also used in the present study. These patients had been hospitalized and followed up at Nagoya University Hospital and its affiliated hospitals during the past 25 years. Informed consent was obtained to use the tissues for research purposes. Paraffin-embedded sections of the spinal cord and brain were obtained and examined in the same way as those from transgenic mice.

Quantification of 1C2-positive cells. For assessment of 1C2-positive cells in the ventral horn of the spinal cord, 50 consecutive transverse sections of the thoracic spinal cord were prepared from each individual mouse, and 1C2-positive cells within the ventral horn of every fifth section were counted as described previously (Adachi et al., 2001). Populations of 1C2-positive cells were expressed as number/mm^2. For assessment of 1C2-positive cells in muscle, the number of 1C2-positive cells was calculated from counts of >500 fibers in randomly selected areas of individual mice and expressed as the number per 100 muscle fibers. The quantitative data of six individual mice were expressed as mean ± SEM.

Protein expression analysis and ubiquitination assay. Forty-eight hours after transfection, cells were lysed in CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma, St. Louis, MO) with 1 mM PMSF and 6 μg/ml aprotinin and centrifuged at 15,000 × *g* for 15 min at 4°C. Sixteen-week-old mice were exsanguinated under ketamine–xylazine anesthesia, and tissues were snap frozen with powdered CO_2 in acetone. The tissues were homogenized in CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma) with 1 mM PMSF and 6 μg/ml aprotinin and centrifuged at

2500 × *g* for 15 min at 4°C. Supernatant fraction protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA). Aliquots of supernatant fractions were loaded on 5–20% SDS-PAGE gels, each lane containing 10 μg of protein for cells, 160 μg for neural tissue, and 80 μg for muscle tissue, and then transferred to Hybond-P membranes (GE Healthcare, Buckinghamshire, UK), using 25 mM Tris, 192 mM glycine, 0.1% SDS, and 10% methanol as transfer buffer. Primary antibodies were used at the following concentrations: rabbit anti-AR, 1:1000 (N20; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-AR, 1:1000 (H280; Santa Cruz Biotechnology); mouse anti-Hsp70, 1:1000 (SPA-810; Stressgen Biotechnologies, San Diego, CA); rabbit anti-Hsp40, 1:5000 (SPA-400; Stressgen Biotechnologies); mouse anti-Hsp90, 1:1000 (F8; Santa Cruz Biotechnology); and mouse anti-α-tubulin, 1:5000 (T9026; Sigma). Primary antibodies were probed using HRP-conjugated anti-rabbit Ig $F(ab')_2$ and anti-mouse Ig $F(ab')_2$ (1:5000; GE Healthcare) secondary antibodies and detected with the ECL Plus kit (GE Healthcare). An LAS-3000 imaging system was used to produce digital images and to quantify band intensities, which were then analyzed with Image Gauge software version 4.22 (Fujifilm, Tokyo, Japan). Densitometric values of AR were normalized to those of endogenous α-tubulin. Relative signal intensity (RSI) was computed as the signal intensity of each sample divided by that of mock-transfected cells (see Fig. 1) or AR-24Q/CHIP$^{(-/-)}$ or AR-97Q/CHIP$^{(-/-)}$ mice (see Fig. 6).

Immunoprecipitation from mouse tissues was performed using 1 mg of total protein lysed in CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma), 15 μl of protein G Sepharose (GE Healthcare) and 2.5 μl of anti-myc antibody (Medical and Biological Laboratories). Protein was eluted from beads by boiling for 5 min in 15 μl of elution buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 60 μl/ml 2-mercaptoethanol, 10% glycerol) and loaded on SDS-polyacrylamide gels.

For the AR ubiquitination assay, full-length ARs were constructed by subcloning AR inserts derived from pCR-AR24 (24 CAG repeats) or pCR-AR97 (97 CAG repeats) into the pDsRed monomer mammalian expression vector (Takara Bio, Otsu, Japan). SH-SY5Y cells were seeded into 60 mm plates and cotransfected with plasmids encoding DsRed-AR and either CHIP or mock. Cells were exposed to MG132 (5 μM) for a 24 h period. Extracts were prepared, and AR was immunoprecipitated with anti-DsRed antibody. Blots were probed as described for Western blots with ubiquitin antibody (1B3; Medical and Biological Laboratories).

Filter trap assay. To quantify the large-molecular aggregated and soluble forms of the mutant AR protein, filter trap assays of total tissue homogenates from the spinal cord and muscle of male AR-24Q or 97Q mice (16 weeks of age) were performed as described previously (Adachi et al., 2003). Proteins were filtered through a 0.2 μm cellulose acetate membrane (Sartorius, Goettingen, Germany) using a slot-blot apparatus (Bio-Rad). We also put 0.45 μm nitrocellulose membranes (Bio-Rad) under the cellulose acetate membrane to capture the monomeric AR protein passing through this membrane. Only the larger-sized mutant AR protein was retained on the cellulose acetate membrane (pores 0.2 μm in diameter), whereas the nitrocellulose membrane captured protein of all sizes. Samples of protein, 200 μg for spinal cord, and 80 μg for muscle, were prepared in a final volume of 200 μl of lysis buffer, loaded, and gently vacuumed. Slot blots were probed as described for Western blots by an antibody against AR (H-280; Santa Cruz Biotechnology) or α-tubulin (T9026; Sigma).

Pulse-chase labeling assay. Cells were transfected as described above, starved for 1 h in methionine- and cysteine-free DMEM containing 10% dialyzed fetal calf serum, and then labeled for 1 h with 150 μCi of Redivue Pro-Mix L-[^{35}S] *in vitro* cell-labeling mix (GE Healthcare) per milliliter. After washing in PBS, the cells were chased for the indicated time intervals in complete medium. Immunoprecipitation was performed using equivalent amounts of protein lysates, 10 μl of protein G Sepharose (GE Healthcare), and 5 μl of anti-AR antibody (N20; Santa Cruz Biotechnology) as described above. Each sample was separated by 5–20% SDS-PAGE, and analyzed by phosphorimaging (Typhoon 8600 PhosphorImager; GE Healthcare) and Image Gauge software version 4.22 (Fujifilm).

Quantitative real-time reverse transcription-PCR. The levels of AR mRNA were determined by real-time Taqman PCR as described previously (Ishigaki et al., 2002). Total RNA was isolated from SH-SY5Y cells using the RNeasy Mini kit (Qiagen, Valencia, CA) and from transgenic mouse spinal cord and muscle by homogenizing in Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (5 μg) from cells and mouse spinal cord and muscle were reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Real-time Taqman reverse transcription (RT)-PCR was performed in a total volume of 50 μl, containing 25 μl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen) and 10 μM each primer. PCR products were detected by the iCycler system (Bio-Rad). The reaction conditions were 95°C for 15 min and then 45 cycles of 15 s at 94°C, 30 s at 55°C, and 30 s at 72°C. As an internal standard control, the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously quantified. PCR primers were designed as described previously (Waza et al., 2005). RSI was computed as the signal intensity of each sample divided by that of mock-transfected cells (see Fig. 1*E*) or AR-24Q/CHIP$^{(-/-)}$ or AR-97Q/CHIP$^{(-/-)}$ mice (see Fig. 6*C*).

Statistical analysis. Data were analyzed by unpaired *t* tests in the experiments shown in Figures 1, *B* and *E*, and 6 and log-rank tests for survival rate in that shown in Figure 4*D* using Statview software version 5 (Hulinks, Tokyo, Japan). Statistical significance of the data shown in Figures 1*A*, 4, *A–C* and *F*, and 5*G–H* was examined by the Williams test for multiple comparisons using Microsoft Excel 2004 (Microsoft, Redmond, WA).

Results

Effect of CHIP overexpression on expression and ubiquitination of AR *in vitro*

CHIP directly interacts with and degrades the wild-type AR through its N-terminal conserved motif (He et al., 2004) and induces wild-type AR ubiquitination (Cardozo et al., 2003). To address the question of whether CHIP overexpression promotes the degradation of polyQ-expanded AR, we transfected SH-SY5Y cells stably expressing the wild-type (AR-24Q) or mutant (AR-65Q) AR with varying amounts of CHIP or mock as control. Although immunoblot analysis demonstrated a dose-dependent decline in both wild-type and mutant AR expression after CHIP overexpression (Fig. 1*A*), the monomeric mutant AR decreased significantly more than did the wild type ($p < 0.05$) (Fig. 1*B*), suggesting that the mutant AR is more sensitive to CHIP than is the wild type. CHIP was reported to interact with HSF1 and increase Hsp chaperone levels (Dai et al., 2003; Qian et al., 2006); however, the expression levels of Hsp90, Hsp70, and Hsp40 were not changed after CHIP overexpression (Fig. 1*A*). This finding is consistent with a previous report (Miller et al., 2005) and suggests that the stress-induced response is different among different cell types.

To determine whether the enhanced degradation of mutant AR was attributable to protein degradation or to changes in RNA expression, the turnover of wild-type and mutant AR was then assessed with a pulse-chase labeling assay. SH-SY5Y stable cells were transiently transfected with mock or CHIP constructs. Without CHIP, the wild-type and mutant ARs were degraded almost equally, as reported previously (Bailey et al., 2002; Lieberman et al., 2002). In the presence of overexpressed CHIP, however, the wild-type and mutant ARs had half-lives of 3.6 and 2.7 h, respectively (Fig. 1*D*), whereas mRNA levels for both the wild-type and mutant AR were quite similar (Fig. 1*E*). These data indicate that CHIP preferentially degrades the mutant AR protein without altering mRNA levels.

The preferential degradation of mutant AR by CHIP suggests that CHIP may promote mutant AR ubiquitination, thereby targeting it for degradation. To assess this possibility, SH-SY5Y cells were transiently cotransfected with CHIP and DsRed-tagged AR-24Q or AR-97Q, and the cell lysates were immunoprecipitated with anti-DsRed. In this experiment, blots were probed with anti-