are responsible for the pathogenesis of polyglutamine diseases. Such clarification would expand therapeutic options for these devastating disorders.

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Field strengths and sequences influence putaminal MRI findings in multiple system atrophy

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Multiple system atrophy (MSA) is a sporadic neurodegenerative disease that is difficult to diagnose. MRI can show signal abnormalities such as putaminal hyperintensity, hyperintense putaminal rim, and significant putaminal hypointensity, strengthening a diagnosis of MSA.[1-7] However, these abnormalities are highly variable among the published cases and reports.[1-7] Differences of magnetic field strengths and sequences and pathologic features could explain such variability of putaminal signal changes, but the precise nature of putaminal signal changes remains unclear.[1-7]

We report an MRI study of the cadaveric tissue from a patient with MSA performed to investigate putaminal signal changes with different magnetic field strengths and then correlated the findings with histopathologic observations.

Case report. A 54-year-old man first noticed gait disturbance at age 48 years and dysuria and erectile dysfunction at age 49 years, all of which steadily worsened. Neurologic examinations at age 50 years revealed urinary dysfunction and parkinsonism poorly responsive to L-dopa, including bradykinesia, rigidity, and postural instability. His condition worsened progressively, and he died of pneumonia 6 years after onset of illness.

Autopsy was performed 3 hours post-mortem. The brain was fixed in 10% buffered formalin. A 2-cm coronal section that included the putamen was used for MRI examination with 0.35-T (OPART, Japan) and 1.5-T (VISART, Japan) scanners using a surface coil. Images were obtained in the coronal plane using T2-weighted fast spin-echo (FSE) at 0.35 T and 1.5 T (2,000/120/5) and T2*-weighted gradient echo (GE) sequences (38/24/5; flip angle, 15) at 1.5 T. For all sequences, a 3-mm slice thickness, a field of view of 75 × 75 mm, and a matrix of 192 × 192 mm were used.

T2-weighted FSE at 0.35 T showed marked putaminal hyperintensity, whereas T2-weighted FSE at 1.5 T showed little signal change in the putamen except for mild hyperintensity in the outer margin. T2*-weighted GE at 1.5 T, representing the most sensitive MRI sequences for evaluation of putaminal iron deposition, showed low intensity (figure, A through C). For histopathologic evaluation, 5-μm-thick paraffin-embedded sections were prepared from the same position and stained with hematoxylin and eosin, Holzer, Gallyas–Braak, Bodian, and Prussian blue stains. Neuronal loss and gliosis were noted, as were glial cytoplasmic inclusions, all typical histopathologic findings for MSA. The Holzer stain showed severe gliosis predominantly in the dorsolateral putamen (figure, D). No calcification was present. Extracellular and intracellular iron deposits were present extensively in this area (figure, E). The high signal intensity on T2-weighted FSE at 0.35 T was highly consistent with the distribution of gliosis. Conversely, the low intensity on T2*-weighted GE at 1.5 T corresponded well to the region of iron deposits.

Discussion. This is the first study to correlate putaminal MRI findings at different magnetic field strengths and sequences in postmortem MSA brain with histologic findings. Although artifact from formalin and varying relaxation rates caused by a different hydration state of the tissue should be considered, direct comparison of post-mortem MRI and histologic findings would be beneficial particularly for understanding MR signal differences in the putamen of patients with MSA because putaminal findings at autopsy could differ from those at examination of MRI during life.

MRI using FSE at 1.5 T, which is used widely for diagnosis of MSA, showed only mild hyperintensity in the outer putaminal margin, which can be observed even in healthy subjects. Conversely, FSE at 0.35 T and GE at 1.5 T revealed significant putaminal hyperintensity and hypointensity. Histopathologic study showed severe gliosis and an increase in ferritin deposition, particularly in the dorsolateral putamen. Gliosis probably increased signal intensity in T2-weighted images, whereas ferritin enhanced T2 relaxation in the order FSE at 0.35 T, FSE at 1.5 T, and GE at 1.5 T. Taken together, topography of gliosis predominantly in dorsolateral parts, severities of gliosis and iron deposits, and magnetic field strengths and sequences would determine the putaminal MRI findings and can result in a normal putaminal appearance under FSE at 1.5 T despite the presence of pathologic

Figure. (A) MRI operated at T2-weighted fast spin-echo (FSE) at 0.35 T. Significant putaminal hyperintensity is evident. (B) MRI operated at T2-weighted FSE at 1.5 T. The greater part of the putamen shows isointensity except for mild hyperintensity of the rim at the outer margin. (C) MRI operated at T2*-weighted gradient echo at 1.5 T. Severe putaminal hypointensity is observed. (D) Holzer stain. Exclusive severe gliosis is seen in the putamen. (E) Prussian blue stain. An increase in ferritin deposits is widespread in the putamen. Original magnification, ×400.

change. In contrast, the higher insensitivity to paramagnetic effects can provide the hyperintensity at 0.35 T.

Putaminal hyperintensity in MRI was first described as a finding typical to MSA.[1] Many subsequent reports have confirmed this, but sensitivity and specificity were found to be highly variable.[2-7] In MR survey of patients with MSA, it should be considered that histopathologic features differentially affect MR findings at different magnetic field strengths and sequences.

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Sweet relief for Huntington disease

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Oral delivery of a simple, nontoxic sugar molecule alleviates symptoms of Huntington disease in a mouse model (pages 148–154).

A CAG repeat was first pinned to a neurological disorder—spinal and bulbar muscular atrophy (SBMA)—in 1991 (ref. 1). The pathological influence of the affected gene, an androgen receptor, eventually paled beside the growing realization that the repeat itself was key to the disease. A number of other neurological diseases have since been linked to glutamine-encoding CAG repeats, most prominent among them Huntington disease.

Despite the excitement they have sparked, these discoveries have as yet done little to improve the lives of patients, including more than 35,000 with Huntington disease in the United States. In this issue, Tanaka *et al.* move us closer to reaping the benefits of this decade of research. The authors report that in mouse models of Huntington disease, treatment with a nontoxic sugar substance can prevent the development of the brain pathology associated with Huntington disease, and can delay the progress of symptoms such as motor dysfunction. The approach stands out as the most exciting therapeutic prospect to date for Huntington disease, and also holds promise for the entire class of polyglutamine disorders.

In addition to Huntington disease and SBMA, other polyglutamine disorders include several forms of spinocerebellar ataxia, as well as dentatorubral pallidoluysian atrophy[2]. Outside of the polyglutamine stretch, each causative protein seems unrelated, and removal of the causative protein genetically or by other means does not result in disease in humans or animal models[2]. Yet the diseases show phenotypic similarities. The clinical features depend at least partially on the number of CAG repeats, and are influenced by the meiotic instability of the repeat length. These clinical and genetic similarities imply that polyglutamines induce toxicity, although loss of the normal function of causative proteins may influence the disease[3].

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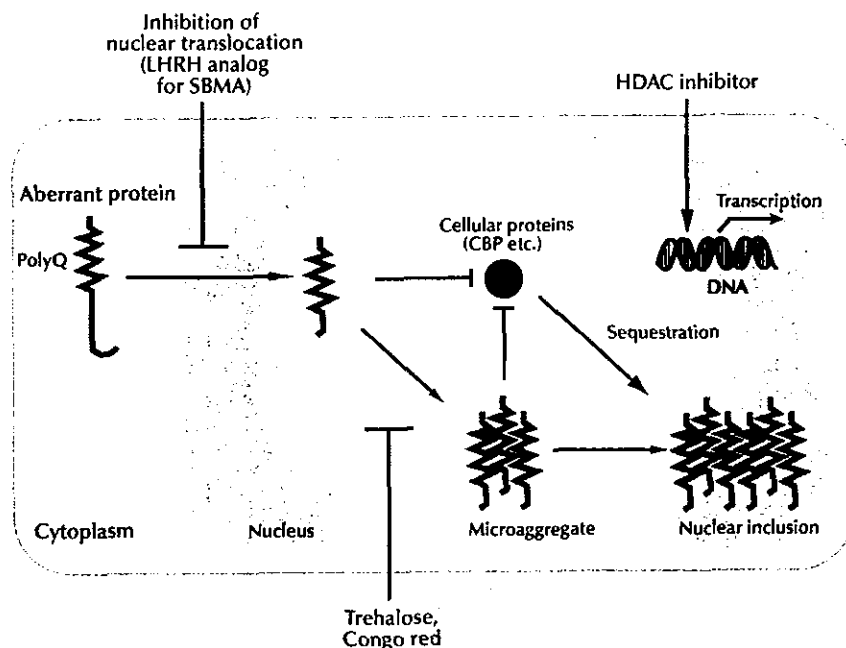e-mail: sobueg@med.nagoya-u.ac.jp

Figure 1 Therapeutic approaches for polyglutamine diseases. Several approaches attempt to mitigate the toxicity of polyglutamine (poly Q) tract–containing proteins. Leuprorelin prevents nuclear uptake of mutant androgen receptor, resulting in the rescue of neuromuscular phenotypes of SBMA. Histone deacetylase inhibitors ameliorate transcription of affected cells. Tanaka *et al.* found that a disaccharide, trehalose, inhibits aggregation of a protein with an expanded polyglutamine tract, especially in the nucleus (as does Congo red). LHRH, luteinizing hormone–releasing hormone; CBP, CREB-binding protein.

How do the glutamine repeats affect the cell? The expansion of the polyglutamine tract alters protein conformation, resulting in the formation of insoluble aggregates. These aggregates sequester normal cellular proteins such as transcription factors, heat shock proteins, ubiquitin, and proteasome components. The propensity of the polyglutamine-containing proteins to aggregate depends on the length of the polyglutamine stretch and is enhanced by protein cleavage through caspase activation.

Patients with polyglutamine diseases show loss of specific types of neurons. The cells that do not die contain inclusions rich in polyglutamine-containing proteins, mainly in the nucleus. These inclusions are found in the vulnerable neurons, implying a direct role in pathogenesis. Whether these aggregates are toxic—a notion supported by a large body of evidence—or reflect a protective response is controversial.

A dramatic study earlier this year bolstered arguments in favor of toxicity. Injection of the dye Congo red prevented formation of nuclear inclusions and alleviated symptoms in a mouse model of Huntington disease[4].

Tanaka *et al.*, favoring the notion that the aggregates are toxic, sought a new aggregation inhibitory therapy for Huntington disease[5]. The authors first used an *in vitro* aggregate formation assay, with myoglobin containing an expanded polyglutamine tract as a target molecule. Using this screening system, they discovered that disaccharides potently inhibited aggregate formation. Trehalose, the most effective disaccharide, selectively stabilized a protein containing a long polyglutamine tract, but not a protein with the normal number of glutamines. The authors confirmed this stabi-

lization in a cell model expressing the aberrant form of huntingtin, the causative protein of Huntington disease. The stabilization of myoglobin with an elongated polyglutamine tract results[6] accounts for the trehalose-mediated suppression of aggregation formation.

The authors went on to test transgenic mouse models of Huntington disease. They found that trehalose-treated mice had fewer nuclear inclusions than untreated mice. Trehalose also improved motor dysfunction and prolonged survival, without any deleterious side effects. The extremely low toxicity and high water-solubility of this compound make this an attractive therapeutic approach, although the therapeutic effects seem to result from prevention of new aggregate formation, not from reversal of the pathology. The data reinforce the rationale of aggregation-inhibitory therapy for polyglutamine diseases (Fig. 1). Trehalose is now ready for phase 1 safety trials in humans.

Other approaches to polyglutamine disease also show promise. Nuclear accumulation of polyglutamine-containing proteins before aggregate formation is probably an essential step in pathogenesis. A mutation that inactivates the nuclear localization signal in ataxin-1, the causative protein in spinocerebellar ataxia-

1, nullifies polyglutamine-induced neurodegeneration in a transgenic mouse model[7]. In cell culture, chemically synthesized polyglutamine peptides induce neuronal cell death only when they are directed into the nucleus[8]. These observations suggest that nuclear-directed transport of mutant proteins is an alternative target of intervention (Fig. 1), although cytosolic events should not be neglected[3].

Androgen deprivation therapy in a transgenic mouse model of SBMA clearly demonstrates the usefulness of this therapeutic strategy[9]. A luteinizing hormone–releasing hormone analog, leuprorelin, prevents nuclear translocation of polyglutamine-containing androgen receptor protein, resulting in a significant improvement of disease[9]. A clinical trial with leuprorelin, currently under way in Japan, should clarify the clinical benefit of this drug for SBMA patients.

Transcriptional dysregulation, an event downstream of polyglutamine aggregation, can also be targeted (Fig. 1). Transcriptional coactivators including CREB-binding protein are sequestrated in the inclusion, and are also enfeebled by interaction with soluble polyglutamine tract–containing proteins[3]. An increase in acetylation of nuclear histone proteins, facilitated by histone deacetylase

inhibitors, ameliorates neurodegeneration in a mouse model of Huntington disease[10,11]. These compounds have also been used for patients with malignancies. Clinical trials of histone deacetylase inhibitors should be planned carefully, however, taking the hazardous side effects into account.

The promising results of these preclinical studies are ushering in a new era in polyglutamine research: the therapeutic stage. The new investigations also encourage us to continue to search for new, clinically applicable compounds such as aggregation inhibitors. The intensive basic research is bearing fruit, and shows promise of continuing to do so as we move into clinical trials.

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PAPER

Multiple regional ^{1}H-MR spectroscopy in multiple system atrophy: NAA/Cr reduction in pontine base as a valuable diagnostic marker

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Objective: We performed ^{1}H-MR spectroscopy (^{1}H-MRS) on multiple brain regions to determine the metabolite pattern and diagnostic utility of ^{1}H-MRS in multiple system atrophy (MSA).
Methods: Examining single voxels at 3.0 T, we studied metabolic findings of the putamen, pontine base, and cerebral white matter in 24 MSA patients (predominant cerebellar ataxia (MSA-C), n = 13), parkinsonism (MSA-P), n = 11), in 11 age and duration matched Parkinson's disease patients (PD) and in 18 age matched control subjects.
Results: The N-acetylaspartate to creatine ratio (NAA/Cr) in MSA patients showed a significant reduction in the pontine base (p<0.0001) and putamen (p = 0.02) compared with controls. NAA/Cr in cerebral white matter also tended to decline in long standing cases. NAA/Cr reduction in the pontine base was prominent in both MSA-P (p<0.0001) and MSA-C (p<0.0001), and putaminal NAA/Cr reduction was significant in MSA-P (p = 0.009). It was also significant in patients who were in an early phase of their disease, and in those who showed no ataxic symptoms or parkinsonism, or did not show any MRI abnormality of the "hot cross bun" sign or hyperintense putaminal rims. NAA/Cr in MSA-P patients was significantly reduced in the pontine base (p = 0.001) and putamen (p = 0.002) compared with PD patients. The combined ^{1}H-MRS in the putamen and pontine base served to distinguish patients with MSA-P from PD more clearly.
Conclusions: ^{1}H-MRS showed widespread neuronal and axonal involvement in MSA. The NAA/Cr reduction in the pontine base proved highly informative in the early diagnosis of MSA prior to MRI changes and even before any clinical manifestation of symptoms.

M ultiple system atrophy (MSA) is a sporadically occurring neurodegenerative disease that presents parkinsonism, cerebellar ataxia, autonomic failure, and pyramidal signs of varying severity during the course of illness.[1-3] Neuropathological findings consist of a varying neuronal loss, gliosis, and demyelination with widespread regional involvement, particularly including the striatonigral, olivopontocerebellar, and autonomic nervous systems.[4-6] The tempo and progression of multiple system involvement vary widely among individual MSA patients and have been closely related to both functional deterioration and prognosis by clinical evaluation.[7] Thus, assessing the multi-regional involvement in MSA is essential for accurate diagnosis, counselling of patients and families, optimal management of symptoms, and the usefulness of future therapeutic trials.

Proton magnetic resonance spectroscopy (^{1}H-MRS) is a valuable non-invasive MR technique for monitoring brain metabolism in vivo.[8-18] The major peaks of the ^{1}H-MRS spectrum, corresponding to N-acetylaspartate (NAA), creatine (Cr), and choline (Cho) containing phospholipids, have been used to evaluate neuronal loss and active myelin breakdown. The ratio of NAA to Cr (NAA/Cr) is considered a metabolic marker reflecting the functional status of neurones and axons in the brain, with a decrease indicating neuronal or axonal loss or dysfunction. Previous studies using ^{1}H-MRS in MSA with predominant parkinsonism (MSA-P) reported a significant NAA/Cr reduction in the striatum compared with Parkinson's disease (PD) patients and normal subjects.[11-14] However, the pontine base and cerebral white matter, which are also pathologically involved

in MSA, have not been fully assessed by ^{1}H-MRS. Recent technical innovations have permitted ^{1}H-MRS at higher magnetic field strengths.[19-21] Multi-regional data can be obtained from single voxel ^{1}H-MRS within a short examination time with increasing signal to noise ratio (SNR).

Our purpose was to assess the extent of multiple system involvement in patients with MSA by using multiple regional single voxel ^{1}H-MRS including the putamen, pontine base, and cerebral white matter (CWM), and to further assess the diagnostic value of the regional ^{1}H-MRS.

METHODS

All patients and control subjects gave written informed consent. The MR protocol was approved by the Ethics Committee of the Nagoya University School of Medicine. Twenty four patients with MSA (12M, 12F; mean (SD) age 61 (7) years old), 11 patients with PD (5M, 6F; 63 (9) years old), and 18 control subjects with no history of any neurological disease (10M, 8F; 59 (7) years old) were studied. No significant differences in male to female ratio or age were noted among the three groups. The duration from initial
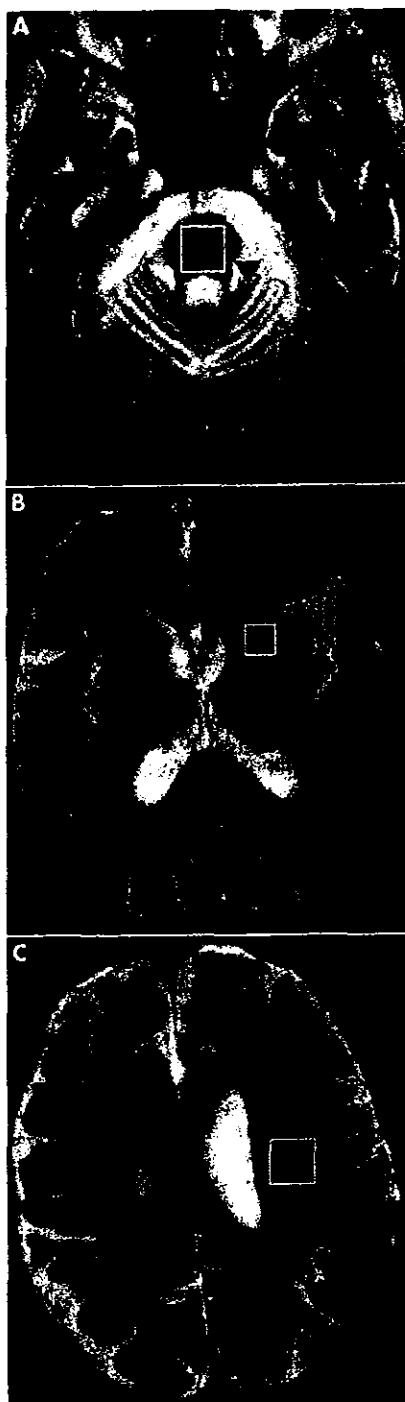
Figure 1 Location of volumes of interest are shown by squares in the pontine base (A), putamen (B), and white matter of the frontal lobe (C). Additionally, in these images, an HCB sign is present in the pons (A), as is a hyperintense putaminal rim (B). Axial T2 weighted images (3.0 T; TR: 3970, TE: 80), with respective findings are indicated by arrowheads.

symptoms to MRI and MRS evaluation also showed no differences between MSA and PD patients (MSA: 3.7 (2.4) years; PD; 4.4 (2.2) years, p>0.4). Diagnoses of all MSA and PD patients were "probable" according to established diagnostic criteria.[1] [3] As for subtypes of MSA,

cerebellar dysfunction (MSA-C) predominated in 13 patients and parkinsonism (MSA-P) in 11. We classified patients into two groups according to the presence of parkinsonian signs in MSA, based on the consensus statement for MSA diagnosis. Patients with bradykinesia plus at least one sign of either rigidity, postural instability, or tremor were considered to manifest parkinsonism and designated as "parkinson-ism+", while others were taken to be "parkinsonism−". As for cerebellar dysfunction, patients with gait ataxia plus at least one sign of ataxic dysarthria, limb ataxia, or sustained gaze evoked nystagmus were considered "ataxia+", and others as "ataxia−" based on the consensus criteria.[3] Six of nine MSA-P patients and all PD patients were taking medication for parkinsonism (benserazide/levodopa 25/100 mg, or carbidopa/levodopa 10/100 mg, two or three times daily). All PD patients showed a good response to treatment.

MRI and ^{1}H-MRS were performed with a 3.0 T system (Bruker, Ettlingen, Germany) using a standard head coil with circular polarisation. The imaging protocol consisted of sagittal T1 weighted spin echo sequences (repetition time (TR), 460 ms; echo time (TE), 14 ms) and transverse T2 weighted sequences (TR, 3970 ms; TE, 80 ms). Slice thickness was 6 mm with a 1.2 mm gap and a 512×384 matrix. We evaluated whether a "hot cross bun" (HCB) sign was present in the pons and whether the putamen showed a hyperintense rim (HPR), according to the criteria described in previous reports (fig 1A, B).[7] [23-26] The spectroscopic volume of interest (VOI) was placed in the pontine base (2.2 to 3.4 cm³), the putamen (1 cm³), and the CWM (3.4 cm³; fig 1A to C). Voxel size was chosen to be as small as possible while maintaining an acceptable SNR in order to minimise the partial volume effect. Care was taken not to incorporate cerebrospinal fluid spaces within a VOI. The VOI in the putamen was placed on the more affected side, and the frontal lobe VOI was ipsilateral to the putaminal VOI. [1]H-MR spectra were acquired using a point resolved spectroscopy sequence with chemical shift selective water suppression. Spectral parameters were as follows: TR: 2000 ms; TE: 30 ms; averages: 256 in the putamen, and 64 each in the centrum semiovale and pons; data points: 1024. A shimming procedure focused on the water signal was performed to obtain a uniform and homogenous magnetic field. After Fourier transformation and zero order phase correction, relative metabolite concentrations for NAA at 2.0 ppm, Cr at 3.0 ppm, and Cho at 3.2 ppm were determined by Lorentzian curve fitting of the corresponding resonance in the frequency spectra. The baseline was corrected for purposes of data presentation. From these data, the metabolite ratios NAA/Cr, and Cho/Cr were determined as semiquantitative values. Post-procedural processing was performed by the same radiologist (HF). All preconditioning, spectroscopic measurements, and processing were performed with Paravision 2.01 software (Bruker). Total examination time including MRI and ^{1}H-MRS was <1 hour. One MSA-C patient with severe pontine atrophy was excluded because a good pontine spectrum could not be obtained.

Values obtained were entered into a database for further statistical analysis. The Mann-Whitney U test and the Kruskal-Wallis test for nonparametric statistics were performed as appropriate. When the Kruskal-Wallis test indicated differences among groups, in a multiple comparison analysis, Scheffé's test was used to identify which group differences accounted for the significant p value. Relationships of NAA/Cr reduction to duration of illness were analysed using Pearson's correlation coefficient. Calculations were performed using the Stat View statistical software package (Abacus Concepts, Berkeley, CA, USA). Statistical significance was defined as p<0.05.
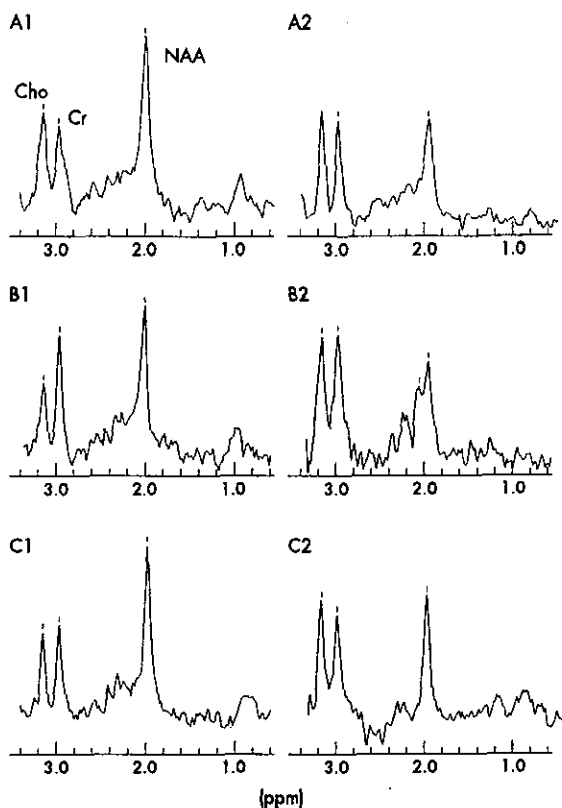
Figure 2 Representative ¹H-MRS spectra from control and MSA subjects. A1, B1, and C1 represent spectra from a control subject's pontine base, putamen, and cerebral white matter, respectively. A2, B2, and C2 represent spectra from those same three regions in an MSA patient. NAA, N-acetylaspartate; Cho, choline; Cr, creatine; MSA, multiple system atrophy; CWM, cerebral white matter.

RESULTS

Widespread NAA/Cr reduction in MSA in multiple regional ¹H-MRS

A representative MSA patient (fig 2) showed a marked reduction of the NAA peak in the pontine base, putamen, and cerebral white matter compared with controls. NAA/Cr was significantly reduced in the pontine base of MSA patients (p<0.0001) and in the putamen (p = 0.02) compared with controls. MSA patients also showed a lower NAA/Cr in cerebral white matter than controls, but this difference was not statistically significant (p = 0.12). Cho/Cr was only slightly increased in MSA, and no significant differences were found among the three groups for the pontine base, putamen, and CWM.

Prominent NAA/Cr reduction in pontine base in both MSA-C and MSA-P

Significant reductions of NAA/Cr were evident in the pontine base, putamen, and CWM in MSA-C and MSA-P compared with controls (fig 3A–C). MSA-C patients showed a significant reduction of NAA/Cr in the pontine base (p<0.0001) and CWM (p = 0.02), but not in the putamen. MSA-P patients showed a significant reduction of NAA/Cr in the pontine base (p<0.0001) and putamen (p = 0.009) but not in the CWM. These observations indicate that the NAA/Cr reduction in the pontine base was significant in both MSA-C and MSA-P. Cho/Cr
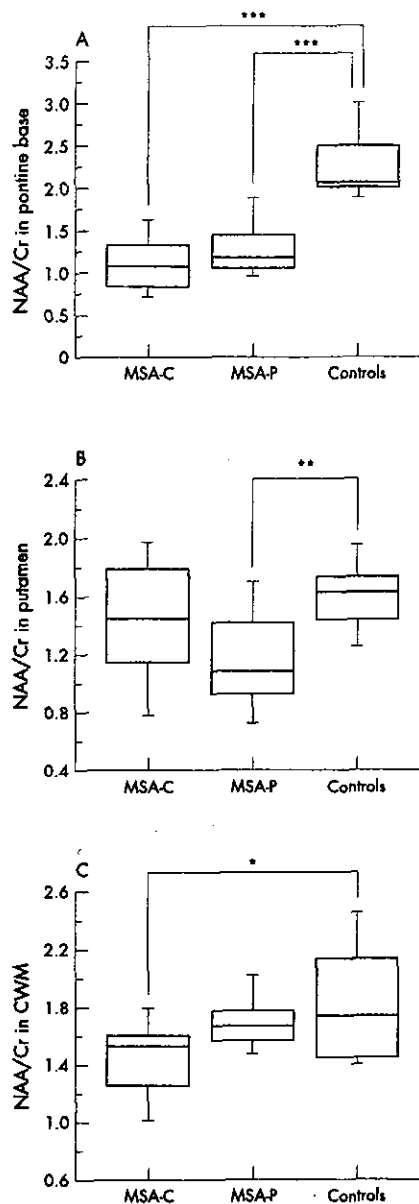

Figure 3 Box and whisker plot of the NAA/Cr ratio. Horizontal lines indicate median values. Boxes extend from the 25th to the 75th percentile. A, B, and C respectively show NAA/Cr in the pontine base, putamen, and cerebral white matter, comparing MSA-C, MSA-P, and control subjects. *p=0.02, **p=0.009, and ***p<0.0001 by Scheffé's test, respectively. NAA, N-acetylaspartate; Cr, creatine; Cho, choline containing component; MSA-C, multiple system atrophy with cerebellar ataxia predominant; MSA-P, multiple system atrophy with parkinsonism predominant.

was not changed in MSA-P or MSA-C compared with controls.

Relation of NAA/Cr reduction in pontine base with disease phase, motor symptoms and MRI abnormalities in MSA

In terms of disease duration, the NAA/Cr reduction was most significant in the pontine base of patients with MSA even in an early phase of illness (fig 4). A tendency toward an inverse
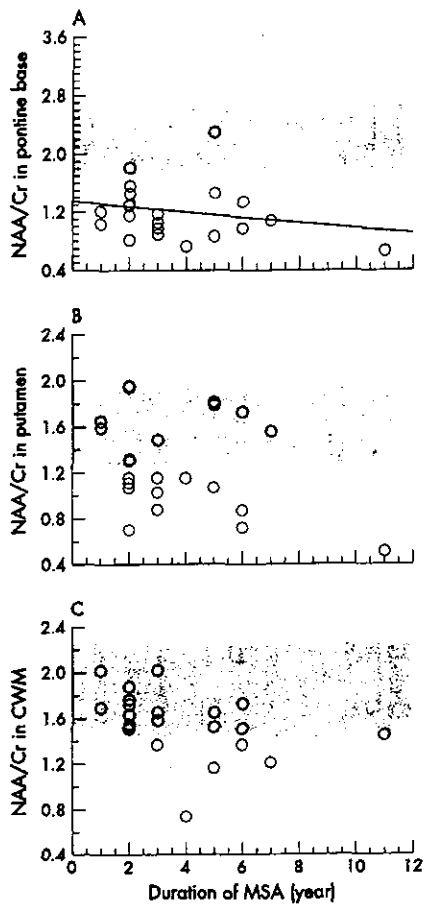
Figure 4 Correlation with duration of MSA of individual NAA/Cr ratios in the pontine base (A), putamen (B), and cerebral white matter (C). The shaded area corresponds to the mean (SD) of NAA/Cr in control subjects. NAA, N-acetylaspartate; Cr, creatine; CWM, cerebral white matter; MSA, multiple system atrophy.

relationship between disease duration and NAA/Cr in the three regions was observed, but did not attain significance (pontine base: $r = -0.24$, $p = 0.29$; putamen: $r = -0.32$, $p = 0.14$; CWM: $r = -0.41$, $p = 0.06$). NAA/Cr in the pontine base was significantly reduced compared with controls even in patients who did not show ataxic symptoms ($p = 0.0006$, fig 5A-1). However, NAA/Cr in the putamen and white matter was not reduced in patients with ataxia (fig 5B-1, C-1). NAA/Cr in the putamen was markedly decreased in MSA patients with parkinsonism ($p = 0.02$, Fig 5B-2), whereas patients without it exhibited no significant reduction compared with controls. NAA/Cr reduction in the pontine base, on the other hand, was significant ($p < 0.0001$) irrespective of parkinsonism (Fig 5A-2).

The MRI revealed the HCB sign in the pontine base in eleven MSA patients (46%) and the HPR sign in six (25%). A significant reduction of NAA/Cr was seen in the pontine base even in patients without ($p < 0.0001$) as well as in those with an HCB sign ($p < 0.0001$; fig 5A-3). In the putamen and cerebral white matter, NAA/Cr values did not show any significant difference irrespective of the HCB sign (fig 5B-3, C-3). Moreover, NAA/Cr significantly decreased in the pontine base in patients both with and without HPR (fig 5A-4). NAA/Cr in the putamen and cerebral white matter did not show any significant differences

irrespective of HPR signs (fig 5B-4, C-4). Cho/Cr had no significant relationship to ataxic, parkinsonism, or MRI abnormalities.

NAA/Cr in pontine base in MSA-P and PD

NAA/Cr reduction in the pontine base was highly significant in patients with MSA-P compared with both controls and PD ($p < 0.0001$, $p = 0.001$; fig 6A). NAA/Cr in the putamen in MSA-P patients also showed a significant decrease compared with both controls and PD ($p = 0.003$, $p = 0.002$; fig 6B). No significant differences in NAA/Cr were noted in cerebral white matter between MSA-P and PD. These data indicate that the NAA/Cr reduction in the pontine base is a valuable marker to discriminate MSA-P from PD. In addition, combining individual NAA/Cr values for the pontine base and putamen further reduced the overlap between MSA-P and PD (fig 6D), suggesting that a combined assessment of the pontine base and putamen was more effective in discriminating between MSA-P and PD than individual area assessments. Cho/Cr did not display any significant changes in the pontine base, putamen or cerebral white matter.

DISCUSSION

We demonstrated widespread NAA/Cr reduction in the pontine base, putamen and in some cases, in the cerebral hemisphere, but no significant Cho/Cr alteration in patients with MSA using localised ^{1}H-MRS at 3.0 T. In this study, absolute metabolite concentrations were not measured. However, the specific conditions that may change the total Cr signal, such as trauma, hyperosmolar conditions, hypoxia, stroke, and tumours, were not included. Age was matched among MSA, PD, and control groups. Moreover, quantitative studies did not show significant Cr changes between MSA patients and control subjects.[11][16] Thus, the reduction of the NAA/Cr ratio in the present study can be considered due to a selective decrease in NAA levels.

NAA has been immunohistochemically demonstrated to localise almost exclusively within neurones and axons,[27][28] but some in vitro studies have also detected NAA expression in mature, immature, and undifferentiated oligodendrocytes.[29][30] Nevertheless, according to a recent study, in vivo MRS measurements of NAA remain axon specific, with no oligodendrocytes, nonproliferating oligodendrocyte progenitor cells, or myelin contributing to detectable NAA in the mature CNS.[31] This result supports the view that the widespread NAA/Cr reductions observed in this study ultimately reflect widespread neuronal and axonal involvement in MSA, although oligodendrocytes might influence the NAA levels to some degree.

The striking observation in this study is that the NAA/Cr reduction in the pontine base was the most significant among the three regions examined. That reduction was detected in the early phase of illness even in patients with no symptoms of ataxia or parkinsonism, or in patients without MRI abnormality of the HCB sign. Moreover, the pontine NAA/Cr reduction was significant even in MSA-P patients. In addition, NAA/Cr reductions in the pontine base were seen even in patients with no HPR sign in the lateral putamen. These observations suggest that NAA/Cr reduction in the pontine base is an accurate diagnostic marker for MSA even in patients in an early stage and a pre-symptomatic phase of ataxia or parkinsonism. The diagnostic focus of ^{1}H-MRS in MSA has been on the putamen,[11][14] whereas our results unequivocally demonstrated that MRS abnormality can be detected sooner and more universally in the pontine base than in the putamen in the course of the disease. The question is why a significant NAA/Cr reduction can be detected more readily in the pontine base than in the putamen. One reason may be that neuroaxonal degeneration
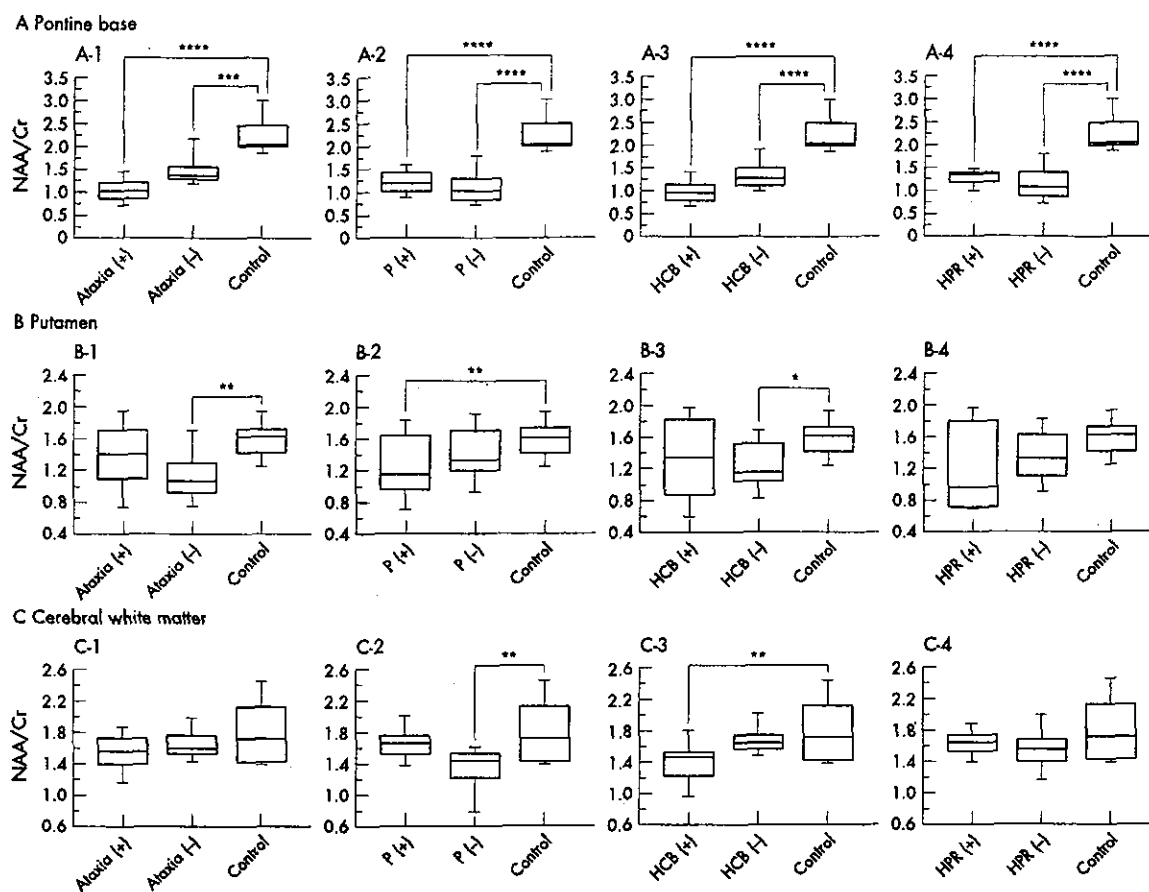
Figure 5 NAA/Cr in the pontine base (A), putamen (B) , and cerebral white matter (C) for MSA patients classified in terms of clinical features of ataxia (+ or −; A-1, B-1, C-1), parkinsonism (P; + or −; A-2, B-2, C-2), HCB on MRI (+ or −; A-3, B-3, C-3), and hyperintense putaminal rim (HPR) on MRI (+ or −; A-4, B-4, C-4). +, Presence; −, absence. *p=0.047, **p=0.02, ***p=0.0006, and ****p<0.0001 by Scheffé's test, respectively. NAA, N-acetylaspartate; Cr, creatine; MSA, multiple system atrophy; CWM, cerebral white matter.

in the pons would be more extensive than in the putamen. As the pontine base consists of the axons and neurones specifically involved in MSA (for example fibres of cerebellar inflow and outflow, corticospinal tracts and transverse pontine tracts), subclinical involvement of such fibres could be detected as a reduction of NAA/Cr. Furthermore, because, as we demonstrated previously, MSA-C is significantly more prevalent in Japan than MSA-P, compared with white populations in the western countries,[7] the cerebellar pontine system should be more profoundly involved in Japanese MSA patients. A second possibility is that the volume effect due to putaminal atrophy would ultimately include the neighbouring normal tissues in the VOI of the MRS, influencing the degree of the NAA/Cr reduction. As atrophy of the putamen is severe in certain patients, the size of the VOI is a limiting factor in ¹H-MRS for maintaining an acceptable SNR. Such volume effects due to putaminal atrophy can result in conflicting data. Clarke and Lowry reported an absence of significant reductions in basal ganglionic NAA/Cr in MSA,[16] precluding the use of NAA/Cr reductions in the striatum for differential diagnosis.[18] Disease duration in their patients averaged 7.9 years.[16] In contrast, mean disease duration in other reports showing significant NAA/Cr reductions in the striatum of MSA patients ranged from 3.2 to 4.5 years,[11-14] similar to the duration in our patients. Because, with longer duration, putaminal atrophy in patients with MSA-P becomes more severe, discrepancies could be explained by

differences in putaminal atrophy that can profoundly influence ¹H-MRS results. By avoiding this volume effect, MRS for the pontine base would provide a more accurate diagnostic marker.

Discriminating clearly between MSA-P and PD has long been a diagnostic problem from both therapeutic and prognostic viewpoints. Putaminal NAA/Cr reduction was significant in MSA-P patients compared with PD and control subjects, as previously reported.[11-14] However, as discussed above, the putaminal volume effect could influence the significance of putaminal NAA/Cr reduction, particularly in patients with advanced disease. Although brainstem and cerebellar involvement is an important and specific finding in differentiating MSA-P from PD,[26 32] the sensitivity of both clinical and MRI evaluations of these abnormalities is relatively low.[7 26] Based on our results, we believe that ¹H-MRS assessment of the pontine base would be of considerable value in the differential diagnosis between MSA-P and PD. However, combined ¹H-MRS study of the pontine base and putamen can provide a more sensitive differentiation between MSA-P and PD than a conventional single regional study, such as that of the putamen.

The cerebral hemisphere is involved more extensively in MSA than previously believed. Recently, Abe et al reported a significant decrease in NAA/Cr in MSA, involving Brodmann's areas 6, 8, and 46.[14] Moreover, Spargo et al
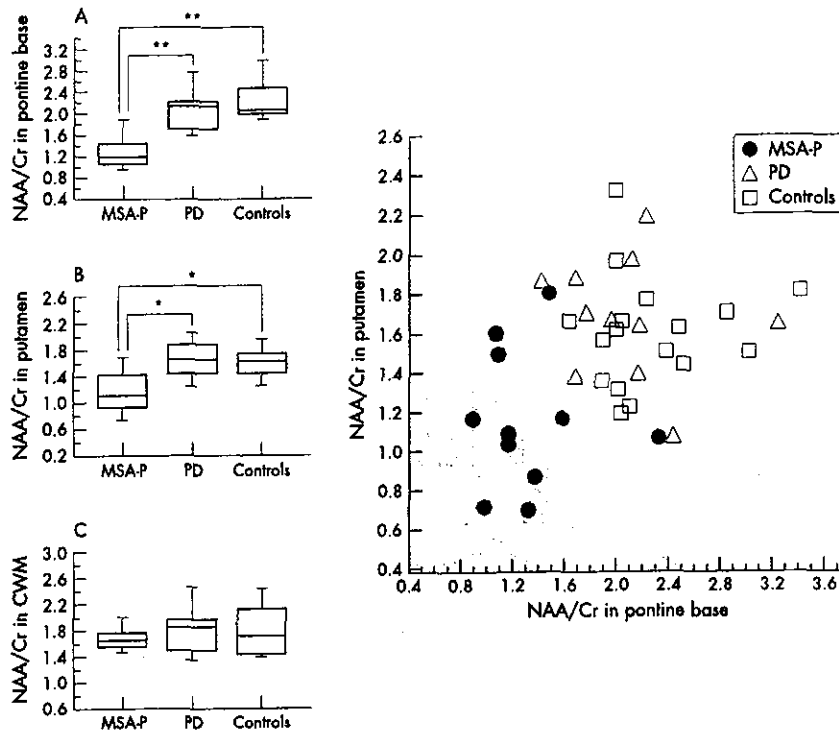
Figure 6 Box and whisker plot of the NAA/Cr ratio in the pontine base (A), putamen (B) and cerebral white matter (CWM, C) compared between MSA-P, PD, and controls. D is a scatter plot of the individual NAA/Cr data in the pontine base v putamen including MSA-P, PD, and control subjects. The scaled area corresponds to the mean−2 SD of NAA/Cr in the pontine base and putamen of control subjects. *p = 0.002, **p < 0.0001 by Scheffé's test, respectively. NAA, N-acetylaspartate; Cr, creatine; MSA-P, multiple system atrophy with parkinsonism predominant.

reported 18.7% and 21.4% neuronal loss in the primary and supplementary motor cortex, respectively." In addition, the degree of atrophy in cerebral hemispheric areas varies between individuals, often becoming severe in long standing cases." We found a mild overall reduction of NAA/Cr in CWM with a more significant NAA/Cr reduction in the subgroup with a longer duration of illness. This finding is in good agreement with previous ^{1}H-MRS reports and pathological observations.

Davie et al[1] reported a significant reduction of Cho/Cr ratio suggesting reduced membrane turnover in the lentiform nucleus in MSA, perhaps as result of cell loss. In the present study, Cho/Cr showed little change throughout the course of disease in the putamen, pontine base, and CWM, in agreement with other reports.[12-14] [16] The relevance of this discrepancy is uncertain. One possible explanation is the difference of technical factors such as size of VOI and echo time. On the other hand, pathological study shows not only cell loss but also widely and variously distributed myelin degeneration in MSA brains that may increase the Cho.[15] Thus, heterogeneity of lesions in association with disease stage also may influence the Cho/Cr result. Further long-itudinal studies and comparison of ^{1}H-MRS with histological findings will be needed to clarify the uncertainty as to the Cho/Cr ratio in MSA.

In conclusion, localised ^{1}H-MRS at 3.0 T in multiple regions showed widespread neuronal and axonal involvement in patients with MSA. NAA/Cr reduction in the pontine base provided a significant diagnostic marker for MSA irrespective of the disease form of MSA-P or MSA-C, disease duration, symptomatic manifestations, or MRI abnormalities. Moreover, combined ^{1}H-MRS study of the pontine base and putamen proved particularly effective in differentiating MSA from PD. We believe that ^{1}H-MRS would provide an early and accurate MSA diagnosis, an enhanced understanding of its pathogenetic mechanism, and the conclusiveness needed for future therapeutic trials.

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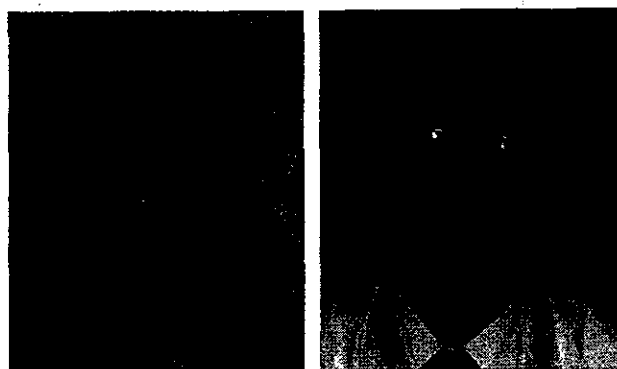
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Spinal and bulbar muscular atrophy: ligand-dependent pathogenesis and therapeutic perspectives

Abstract Spinal and bulbar muscular atrophy (SBMA) is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. SBMA exclusively affects males, while females are usually asymptomatic. The molecular basis of SBMA is the expansion of a trinucleotide CAG repeat, which encodes the polyglutamine (polyQ) tract in the first exon of the androgen receptor (AR) gene. The histopathological hallmark is the presence of nuclear inclusions containing mutant truncated ARs with expanded polyQ tracts in the residual motor neurons in the brainstem and spinal cord, as well as in some other visceral organs. The AR ligand, testosterone, accelerates AR dissociation from heat shock proteins and thus its nuclear translocation. Ligand-dependent nuclear accumulation of mutant ARs has been implicated in the pathogenesis of SBMA. Transgenic mice carrying the full-length human AR gene with an expanded polyQ tract demonstrate neuromuscular phenotypes, which are profound in males. Their SBMA-like phenotypes are rescued by castration, and aggravated by testosterone administration. Leuprorelin, an LHRH agonist that reduces testosterone release from the testis, inhibits nuclear accumulation of mutant ARs, resulting in the rescue of motor dysfunction in the male transgenic mice. However, flutamide, an androgen antagonist promoting nuclear translocation of the AR, yielded no therapeutic effect. The degradation and cleavage of the AR protein are also influenced by the ligand, contributing to the pathogenesis. Testosterone thus appears to be the key molecule in the pathogenesis of SBMA, as well as main therapeutic target of this disease.

Keywords Spinal and bulbar muscular atrophy ·
Polyglutamine · Androgen receptor · Ligand ·
Testosterone · Heat shock protein

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Introduction

More than a hundred years have elapsed since the first description of spinal and bulbar muscular atrophy (SBMA) [1, 2]. Early case reports [3, 4, 5, 6, 7, 8] were followed by the clinical and genetic description of 11 cases from two families by Kennedy and colleagues [9], which established the clinical entity of SBMA. Since the discovery of abnormal CAG triplet expansion in the androgen receptor (AR) gene as the cause of SBMA [10], molecular biological approaches have been undertaken to elucidate the pathogenesis of the disease and develop

approaches for its treatment. Here we review the research findings from which the ligand-dependent pathophysiology of SBMA has emerged, and discuss its therapeutic approaches.

Clinical features of SBMA

SBMA, also known as Kennedy's disease, is an inherited motor neuron disease characterized by adult-onset proximal muscle atrophy, weakness, fasciculations, and bulbar involvement [9, 11]. Fasciculations often manifest upon muscle contraction, and have been described as contraction fasciculations. The onset of weakness is usually between 30 and 50 years, but is often preceded by nonspecific symptoms such as tremor, muscle cramps and fatigue [12, 13]. Deep tendon reflex is diminished or absent, with no pathological reflex. Sensory involvement is largely restricted to that of vibration, which is affected distally in the legs [11]. Male patients occasionally demonstrate signs of androgen insensitivity such as gynecomastia, testicular atrophy, erectile dysfunction and decreased fertility [14, 15, 16], and some of these symptoms may be detected before the onset of motor symptoms. Endocrinological examinations frequently reveal partial androgen resistance with an elevated serum testosterone level [17]. Examination by electromyogram shows neurogenic abnormalities, and distal motor latencies are often prolonged in nerve conduction studies. Both the sensory nerve action potential and sensory evoked potential are reduced or absent [18, 19]. Serum creatine kinase levels are elevated in the majority of patients. Hyperlipidemia, liver dysfunction and glucose intolerance are also detected in some patients [12, 20]. SBMA exclusively affects males, and thus has been reported as an X-linked hereditary disease. The prevalence of SBMA has been estimated 1 in 40,000 in areas with where the diagnosis is efficient [21], although considerable numbers of patients may have been under-diagnosed [22, 23]. Profound facial fasciculations, bulbar signs, gynecomastia, and sensory disturbance are the main clinical features distinguishing SBMA from other motor neuron diseases, although genetic analysis is indispensable for diagnosis. Female patients are usually asymptomatic, but some express subclinical phenotypes including high amplitude motor unit potentials on electromyography [24, 25].

In the histopathology of SBMA, lower motor neurons are markedly depleted through all spinal segments and in the brainstem motor nuclei except the third, fourth and sixth cranial nerves [11, 26]. 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 the large myelinated fibers demonstrate a distally accentuated sensory axonopathy in the peripheral nervous system [27]. Neurons in the Onufrowicz nuclei, intermediolateral columns and Clarke's columns of the spinal cord are generally well preserved. Muscle histopathology shows 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.

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 [13]. No specific treatment for SBMA has been established. Testosterone has been used in some patients, although it has no effects on the progression of the disease [28, 29, 30].

Molecular pathogenesis of SBMA

The molecular basis of SBMA is the expansion of a trinucleotide CAG repeat, which encodes the polyglutamine (polyQ) tract, in the first exon of the AR gene [10] (Fig. 1). The CAG repeat within AR ranges in size from 11 to 35 repeats in normal subjects, but from 40 to 62 in SBMA patients [10, 21, 31]. Multiple founder effects have been reported in Japan, Europe and Australia [31, 32]. Expanded polyQ tracts have been found to cause several neurodegenerative diseases including SBMA, Huntington's disease (HD), several forms of spinocerebellar ataxia, and dentatorubral and pallidoluysian atrophy (DRPLA) [33, 34, 35]. These disorders, known as polyQ diseases, share salient clinical features such as anticipation [36], somatic mosaicism [37], and selective neuronal and non-neuronal involvement despite widespread expression of the mutant gene [38]. There is also an inverse correlation between the CAG repeat size and the age at onset, or the disease severity adjusted by age at examination in SBMA [36, 39], as well as in other polyQ diseases [33, 40]. These observations suggest that common mechanisms underlie the pathogenesis of polyQ diseases, although the nature of each causative protein is discrete apart from the existence of the polyglutamine stretch.

A striking pathological hallmark of most polyQ diseases is the presence of nuclear inclusions (NIs), which have been considered relevant to the pathophysiology [33]. In SBMA patients, NIs containing the mutant,
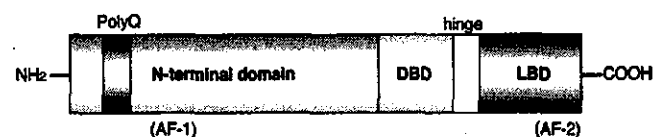
Fig. 1 Structure of the androgen receptor (AR) protein. The AR, a ligand-dependent transcriptional factor, is a member of the steroid/thyroid hormone receptor family. The AR protein consists of three major domains: an N-terminal transactivating domain, a DNA-binding domain, and a ligand-binding domain. The polyglutamine tract is located in the N-terminal domain, which possesses the major transactivating function (AF-1). The DNA binding domain (DBD) contains zinc finger structures, facilitating AR binding to DNA. The ligand binding domain (LBD) in the C-terminus also contains weak transactivating function (AF-2)

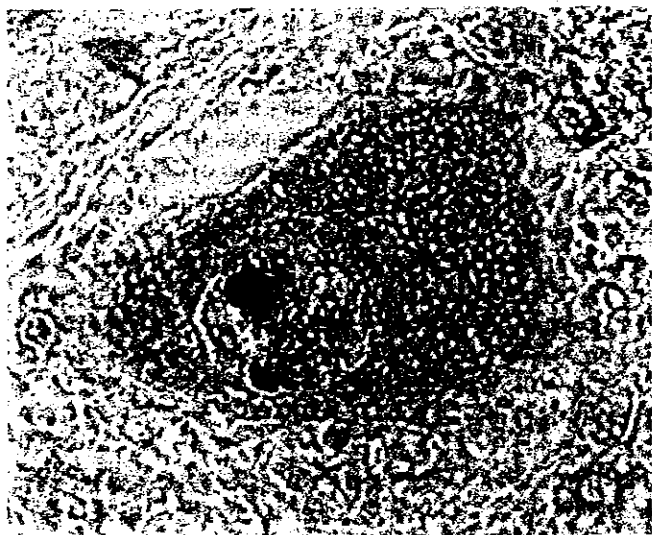Fig. 2 Nuclear inclusion of spinal and bulbar muscular atrophy (SBMA). A residual motor neuron in the lumbar anterior horn shows a nuclear inclusion detected by an anti-polyglutamine antibody

truncated AR are detected in the residual motor neurons in the brainstem and spinal cord [41] (Fig. 2) as well as in the skin, testis and some other visceral organs [42]. These inclusions have similar epitope features detectable by antibodies that recognize a small portion of the N-terminus of the AR protein only, and they are ubiquitinated. Although considerable controversy surrounds the importance of NIs in the pathophysiology of the polyQ diseases [43], several studies have implied that the nuclear localization of the mutant protein is essential for inducing neuronal cell dysfunction and degeneration in the majority of polyQ diseases [34]. This hypothesis is supported by the fact that several transcriptional regulatory proteins are sequestrated into NIs [44, 45].

SBMA is unique among polyQ diseases in that the mutant protein, the AR, has a specific ligand, testosterone, and this ligand alters the subcellular localization of the protein by favoring its nuclear uptake. The AR is normally confined to a multi-heteromeric inactive complex in the cell cytoplasm, and translocates into the nucleus in a ligand-dependent manner [46]. This intra-cellular trafficking of AR and other ligand-related mechanisms appear to play important roles in the pathogenesis of SBMA.

Ligand effects in a mouse model of SBMA

In order to investigate ligand effects in SBMA, we generated transgenic mice expressing the full-length human AR containing either 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken β-actin promoter [47]. This model recapitulated not only the neurological disorder, but also the phenotypic differences with gender, which is a specific feature of SBMA.

The lines with 97 CAG repeats (AR-97Q) exhibited progressive motor impairment, although no lines with 24 CAG repeats showed any phenotypes. All symptomatic lines showed small body size, short lifespan, progressive muscle atrophy and weakness, as well as reduced cage activity, all of which were pronounced and markedly accelerated in the male AR-97Q mice, but were either not observed, or far less severe, in the female AR-97Q mice, regardless of the line. The onset of motor impairment detected by the rotarod task was between 8 and 9 weeks of age in the male AR-97Q mice, and at 16 weeks or more in the females. The 50% mortality ranged from 66 to 132 days in the male AR-97Q mice, whereas the mortality of the females remained only 10–30% at more than 210 days. Western blot analysis revealed the transgenic protein smearing from the top of the gel, indicating the presence of insoluble AR fragments, in tissues such as the spinal cord, cerebrum, heart, muscle and pancreas. Although the male AR-97Q mice had more smearing protein than their female counterparts, the female AR-97Q mice had more monomeric AR protein. The nuclear fraction contained the most of smearing mutant AR protein. Diffuse nuclear staining and less frequent NIs detected by 1C2, an antibody specifically recognizing the expanded polyQ, were demonstrated in the neurons of the spinal cord, cerebrum, cerebellum, brainstem and dorsal root ganglia as well as non-neuronal tissue such as the heart, muscle and pancreas. Male AR-97Q mice showed markedly more abundant diffuse nuclear staining and NIs than females, in agreement with the symptomatic and Western blot profile differences with gender. Despite the profound sexual differences of mutant AR protein expression, there was no significant difference in the expression of the transgene mRNA between the male and female AR-97Q mice. These observations indicate that the testosterone level plays an important role in the gender-specific differences in the phenotypes, especially in post-transcriptional regulation of the mutant AR. Gender-specific phenotypes have also been demonstrated in another transgenic mouse model of SBMA carrying the full-length AR with 120 CAG repeats driven by a cytomegalovirus promoter [48].

The dramatic sexual difference of phenotypes led us to trial hormonal interventions in our mouse model. First, we castrated male AR-97Q mice in order to decrease their testosterone level. Castrated males showed profound improvement of their symptoms, histopathological findings, and nuclear localization of the mutant AR compared with sham-operated males. The body weight, motor function, and lifespan of these mice were significantly improved by castration. Western blot analysis and histopathology revealed diminished nuclear accumulation of mutant AR in the castrated males compared with the sham-operated males. Next, we administered testosterone to female AR-97Q mice. In contrast to castration of the male mice, testosterone caused a significant aggravation of symptoms, histopathological features, and nuclear localization of the mutant AR in female mice. Since the nuclear translocation of AR is testosterone-dependent,

testosterone appears to show toxic effects in the female AR-97Q mice by accelerating nuclear translocation of the mutant AR. Hence, castration of the males prevented the nuclear localization of the mutant AR by reducing the testosterone level. Nuclear localization of the mutant protein with an expanded polyQ tract is likely to be important in inducing neuronal cell dysfunction and degeneration in the majority of the polyQ diseases. Addition of a nuclear export signal to the mutant huntingtin protein eliminates aggregate formation and cell death in cell models of HD [49, 50], whereas a nuclear localization signal has the opposite effect [50]. When its nuclear localization signal is mutated, atxaxin-1, the causative protein of SCA-1, is distributed in the cytoplasm and does not cause any neurological disorders in SCA1 transgenic mice [43]. Addition of a nuclear export signal to the mouse Hprt protein containing expanded polyQ reduces NIs and delays the onset of behavioral abnormalities [51]. These findings suggest that reduction in the testosterone level improved phenotypic expression by preventing nuclear localization of the mutant AR. In support of this hypothesis, the ligand-dependent neurodegeneration has also been revealed in a fruit fly model of SBMA [52]. Alternatively, castration may enhance the protective effects of heat shock proteins, which are normally associated with the AR and dissociate upon ligand binding. Although ligand-induced neuronal dysfunction is apparent in the mouse model, testosterone administration does not worsen the symptoms of SMBA patients in preliminary clinical trials [28, 29]. This inconsistency may be explained by several reasons. The treatment duration may not be long enough to show its negative effects on disease progression. The negative effect may be saturated by the endogenous level of testosterone. The anabolic effects of androgens on the muscle may attenuate the motor symptoms induced by anterior horn cell degeneration in SBMA.

Successful treatment of AR-97Q mice by castration inspired us to trial testosterone blockade therapies, using a LHRH analogue and an AR antagonist used in the treatment of prostate cancer [53]. AR-97Q mice treated with leuprorelin, a LHRH analogue which reduces testosterone release from the testis, showed a marked amelioration of symptoms, histopathological findings, and nuclear localization of the mutant AR compared with vehicle-treated mice (Fig. 3). Leuprorelin initially increased the serum testosterone level by upregulating the LHRH receptor, but this effect was subsequently reduced to undetectable levels. Androgen blockade effects were also confirmed by reduced weights of the prostate and the seminal vesicle. The leuprorelin-treated AR-97Q mice showed significant improvements in lifespan, muscle atrophy and reduced body size as well as motor impairment as assessed by the rotarod task and cage activity. Although the negative effect on fertility was mitigated by reducing the dosage, the therapeutic effects on neuromuscular phenotypes were insufficient at a lower dose of leuprorelin. In the Western blot analysis and anti-polyglutamine immunostaining, the leuprorelin-treated male

AR-97Q mice had markedly diminished levels of mutant AR in the nucleus, suggesting that leuprorelin successfully reduced nuclear AR accumulation. Testosterone, which was given from 13 weeks of age, markedly aggravated the neurological symptoms and pathological findings of the leuprorelin-treated male AR-97Q mice. Leuprorelin prevents testicular testosterone production by down-regulating LHRH receptors in the pituitary, and has extensively been used as medical castration in the therapy of prostate cancer. Leuprorelin appears to improve SBMA-related neuronal dysfunction by preventing ligand-dependent nuclear translocation of the mutant AR in the same way as castration. Given its minimal invasiveness and established safety, leuprorelin is likely to be a promising therapeutic agent for SBMA. Upon clinical trials, however, the patient's desire for fertility should be taken into account, and the appropriate therapeutic dose carefully determined.

Leuprorelin-treated AR-97Q mice showed deterioration in their body weights and rotarod task performance from the age of 8–9 weeks, when serum testosterone initially increased through the agonistic effect of leuprorelin. This change was transient and followed by a sustained amelioration together with consequent suppression of testosterone production. The footprint analyses also revealed a temporary exacerbation of motor impairment. Immunostaining of tail specimens, sampled from the same individual mouse, demonstrated an increase in the number of the muscle fibers with nuclear 1C2 staining after 4 weeks of leuprorelin administration, although this 1C2 staining was diminished after another 4 weeks of treatment. Reversibility of polyQ pathogenesis has also been demonstrated by turning off gene expression in an inducible mouse model of HD [54]. Our results, however, indicate that preventing nuclear translocation of the mutant AR is enough to reverse both the symptomatic and pathological phenotypes in our AR-97Q mice. Since the pathophysiology of AR-97Q mice is neuronal dysfunction without neuronal cell loss [47], our results indicate that polyQ pathogenesis is reversible at least in its dysfunctional stage. We need to determine the early dysfunctional period in human polyQ diseases.

By contrast, flutamide, an AR antagonist, did not ameliorate the symptoms, pathological features, or nuclear localization of the mutant AR in the male AR-97Q mice, although there was no significant difference between flutamide and leuprorelin in terms of androgen blockade. Flutamide, the first androgen antagonist discovered, has a highly specific affinity for the 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 agonist, in order to block the action of adrenal testosterone. Although flutamide suppresses androgen-dependent transactivation, it does not reduce the plasma levels of testosterone. Furthermore, flutamide does not inhibit, but may even facilitate, the nuclear translocation of the AR [55, 56]. Flutamide also promotes nuclear translocation of mutant ARs containing expanded polyQ tracts in both cell and fly models of
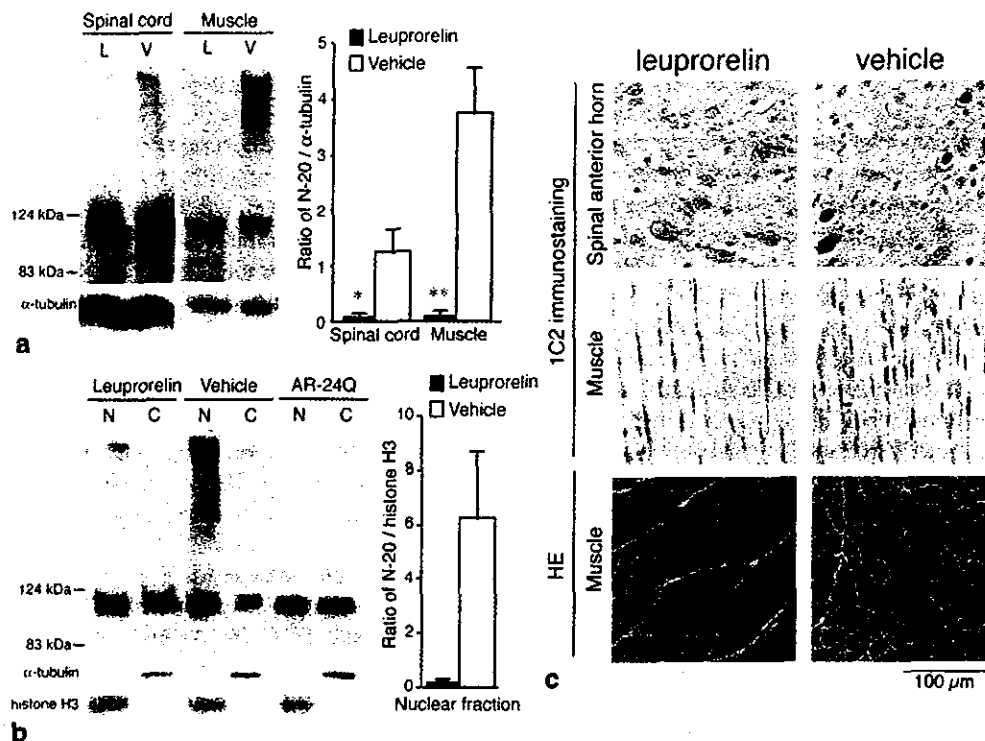
Fig. 3a–c Effects of leuprorelin on mutant AR expression and neuropathology of male AR-97Q mice. **a** Western blot analysis with an anti-AR antibody (N-20) of total homogenates from the spinal cord and muscle of the leuprorelin-treated (*L*) and vehicle-treated (*V*) male AR-97Q mice. Densitometric analysis demonstrated that leuprorelin significantly diminished the amount of mutant AR smearing from the top of the gel. (*P*=0.011, **P*=0.015). **b** Western blot analysis (with N-20) of the nuclear (*N*) and cytoplasmic (*C*) fractions from muscle tissues of the male mice given leuprorelin (*L*) and vehicle (*V*), and a transgenic mouse carrying an AR with 24 CAG repeats (*AR-24Q*). Smearing mutant AR, which decreased with leuprorelin treatment, was contained in the nuclear fraction. In the densitometric analysis, leuprorelin significantly reduced the amount of smearing mutant AR in the nuclear fraction. (*P*=0.014). **c** Immunohistochemical studies using 1C2 showed marked differences in diffuse nuclear staining and nuclear inclusions between the leuprorelin-treated and vehicle-treated AR-97Q male mice in the spinal anterior horn and the muscle. Hematoxylin and eosin staining of the muscle in the vehicle-treated male mouse revealed apparent grouped atrophy and small angulated fibers, which were not seen in the leuprorelin-treated mice. This figure is reproduced from [53]

SBMA [52, 57]. This may be the reason why flutamide showed no therapeutic effect in our transgenic mouse model of SBMA, and hence is not likely to be a useful therapeutic agent for SBMA.

The castrated or leuprorelin-treated AR-97Q mice showed phenotypes similar to those of the female AR-97Q mice, implying that the motor impairment of SBMA patients can be reduced to the level seen in females. SBMA has been considered an X-linked disease, whereas other polyQ diseases show autosomal dominant inheritance. In fact, female SBMA patients hardly manifest clinical phenotypes, although they possess similar number of CAG repeats in the disease allele as their male siblings with SBMA [24, 25]. Indeed the lower level of mutant AR expression in females due to X-inactivation may cause the escape from phenotypic manifestations, but hormonal interventions in mouse and fly models strongly suggest that low levels of testosterone prevent the nuclear accumulation of the mutant AR, resulting in a lack of phenotypic manifestations in females. This hypothesis is supported by the finding that manifestation of symptoms is minimal, even in females homozygous for SBMA [58].

In another case report, an 85-year-old woman with 38/51 CAG repeats was also asymptomatic [59]. It would appear logical that SBMA is not an X-linked recessive inherited disease, but rather that its phenotype depends on testosterone concentration.

Ligand-dependent modifications of the AR

In addition to nuclear translocation, ligand binding modulates AR function in various ways. These ligand effects are also likely to influence the pathogenesis of SBMA (Fig. 4). Many components of the ubiquitin-proteasome and molecular chaperones are known to co-localize with polyQ-containing NIs [60, 61, 62]. These chaperones and proteasomes facilitate refolding or degradation of the mutant protein, and may play a role in protecting neuronal cells against the toxicity of the expanded polyQ tract [60, 63]. Over-expression of heat shock proteins (HSPs) decreases aggregate formation of mutant ARs and markedly prevents the cell death in a neuronal cell model of SBMA [64]. HSP70 over-expres-
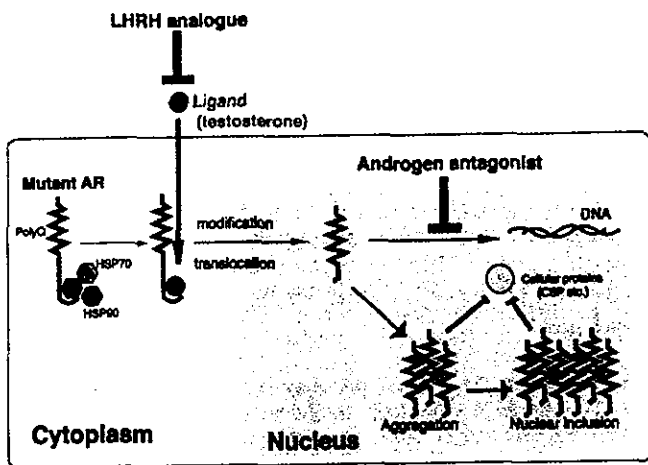
Fig. 4 Hypothetical relationship between the ligand and the mutant AR. In the absence of ligand, the mutant AR is confined to a multi-heteromeric inactive complex with heat shock proteins (*HSP*) in the cell cytoplasm. Ligand-binding facilitates its dissociation from this complex and translocation into the nucleus. Ligand also induces AR modifications such as conformational change, phosphorylation and proteolytic cleavage. In the nucleus, the mutant AR aggregates in a fashion partially dependent on transglutaminase activity, and forms nuclear inclusions as a consequence. The function of critical cellular proteins such as CREB-binding protein is inhibited by mutant the AR, resulting in transcriptional dysregulation. On the other hand, the decreased transactivation function of the mutant AR may contribute to the androgen insensitivity and neurodegeneration in SBMA. LHRH agonist, but not androgen antagonist, inhibits nuclear accumulation of mutant AR, resulting in the improvement of SBMA phenotypes in model mice. This figure is modified from [103]

sion enhances both the solubility and the degradation of mutant ARs in vitro [65]. These protective effects of the HSPs have also been reported in a wide range of polyQ disease models [60, 66, 67, 68]. Over-expression of the inducible form of human HSP70 markedly ameliorated the symptomatic and histopathological phenotypes in our transgenic mouse model, and this amelioration was correlated with the reduction in the amount of nuclear-localized mutant AR protein [69]. It should be noted that the soluble form of the mutant AR was also significantly decreased by HSP70 over-expression, suggesting that the degradation of the mutant AR may have been accelerated by over-expression of HSP70. Without ligand stimulation, the AR is associated with HSP70 and HSP90, and exists as an inactivated complex in the cell cytoplasm. Once ligand binds to the AR, the receptor is released from this complex and translocates into the nucleus. Ligand-dependent dissociation from HSPs is likely to enhance the toxic properties of mutant ARs, and thus contributes to the pathogenesis of SBMA. Supporting this hypothesis, ligand induces mutant AR aggregation even in the cytoplasm in a cell model of SBMA [70].

Proteolytic cleavage or truncation of mutant proteins appears to be of importance for the pathogenesis of polyQ diseases. In transgenic mouse models of HD and those of Machado-Joseph disease, or SCA3, the truncated protein has a particularly pronounced effect on the disease

manifestation [71, 72]. NIs are detected by antibodies against an N-terminal epitope, but not by antibodies against a C-terminal epitope in SBMA [41, 42] as well as in other polyQ diseases [73]. These findings suggest that truncated polyQ-containing proteins confer the toxicity in polyQ diseases. It should be noted that a truncated mutant AR is more toxic than the full-length AR in a SBMA cell model [74]. Additionally, in vitro translated full-length AR protein with an expanded polyQ tract is cleaved by caspase-3, liberating a polyQ-containing fragment, and the susceptibility to cleavage is polyQ repeat length-dependent [75]. Thus, cleavage of polyQ-containing proteins is likely to contribute to the toxicity of polyQ tracts. Testosterone induces a conformation change in the AR, resulting in an altered propensity for proteolytic cleavage. It should be noted that the AR antagonist flutamide exerts the same effect as testosterone on mutant AR cleavage in a fly model of SBMA [52]. The activity of caspase-3 is also enhanced by phosphorylation of the AR, resulting in enhanced cytotoxicity of the polyQ tract [76]. Phosphorylation of the mutant protein is required for polyQ-induced neurodegeneration in both SCA-1 cell and fly models of SMBA [77, 78]. Phosphorylation of the AR is known to be modulated by ligand [79], indicating that ligand-dependent phosphorylation of mutant ARs would appear to contribute to the pathogenesis of SBMA. The exact role of AR phosphorylation in SBMA should be elucidated.

Transglutaminase has also been hypothesized to enhance polyQ toxicity [80, 81]. Mutant proteins with an expanded polyQ tract are preferential substrates for transglutaminase, which catalyzes glutamyl-lysine cross-linking, resulting in the formation of proteolysis-resistant aggregates. The AR is a substrate for transglutaminase, which induces cross-linking of mutant ARs and alters their epitope properties in vitro [82]. Transglutaminase bond formation is found in the tissues of SBMA transgenic mice, suggesting its involvement in pathogenesis. Mutant AR and transglutaminase both induce a perturbation of proteasomal function in the presence of testosterone. The degradation of the AR protein is markedly retarded by its ligands [83]. Taken together, these findings suggest that the disruption of the proteasome, which has been implicated in the pathogenesis of SBMA and other polyQ diseases [84, 85], also appears to be ligand-dependent in SBMA.

As in other polyQ diseases, a toxic gain-of-function mutation has been implicated in the pathophysiology of SBMA. Although the expansion of its polyQ tract mildly suppresses the transcriptional activities of the AR [86, 87], motor impairment has never been observed in severe testicular feminization patients lacking AR function [88] or in AR knockout mice [89]. A transgenic mouse model carrying 239 CAG repeat driven by the human AR promoter demonstrated motor impairment, suggesting that the presence of a polyQ tract is sufficient to induce the pathogenic process of SBMA [62]. Thus, the neurological impairment in SBMA is not to be attributed to the loss of AR function, a reason why testosterone shows transient

and insufficient effects when used as a therapeutic agent for SBMA [28, 29, 30]. However, recent data have demonstrated that loss of normal protein function also plays a role in the neurodegeneration in polyQ diseases. As for the AR, there are several lines of evidence in favor of ligand-induced neuroprotective effects. Exogenous administration of testosterone immediately after nerve injury impacts positively on functional recovery through actions mediated by AR [90, 91]. In a cell culture model, a mutant AR with 24 CAG repeats shows trophic effects upon ligand treatment, whereas an AR with 65 CAG repeats does not demonstrate any neuroprotection [92]. Ligand effects on AR function should be further studied using animal models of SBMA.

Therapeutic perspectives in SBMA and other polyQ diseases

As mentioned above, our recent study indicated that leuprorelin exerts a therapeutic effect in the SBMA transgenic mouse model. This approach can easily be applied to human SBMA therapy, because this drug has extensively been used for medical castration in the therapy of prostate cancer [93]. When we move on to clinical trials of hormonal therapy, it is necessary to determine clinical and laboratory markers reflecting the disease activity. Muscle strength, muscle volume and bulbar function would be reasonable parameters, although they must be quantified. Early symptoms, including hand tremor, muscle cramps or mild weakness could be the key for treatment initiation, only if the patients have no wish to have children. Although no specific ligand for the mutant protein has been revealed in other polyQ diseases, the striking therapeutic effects of leuprorelin in our SBMA mice further suggest that patients with other forms of polyQ disease can be treated by preventing the nuclear translocation of the mutant protein in question. Our studies with transgenic mice also indicate that over-expression or activation of HSP would be of therapeutic benefit in polyQ diseases. Medical approaches to enhance HSP function should be further investigated in vivo.

Besides these strategies, various molecular mechanisms are likely to be the therapeutic targets in polyQ diseases. The function of transcription factors such as CREB-binding protein (CBP) are inhibited through protein-protein interaction in mouse models and patients with polyQ diseases [94, 95]. HDAC inhibitors, which restore histone acetylation by CBP, improve transcriptional activity and ameliorate polyQ-induced neurodegeneration in a cell model of SBMA [96] as well as in a fly model of HD [97], although this is of limited therapeutic benefit in a mouse model of HD due to toxicity [98]. Protein aggregation has been shown to render polyQ-containing proteins toxic in numerous cell models of SBMA and other polyQ diseases [99, 100]. The azo-dye Congo Red inhibits oligomerization of mutant huntingtin, and ameliorates motor function and survival of in a transgenic mouse model of HD [101]. Cystamine, a transglutaminase

inhibitor, has been shown to mitigate polyQ toxicity in a cell model of SBMA [82] as well as in a HD mouse model [102]. Clinical applications of these therapeutic approaches are awaited.

The ideal treatment for polyQ diseases appears to be a combination of these and other therapeutic strategies, since each approach has adverse effect and long-term treatment is unavoidable. Elucidation ofpathophysiology, high-throughput drug screening and intensive clinical trials are necessary for establishing effective therapeutic strategies.

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