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Abundant expression and cytoplasmic aggregations of α1A voltage-dependent calcium channel protein associated with neurodegeneration in spinocerebellar ataxia type 6

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Spinocerebellar ataxia type 6 (SCA6) is one of the eight neurodegenerative diseases caused by a trinucleotide (CAG) repeat expansion coding polyglutamine (CAG repeat/polyglutamine diseases) and is characterized by late onset autosomal dominant cerebellar ataxia and predominant loss of cerebellar Purkinje cells. Although the causative, small and stable CAG repeat expansion for this disease has been identified in the α1A voltage-dependent calcium channel gene (CACNA1A), the mechanism which leads to predominant Purkinje cell degeneration is totally unknown. In this study, we show that the calcium channel mRNA/protein containing the CAG repeat/polyglutamine tract is most intensely expressed in Purkinje cells of human brains. In SCA6 brains, numerous oval or rod-shaped aggregates were seen exclusively in the cytoplasm of Purkinje cells. These cytoplasmic inclusions were not ubiquitinated, which contrasts with the neuronal intranuclear inclusions of other CAG repeat/polyglutamine diseases. In cultured cells, formation of perinuclear aggregates of the channel protein and apoptotic cell death were seen when transfected with full-length CACNA1A coding an expanded polyglutamine tract. The present study indicates that the mechanism of neurodegeneration in SCA6 is associated with cytoplasmic aggregations of the α1A calcium channel protein caused by a small CAG repeat/polyglutamine expansion in CACNA1A.

INTRODUCTION

Spinocerebellar ataxia type 6 (SCA6) is an autosomal-dominant cerebellar ataxia characterized by late onset rather pure cerebellar ataxia and predominant degeneration of Purkinje cells (1–3). The causative gene for SCA6 has been identified as a trinucleotide (CAG) repeat expansion in the 3' region of the α1A voltage-dependent calcium channel gene (CACNA1A) (1). The expansion in CACNA1A is inversely correlated with the age of onset of the disease (3), indicating that the CAG repeat expansion, although small, is strongly associated with the pathogenic mechanism of SCA6. Thus, SCA6 is included in a group of neurodegenerative diseases caused by CAG repeat expansions coding polyglutamine (CAG repeat/polyglutamine diseases), namely spinal and bulbar muscular atrophy [SBMA (4)], Huntington's disease [HD (5)], SCA1 (6), dentatorubral-pallidoluysian atrophy [DRPLA (7,8)], Machado–Joseph disease (MJD)/SCA3 (9), SCA2 (10–12) and SCA7 (13).

The mechanism by which the CAG repeat expansion in CACNA1A leads to predominant Purkinje cell degeneration is totally unknown. The fact that the size of expansion in SCA6 is small [normal individuals, 4–20 repeats; SCA6 patients, 21–33 repeats (1,3,14)] and falls within the normal range of other CAG repeat expansions raises the possibility that the mechanism in SCA6 is different from those in other CAG repeat/polyglutamine diseases. In a number of CAG repeat/polyglutamine diseases (15–21), neuronal intranuclear inclusions composed

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of each mutant protein are commonly found to be associated with their pathogenic mechanism. Therefore, it is particularly important to address whether the CAG expansion in CACNA1A also results in aggregation of mutant α1A calcium channel protein. To gain further insights into the mechanism of neurodegeneration in SCA6, we conducted expression analysis of the α1A calcium channel mRNA/protein in brains of normal and SCA6 individuals. Here we provide evidence that mutant α1A calcium channel protein, intensely expressed in Purkinje cells, is selectively aggregated in the cytoplasm of Purkinje cells.

RESULTS

Levels of CACNA1A mRNA containing a CAG repeat expansion

We first cloned and sequenced the full-length CACNA1A cDNA from both normal and SCA6 cerebella and found that the length of the CAG repeat in exon 47 is the major difference in the nucleotide sequences of the normal and mutated CACNA1A mRNA. We also confirmed that the CAG repeat is predicted to be translated into a polyglutamine tract in the splice variant with the five base GGCAG insertion in the 5' terminus of exon 47 (1). We therefore examined the differences in the levels of mRNAs spanning the GGCAG insertion site and the CAG repeat by semi-quantitative RT–PCR analysis.

In the control brains, the amount of RT–PCR product in the CACNA1A mRNA was 10- to 20-fold higher in the cerebellar cortex than those in cerebral cortices, thalamus, hippocampus or the pontine base (Fig. 1A). In the SCA6 brains, levels of mRNA in the cerebellar cortex also far exceeded those of other brain regions. The electropherogram of the RT–PCR product revealed that mRNAs both with expanded and normal CAG repeats were amplified from the SCA6 cerebellar cortex (Fig. 1B). The splice variant with the GGCAG insertion, coding the polyglutamine tract, was predominantly amplified from the cerebellar CACNA1A mRNA with a normal CAG repeat compared with the other variant without the insertion, suggesting that the isoform with polyglutamine is predominantly expressed in the human α1A calcium channel. Furthermore, this predominant amplification was more pronounced in the CACNA1A mRNA with an expanded CAG repeat in the SCA6 cerebellum: a peak of the RT–PCR product corresponding to the splice variant without the insertion was not obvious in the mRNA with an expanded CAG repeat (Fig. 1B). In accordance with these results, sequence analysis of subcloned plasmids (n = 12) with expanded CAG repeats revealed that all the clones had the GGCAG insertion. This predominant transcription of the splice variant with the GGCAG insertion was also observed in other SCA6 brain regions examined.

Cellular expression of CACNA1A mRNA containing a CAG repeat

The cellular expression profile of the exons 46–47 CACNA1A mRNA was examined by *in situ* hybridization. The highest hybridization signal was detected in the cell body of Purkinje cells (Fig. 2A), while various types of neurons throughout the brain showed less intense signals (Fig. 2B–D). Taking the data from RT–PCR and *in situ* hybridization, we conclude that the CAG repeat in CACNA1A mRNA is most intensely expressed in Purkinje cells.

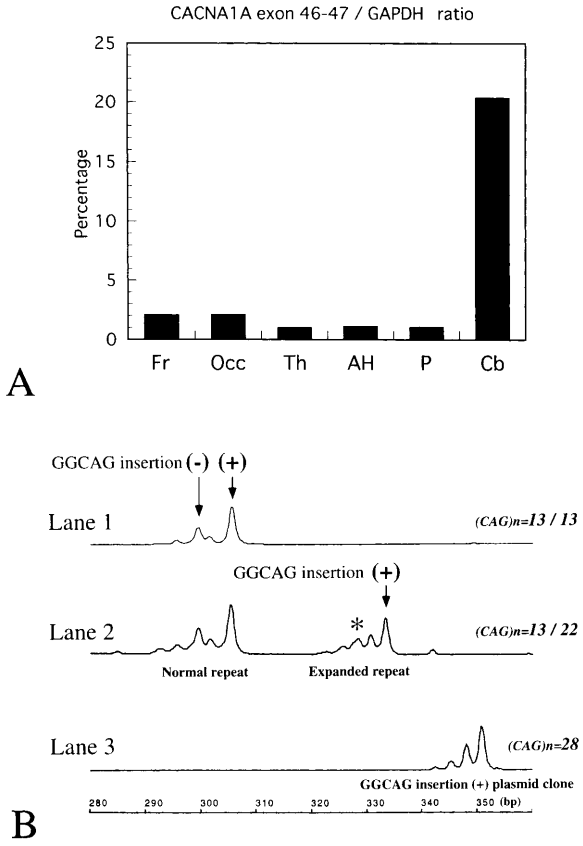

Figure 1. (**A**) α1A calcium channel (CACNA1A) mRNA is highly abundant in the cerebellum. The expression levels of exons 46–47 CACNA1A mRNA relative to those of glyceraldehyde 3-phosphate (GAPDH) mRNA in various regions of a control brain are shown. Fr, frontal cortex (Brodmann's area 6); Occ, occipital cortex (Brodmann's area 17); Th, thalamus; AH, hippocampus; P, pontine base; Cb, cerebellar hemispheric cortex. (**B**) An electropherogram of RT–PCR products demonstrating that mRNAs both with expanded and normal CAG repeats are transcribed in the SCA6 cerebellar cortex. Lane 1, RT–PCR product of cerebellar CACNA1A mRNA from a control individual with a homozygous 13 CAG repeat; lane 2, RT–PCR product of cerebellar CACNA1A mRNA from a SCA6 individual with 13 (normal) and 22 (expanded) CAG repeats; lane 3, the PCR product of the plasmid clone containing the GGCAG insertion and 28 CAG repeat. The peak corresponding to the splice variant with the GGCAG insertion is higher than that without the insertion, suggesting that the splice variant with the GGCAG insertion coding polyglutamine is predominantly transcribed from the mRNAs with normal CAG repeat (lanes 1 and 2, normal repeat). This predominance is more obvious in the CACNA1A mRNA with an expanded CAG repeat in the SCA6 cerebellum. *The peak corresponding to the splice variant without the insertion; lane 2, expanded repeat. Note that the pattern of RT–PCR product from the CACNA1A mRNA with an expanded CAG repeat (lane 2, expanded repeat) is similar to that of PCR product from the plasmid clone containing the GGCAG insertion (lane 3).

Identification of cytoplasmic aggregation of α1A calcium channel protein in SCA6 brains

We next examined expression of the α1A calcium channel protein using antibodies raised against synthetic peptides corresponding to the region between domains II and III (A6RPT-N) or to the C-terminal region (A6RPT-C) of the channel protein. We confirmed that the antibody A6RPT-C specifically recog-

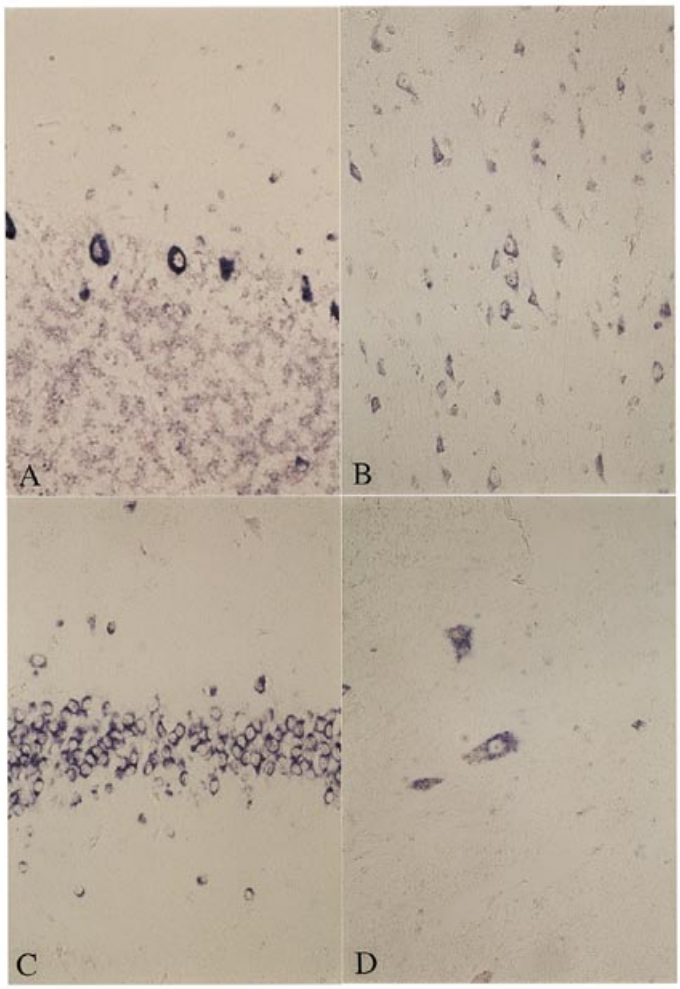

Figure 2. *In situ* hybridization for the exons 46–47 CACNA1A mRNA in a control human brain. The strongest hybridization signal is seen in the Purkinje cell in the cerebellum (**A**), although a considerable level of expression is also seen in various neurons throughout the brain. (**B**) Frontal cortex; (**C**) dentate gyrus of the hippocampus; (**D**) anterior horn cells in the thoracic spinal cord. Magnification: ×66.

nizes the extended C-terminal region of the α1A calcium channel protein due to the GGCAG insertion which disrupts the premature stop codon upstream of the CAG repeat sequence (1) (Fig. 3A).

Immunoreactivities for the two antibodies were fundamentally identical. In control brains, substantial levels of immunoreactivity were observed in various neurons throughout the brain, but the most intense immunoreaction was detected in the cell body of Purkinje cells (Fig. 3B).

In the SCA6 brains, the immunoreactivities were mostly similar to those in control brains. The most remarkable difference was the presence of densely immunoreactive, oval or rod-shaped structures in the cytoplasm of the SCA6 Purkinje cells (Fig. 3C). These cytoplasmic inclusions were seen in roughly more than half of the remaining Purkinje cells in a single specimen. The inclusions were most often localized in the peripheral perikaryal cytoplasm, but also in the proximal dendrites, and tended to show a more robust appearance in Purkinje cells with dysmorphic features (Fig. 3D). In contrast to the neuronal intranuclear inclusions of other CAG repeat/polyglutamine diseases (15–22), aggregates of the channel protein were not obviously recognized in the nucleus of SCA6 Purkinje cells (Fig. 3C and D). The inclusions were also recognized by A6RPT-N (Fig. 3E), suggesting that the entire α1A calcium channel protein is involved. The cytoplasmic aggregates were not positive for ubiquitin (Fig. 3F–H), which was also confirmed by the ABC method. In addition, proteasomes, heat shock protein and cathepsin D were also negative, which contrasts with the aggregates in other CAG repeat/polyglutamine diseases (23,24).

The second remarkable difference found in the SCA6 cerebellum compared with the controls was a mild reduction in the immunoreactivity against calcium channel antibodies in the cell body of Purkinje cells and in the molecular layer (Fig. 3C). The immunoreactivity in the amiculum of the cerebellar dentate nucleus also appeared to be reduced.

In an electron microscope, the SCA6 cytoplasmic inclusions were seen as bundles of filamentous structures often associated with granular material (Fig. 4). Although the inclusions were not associated with particular organelles, smooth or rough endoplasmic reticulum and free ribosomes were frequently seen near the inclusions.

Polyglutamine expansion in the α1A-calcium channel protein causes apoptotic cell death

Green fluorescent protein (GFP)–full-length α1A fusion proteins with normal length or expanded CAG repeats (normal, 13 repeats; expanded, 28 repeats), with or without the GGCAG insertion, were transiently expressed in cultured HEK-293 cells. The expression of fusion proteins without a polyglutamine tract or with normal length glutamines (GFP–FLQ13) were seen mainly in the cytoplasm, but also weakly in the nucleus (Fig. 5A–C). In contrast, the fusion protein with expanded glutamines (GFP–FLQ28) was densely expressed mainly in the perinuclear cytoplasm (Fig. 5D–F), but also sometimes in the nucleus (Fig. 5G). A similar pattern of staining was observed in PC12 cell lines expressing GFP–FLQ28 and the level of protein expression was comparable between the different constructs (data not shown). Remarkably, the cells expressing GFP–FLQ28 underwent cell death with apoptotic features (Fig. 5H). The aggregates in transfected cells were again ubiquitin negative. Since the expression plasmids are identical except for the length of the polyglutamine tract, the differences in aggregate formation and apoptotic cell death are both due specifically to the difference in the length of the polyglutamine tract.

DISCUSSION

We showed that the expression of α1A calcium channel mRNA/protein containing a CAG repeat/polyglutamine tract was most abundant in Purkinje cells in human brains. Although this finding was expected from data on other species (25–27), the present study is the first to show expression of the C-terminal portion of α1A calcium channel protein, which is unique to the human (28). Expression of the α1A calcium channel 3'-mRNA and C-terminal protein regions in the normal controls were also similar to that of the intracellular loop region between domains IIS6 and IIIS1 of the α1A calcium channel in

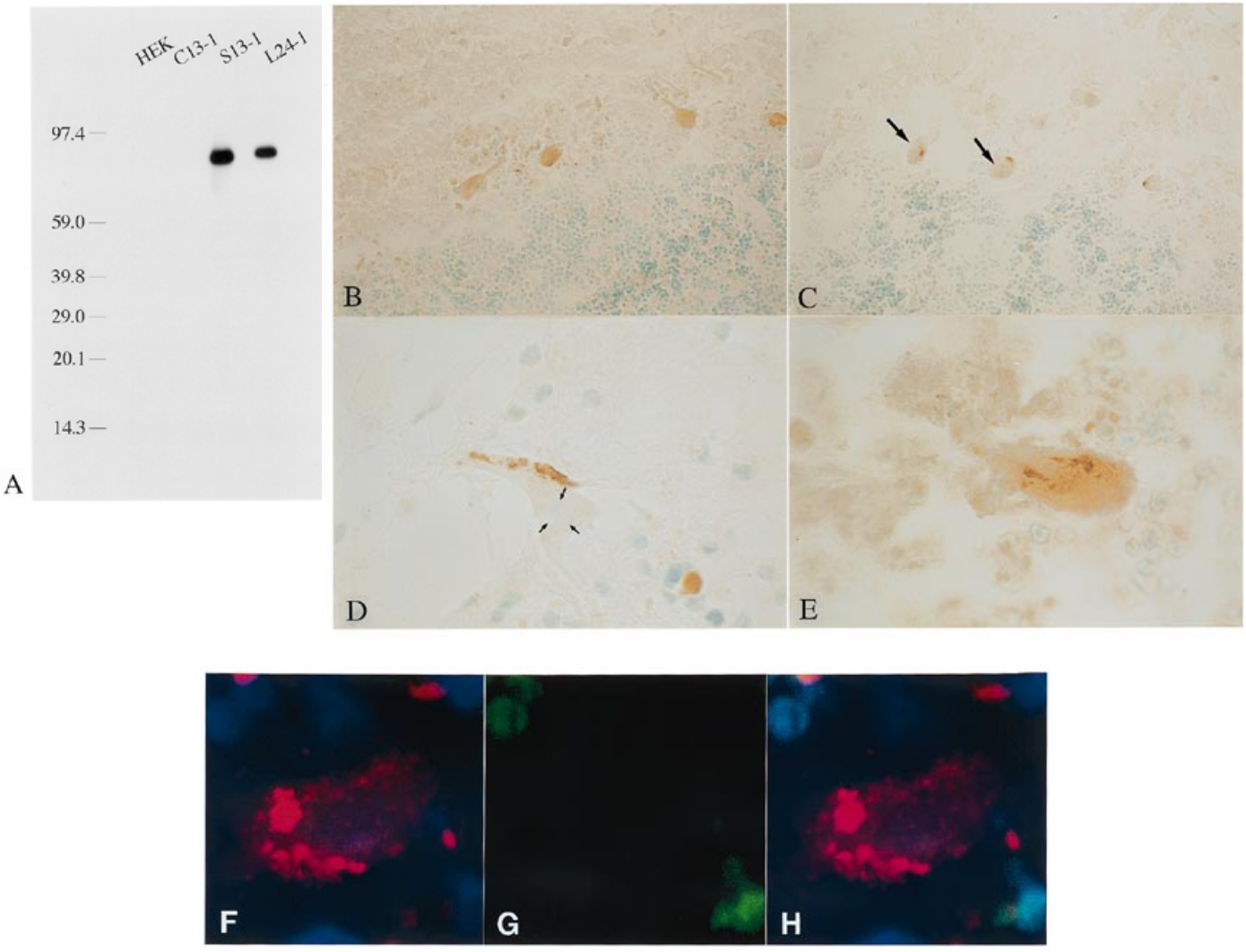

Figure 3. The immunohistochemical analysis of α1A calcium channel protein. (**A**) Western blotting showing that the antibody (A6RPT-C) raised against the C-terminal portion of the α1A calcium channel protein recognizes a single 85–90 kDa fusion protein (residues 1957–2519) in transfected cell lines. HEK, HEK-293 cells without expression vector; C13-1, HEK-293 cells expressing a GFP fusion protein without a polyglutamine tract, due to the absence of the five base GGCAG insertion in the construct; S13-1, HEK-293 cells expressing a fusion protein with 13 glutamines; L24-1, HEK-293 cells expressing a fusion protein with 24 glutamines. Note that the antibody specifically recognizes the extended C-terminal portion of the channel protein in the S13-1 and L24-1 constructs, made by the GGCAG insertion. (**B**) Immunohistochemistry for A6RPT-C in the control cerebellum counterstained with methyl green. The most intense immunoreactivity could be seen in the cell body and proximal dendrite of Purkinje cells (×66). (**C**) In the SCA6 cerebellum, strong immunoreactive structures (i.e. the inclusions) are seen in the cytoplasm of most of the remaining Purkinje cells. No obvious aggregates are seen in the nuclei (arrows). Also note that the immunoreactivity is generally weaker in the cell body of Purkinje cells and in the molecular layer than that in the control (B) (stained with A6RPT-C, ×66, patient 1). (**D**) A robust cytoplasmic inclusion is seen in a Purkinje cell with dysmorphic features. No obvious immunoreactive structures, indicating nuclear aggregates, are seen within the nucleus (small arrows) (stained with A6RPT-C, ×250, patient 2). (**E**) The cytoplasmic inclusion is also recognized with A6RPT-N. The inclusions are often multiple and recognized as clusters of granular immunoreactive structures (×250, patient 1). (**F**–**H**) Double immunofluorescence study against A6RPT-C (red) and mouse monoclonal ubiquitin (green) in a SCA6 Purkinje cell, counterstained with Hoechst 33258 (blue). (F) A6RPT-C and Hoechst 33258; (G) ubiquitin; (H) composite. SCA6 cytoplasmic inclusions are not obviously ubiquitin-immunoreactive (×500, patient 3).

the cerebellum (29). In SCA6 brains, the α1A calcium channel mRNA/protein containing a CAG repeat/polyglutamine tract was expressed most abundantly in Purkinje cells, the main target neurons in SCA6. This would suggest that the level of expression of the mutant gene/protein is an important factor for the mechanism of selective neurodegeneration in SCA6. In addition, the splice variant with the GGCAG insertion coding a polyglutamine tract was transcribed preferentially in the CACNA1A mRNA with an expanded CAG repeat compared with the CACNA1A mRNA with a normal CAG repeat. This phenomenon would further enhance the pathogenic effect of the expanded polyglutamine tract. However, expression of the expanded CAG repeat/polyglutamine tract was also seen in various neurons throughout the brain, indicating that other factors that influence the selectivity of neuronal degeneration may also be present.

The most intriguing point we demonstrated was the identification of cytoplasmic aggregation of the α1A calcium channel protein exclusively in Purkinje cells. Inclusions were observed in >50% the neurons and appeared to be more prominent than

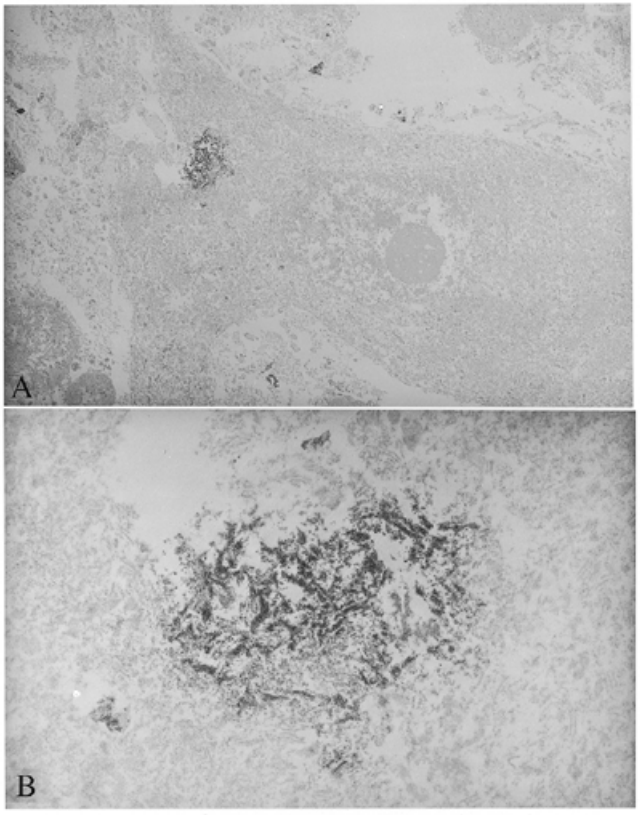

Figure 4. An immunoelectron micrograph of the cytoplasmic inclusion in a Purkinje cell of a SCA6 brain. Aggregates immunoreactive for A6RPT-C (**A**) are mainly composed of bundles of filamentous structures often associated with granular materials (**B**). Note that endoplasmic reticulum, not immunoreactive for A6RPT-C, is seen near the inclusion. Magnification: ×2000 (A); ×10 000 (B).

the neuronal intranuclear inclusions in MJD (17), HD (30) and SBMA (21), although further studies employing a larger number of SCA6 brains are needed. In addition, the full-length α1A calcium channel protein, which is essential for normal Purkinje cell function (31), is likely to be involved in the inclusions. Furthermore, in cultured cells, full-length α1A calcium channel fusion protein with an expanded polyglutamine tract formed dense accumulations in the perinuclear and intranuclear spaces which were associated with apoptotic cell death. These observations strongly suggest that aggregation of α1A calcium channel protein is associated with the pathogenic mechanism in SCA6.

Compared with aggregations of mutant proteins in other CAG repeat/polyglutamine diseases, several differences are noted in the aggregates of SCA6. In affected brains, aggregation of the calcium channel protein was seen in the cytoplasm of Purkinje cells, which contrasts with the intranuclear location of aggregates, frequently ubiquitinated, in other CAG repeat/polyglutamine diseases (15–22). Cytoplasmic aggregations of the mutant protein have been described in HD (18,24) and ubiquitin-positive cytoplasmic labeling is also observed in MJD (17) and DRPLA (32) brains. In contrast to these, the cytoplasmic aggregation in SCA6 brains was not ubiquitinated. This might suggest that ubiquitin-mediated protein degradation, claimed in other CAG repeat/polyglutamine diseases, is not evident in SCA6 or that the protein misfolding that is believed to induce ubiquitin-mediated protein degradation (23) is not strong enough in the SCA6 cytoplasmic inclusion due to the small polyglutamine expansion. In cultured cells, the full-length mutated calcium channel protein formed dense accumulations despite the small expansion, whereas aggregation is generally seen with the truncated forms of the mutated proteins in other CAG repeat/polyglutamine diseases (33,34). This might imply that the rest of the protein sequence of the α1A calcium channel as well as the length of the polyglutamine tract is critical for aggregate formation.

The differences in the intracellular location and ubiquitination of the aggregates found between SCA6 and other CAG repeat/polyglutamine diseases do not preclude the possibility that the mutant calcium channel protein acts in the nucleus of the target neurons, as suggested in SCA1 (35) and in HD (36). The present observation that some accumulations were in the nucleus in cultured cells and appeared to be associated with apoptotic cell death might imply that the mutant calcium channel protein could translocate into the nucleus and cause cell death of Purkinje cells. However, we could not demonstrate abnormal aggregation of the channel protein in the nucleus of SCA6 Purkinje cells, although subtle accumulation without forming discernible aggregates in the nucleus may still be present. It should also be noted that intracellular trafficking of the α1A calcium channel protein in our cultured cells could be different from that in human brains. The absence of other auxiliary calcium channel subunits, which are important for the α1A subunit to be targeted to the plasma membrane (37), may enable the fusion protein to translocate into the nucleus. Further studies, such as expressing α1A calcium channel protein with auxiliary subunits as in the native state, are needed to clarify whether translocation of the mutant α1A calcium channel protein is essential for pathogenicity.

Alternatively, cytoplasmic aggregation of the α1A calcium channel protein may interfere with intracellular trafficking or metabolism of the channel protein, which may result in reduced expression of the protein in the plasma membrane. Reduced immunoreactivity against α1A calcium channel antibodies in the cell body of SCA6 Purkinje cells would reflect reduced expression of both normal and mutated α1A calcium channel protein, although a functional consequence of global dysfunction of the Purkinje cell cannot be excluded. Interestingly, neither reduced expression nor cytoplasmic aggregation of the α1A calcium channel was observed in the neurological mutant mouse *leaner* (38,39), even though the P-type calcium channel current is reduced to ~35% of normal current (31). If expression of the α1A calcium channel protein in the plasma membrane is indeed reduced in SCA6 Purkinje cells, it is probable that normal physiological function governed by the calcium channel is altered, which would lead to neuronal degeneration. Therefore, it is particularly important to address whether the aggregated calcium channel protein alters expression of the α1A calcium channel protein in the plasma membrane. Obviously, it is also important to address whether mutant α1A calcium channel protein, if properly expressed in the plasma membrane, shows altered channel properties. Elucidating how the mutant α1A calcium channel protein aggregates in the cytoplasm as well as whether it acts in the nucleus could be important for understanding the mechanisms leading to neurodegeneration in SCA6.

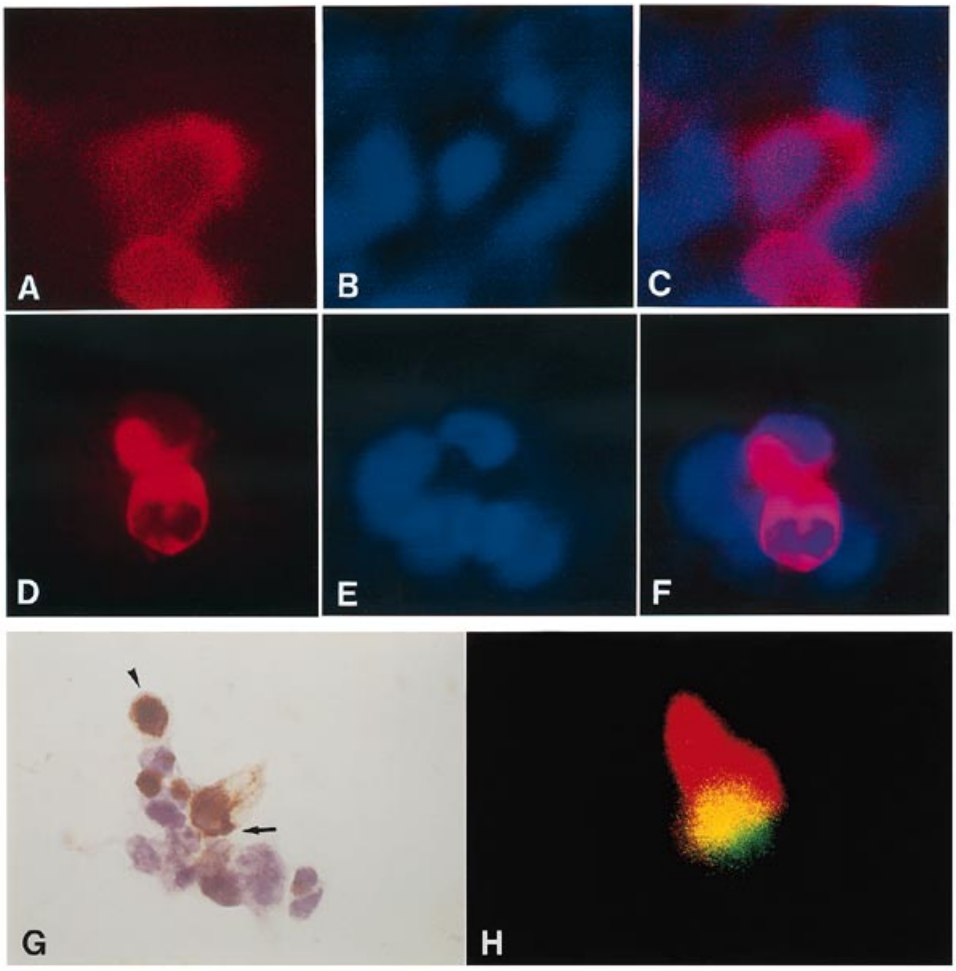

Figure 5. (**A**–**F**) Expression of GFP–full-length α1A calcium channel fusion proteins in transfected HEK-293 cells visualized by immunofluorescent staining: (A and D) A6RPT-C in red; (B and E) Hoechst 33258 in blue; (C and F) composite. The expression of fusion protein with a normal length polyglutamine tract (13 glutamines) was mainly localized diffusely in the cytoplasm but weak expression was additionally seen in the nucleus (center) (A–C). On the other hand, the fusion protein with an expanded polyglutamine tract (28 glutamines) was seen as dense accumulations mainly localized in the perinuclear cytoplasm (D–F). Magnification: ×500. (**G**) Besides perinuclear accumulation (arrow), some dense accumulations are seen within the nucleus (arrowhead) (immunoperoxidase staining against GFP and counterstained with hematoxylin, ×250). (**H**) The cells containing aggregates of α1A calcium channels (red) were stained positively in the TUNEL reaction using FITC-labeled dUTP (green) (×500).

MATERIALS AND METHODS

Patients

Three patients with SCA6, harboring 22 CAG repeats (40), and three control individuals dying from non-neurological diseases were examined. The three SCA6 patients were also described previously as 'familial cortical cerebellar atrophy' (41,42).

Semi-quantitative RT–PCR and sequencing analysis

Total RNA was extracted from six different frozen brain tissues (2 g each) where CACNA1A mRNA is known to be expressed: the frontal cortex (Brodmann's area 6), occipital cortex (Brodmann's area 17), hippocampus, thalamus, pons and cerebellar hemispheric cortex. Poly(A)$^+$ RNA (250 ng), prepared using Oligo(dT) Latex (Takara, Ohtsu, Japan), was reverse-transcribed with random hexamer (Perkin Elmer, Pomona, CA) and a SuperScriptII preamplification kit (Gibco BRL, Gaithersburg, MD). PCR primers for amplifying exons 46–47 of CACNA1A mRNA were as follows: RT-CAG1-F, 5'-GGCACGGGCTCGGGACCAGC-3'; S5-R1, 5'-[6-FAM]-TGGGTACCTCCGAGGGCCGCTGGTGG-3' (5'-[6-FAM]-TGGGTACCTCCGAGGGCCGCTGGTGG-3' from Applied Biosystems, Foster City, CA), amplifying a fragment between 290 and 355 bp. To quantify the relative level of mRNA, glyceraldehyde 3-phosphate cDNA was co-amplified as an internal standard using the following primers: GAPDH-F, 5'-TCATCCCTGCCTCTACTGGC-3'; GAPDH-R, 5'-[6-FAM]-TCCACCACCCTGTTGCTGTA-3', amplifying a fragment of 410 bp. PCR reactions contained RT-CAG primers or GAPDH primers (5 pmol each), dNTP (0.2 mM each) and 0.5 U AmpliTaq DNA polymerase (Perkin Elmer) in a 25 μl reaction volume. A PCR amplification was performed for 23 cycles for GAPDH PCR and 31 cycles for CACNA1A PCR, where amplifications were at the same linear rate. Quantification was performed three times independently using ABI 310 Prism Gene Scan (Applied Biosystems) and finally averaged. For analyzing GGCAG insertion, RT–PCR using primers RT-CAG1-

F and S5-R1 was undertaken in the same six brain regions and examined by ABI 310 Prism Gene Scan. The amplified fragments were also subcloned into pCRII (Invitrogen, San Diego, CA) and cycle sequenced using a M13 reverse primer and an Applied Biosystems Prism dye terminator kit.

Plasmids

Full-length or partial human CACNA1A cDNAs, containing 13 (normal range), 24 (expanded) or 28 (most expanded) CAG repeats, all with or without the GGCAG insertion, were subcloned in the *Sph*I and *Xba*I sites of pcDNA I/Amp (Invitrogen). Normal human cerebellar mRNA was reverse transcribed as described and amplified using primers based on the published CACNA1A sequences (28). Two primers for amplifying the 5'- and 3'-terminal untranslated regions of the CACNA1A mRNA were modified to contain restriction sites in the PCR fragments: the forward primer to amplify the 5'-terminal region was 5'-TTGCATGCCCGCAGCGTAAC-3', which is complementary to nucleotides 239–251 in the CACNA1A cDNA, and the reverse primer for the 3'-terminal region was 5'-GCTCTAGATTAGCACCAATCATCGTCAC-3', which is complementary to nucleotides 7783–7802 in the CACNA1A cDNA. The nucleotide sequences of the constructs were finally confirmed by cycle sequencing.

The GFP–α1A fusion clones were constructed in-frame by blunt-end cloning the *Sph*I–*Xba*I fragments of the α1A calcium channel clones into the blunted *Hin*dIII site of pEGFP-C2 (Clontech, Palo Alto, CA). Other clones for stably expressing the truncated α1A calcium channel (C13-1, S13-1 and L24-1) were also constructed in-frame by cloning *Bgl*II–*Xba*I fragments of the partial α1A calcium channel into the *Bgl*II and *Sma*I sites of pEGFP-C2. Clone C13-1 expresses the C-terminal region of the α1A calcium channel (amino acids 1959–2355), whereas S13-1 and L24-1 express extended C-teminal regions (amino acids 1959–2578 in S13-1 and 1959–2611 in L24-1) containing normal (13 glutamines in S13-1) and expanded (24 glutamines in L24-1) polyglutamine tracts, due to the GGCAG insertion sequence.

Cell cultures and transfections

Subconfluent HEK-293 and PC12 cells were transfected with 3 μg of plasmid DNA using the calcium phosphate precipitation method (Stratagene, La Jolla, CA). Twenty-four hours after transfection, cells were processed for immunocytochemical studies. The results were confirmed by three independent trials.

Generation of anti-α1A calcium channel antibodies and western blotting

Polyclonal antibodies were raised against synthetic peptides corresponding to the sequences of human brain α1A calcium channel protein [for antibody A6RPT-N, CAGSQEAELSREGPYGRE, corresponding to residues C+885–901 in the region between domains II and III (28); for antibody A6RPT-C, SRHGRRLPNGYYPAHGLAR, corresponding to residues 2479–2497 in the C-terminal region]. Aliquots of 200 μg of each synthetic peptide were injected into rabbits with Freund's complete adjuvant. After several boosters, antisera were affinity purified using HiTrap NHS-activated (Amersham Pharmacia Biotech, Uppsala, Sweden).

For western blotting, HEK-293 cells stably expressing GFP–α1A fusion proteins (C13-1, S13-1 and L24-1) were solubilized in lysis buffer (20 mM Tris–HCl, pH 6.8, 3% SDS, 4 M urea, 0.72 M 2-mercaptoethanol) including protease inhibitors (1 mM *p*-amidinophenyl methanesulfonyl fluoride, 5 μg/ml each of leupeptin, antipain, pepstatin A, chymostatin and phosphoramidon) and centrifuged. The supernatant was subjected to western blots as a crude extract. Protein samples (40 μg each) were electrophoresed on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA). Blots were incubated with calcium channel antibodies (1:1000) or anti-GFP antibody (1:1000) (Clontech) in 6% skimmed milk/TBS, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000). Immunoreactive bands were visualized by chemiluminescence (Amersham Pharmacia Biotech).

In situ hybridization and immunohistochemistry

Five control and three SCA6 brains were fixed in 10% buffered formalin and embedded in paraffin. Thick sections (6 μm) from various brain regions were examined. Thick cryostat sections (10 μm) of frozen brain tissues were also used for immunohistochemistry, since the calcium channel antisera worked better on the frozen brain tissues.

For *in situ* hybridization, a riboprobe for exons 46–47 of CACNA1A mRNA was generated from human cerebellar cDNA amplified by primers RT-CAG1-F and P1-Rev (5'-GCTCTAGATTAGCACCAATCATCGTCAC-3'). An antisense cRNA was synthesized with T7 RNA polymerase and a digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed at 55°C in hybridization buffer and hybridized probes were detected with alkaline phosphatase-labeled anti-DIG antibody and finally visualized with NBT and BCIP (Boehringer Mannheim).

Immunohistochemistry was performed using the avidin–biotinylated peroxidase complex (ABC) method as described previously (40), developed with diaminobenzidine (DAB) and counterstained with methyl green. In addition to the calcium channel antisera (1:100), rabbit polyclonal anti-26S proteasome (a gift of Dr K. Tanaka, Tokyo Metropolitan Institute of Medical Science, Japan), rabbit polyclonal cathepsin D (a gift of Dr E. Kominami, Juntendo University, Japan), mouse monoclonal anti-ubiquitin (1:1000) (Chemicon, Temecula, CA), mouse monoclonal anti-Hsp70 (SPA-810; StressGen, Victoria, BC, Canada) and mouse monoclonal anti-HDJ-2/DNAJ Ab-1 (KA2A5.6; Neomarkers, Union City, CA) were used. For double immunofluorescence analysis, primary antibodies were detected by incubation with anti-rabbit–Texas Red and anti-mouse–FITC (1:100) antibodies (Vector, Burlingame, CA) for 2 h at room temperature. Staining was always undertaken with control specimens positively stained with these antibodies. Immunostaining was negative when the calcium channel antibody was pre-absorbed with the peptide antigen and when the primary antibody was omitted, with the entire procedure performed similarly. With the ubiquitin antibody, so-called ubiquitin-positive intranuclear inclusions seen in other CAG/polyglutamine diseases were always detected.

For cultured cell systems, cells were fixed with 4% paraformaldehyde overnight and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. After incubating with blocking solutions, specimens were incubated with polyclonal antibodies (anti-α1A calcium channel or anti-GFP) or mouse monoclonal anti-ubiquitin (1:1000) (Chemicon) overnight in the cold followed by 2 h incubation at room temperature with either anti-rabbit–Texas Red or anti-mouse–FITC (1:100) (Vector). The TUNEL assay was performed using FITC-conjugated dUTP for the terminal deoxynucleotidyl transferase reaction (Promega, Madison, WI). Cells were counterstained with Hoechst 33258 (2.5 μg/ml). Immunofluorescence was examined under a confocal laser scanning microscope (LSM310; Carl Zeiss). The primary antibodies were also detected by the ABC method, developed with DAB, counterstained with hematoxylin and visualized under conventional light microscopy.

Electron microscopic analysis

Immunoelectron microscopic analysis was performed on cryostat sections of the two SCA6 cerebellar cortices immunostained as described and developed with DAB. The sections were then dehydrated and embedded in epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined with a JOEL 100-CX electron microscope.

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REFERENCES

1. Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y. and Lee, C.C. (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nature Genet.*, **15**, 62–69.
2. Subramony, S.H., Fratkin, J.D., Manyam, B.V. and Currier, R.D. (1996) Dominantly inherited cerebello-olivary atrophy is not due to a mutation at the spinocerebellar ataxia-1, Machado–Joseph disease, or dentato-rubro-pallido-luysian atrophy locus. *Mov. Disord.*, **11**, 174–180.
3. Ishikawa, K., Tanaka, H., Saito, M., Ohkoshi, N., Fujita, T., Yoshizawa, K., Ikeuchi, T., Watanabe, M., Hayashi, A., Takiyama, Y., Nishizawa, M., Nakano, I., Matsubayashi, K., Miwa, M., Shoji, S., Kanazawa, I., Tsuji, S. and Mizusawa, H. (1997) Japanese families with autosomal dominant pure cerebellar ataxia map to chromosome 19p13.1–p13.2 and are strongly associated with mild CAG expansions in the spinocerebellar ataxia type 6 gene in chromosome 19p13.1. *Am. J. Hum. Genet.*, **61**, 336–346.
4. La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991) Androgen receptor mutations in X-linked spinal and bulbar muscular atrophy. *Nature*, **352**, 77–79.
5. Huntington's Diseases Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971–983.
6. Orr, H.T., Chung, M., Banfi, S., Kwiatkowski, T.J., Servadio, A., Beaudet, A.L., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zoghbi, H.Y. (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nature Genet.*, **4**, 221–226.
7. Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., Hayashi, T., Saito, M., Tomoda, A., Miike, T., Naito, H., Ikuta, F. and Tsuji, S. (1994) Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). *Nature Genet.*, **6**, 9–13.
8. Nagafuchi, S., Yanagisawa, H., Sato, K., Shirayama, T., Ohsaki, E., Bundo, M., Takeda, T., Tadokoro, K., Kondo, I., Murayama, N., Tanaka, Y., Kikushima, H., Umino, K., Kurosawa, H., Furukawa, T., Nihei, K., Inoue, T., Sano, A., Komure, O., Takahashi, M., Yoshizawa, T., Kanazawa, I. and Yamada, M. (1994) Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p. *Nature Genet.*, **6**, 14–18.
9. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994) CAG expansions in a novel gene for Machado–Joseph disease at chromosome 14q32.1. *Nature Genet.*, **8**, 221–228.
10. Pulst, S.-M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.-N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., DeJong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C. and Sahba, S. (1996) Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nature Genet.*, **14**, 269–276.
11. Sanpei, K., Takano, H., Igarashi, S., Sato, T., Oyake, M., Sasaki, H., Wakisaka, A., Tashiro, K., Ishida, Y., Ikeuchi, T., Koide, R., Saito, M., Sato, A., Tanaka, T., Hanyu, S., Takiyama, Y., Nishizawa, M., Shimizu, N., Nomura, Y., Segawa, M., Iwabuchi, K., Eguchi, I., Tanaka, H., Takahashi, H. and Tsuji, S. (1996) Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. *Nature Genet.*, **14**, 277–284.
12. Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J.-M., Weber, C., Mandel, J.-L., Cancel, G., Abbas, N., Dürr, A., Didierjean, O., Stevanin, G., Agid, Y. and Brice, A. (1996) Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. *Nature Genet.*, **14**, 285–291.
13. David, G., Abbas, N., Stevanin, G., Dürr, A., Yvert, G., Cancel, G., Weber, C., Imbert, G., Saudou, F., Antoniou, E., Drabkin, H., Gemmil, R., Giunti, P., Benomar, A., Wood, N., Ruberg, M., Agid, Y., Mandel, J.-L. and Brice, A. (1997) Cloning of the SCA7 gene reveals a highly unstable CAG repeat expansion. *Nature Genet.*, **17**, 65–70.
14. Yabe, I., Sasaki, H., Matsuura, T., Takada, A., Wakisaka, A., Suzuki, Y., Fukazawa, T., Hamada, T., Oda, T., Ohnishi, A. and Tashiro, K. (1998) SCA6 mutation analysis in a large cohort of the Japanese patients with late-onset pure cerebellar ataxia. *J. Neurol. Sci.*, **156**, 89–95.
15. Tellez-Nagel, I., Johnson, A.B. and Terry, R.D. (1974) Studies on brain biopsies of patients with Huntington's chorea. *J. Neuropathol. Exp. Neurol.*, **33**, 308–332.
16. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunctions in mice transgenic for the HD mutation. *Cell*, **90**, 537–548.
17. Paulson, H.L., Perez, M.K., Trottier, Y., Trojanowski, J.Q., Subramony, S.H., Das, S.S., Vig, P., Mandel, J.-L., Fischbeck, K.H. and Pittman, R.N. (1997) Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron*, **19**, 333–344.
18. DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Aggregation of Huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science*, **277**, 1990–1993.
19. Skinner, P.J., Koshy, B.T., Cummings, C.J., Klement, I.A., Helin, K., Servadio, A., Zoghbi, H.Y. and Orr, H.T. (1997) Ataxin-1 with an expanded tract alters nuclear matrix-associated structures. *Nature*, **389**, 971–974.
20. Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., Sato, A., Egawa, S., Ikeuchi, T., Tanaka, H., Nakano, R., Tanaka, K., Hozumi, I., Inuzuka, T., Takahashi, H. and

Tsuji, S. (1998) Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. *Nature Genet.*, **18**, 111–117.

21. Li, M., Miwa, S., Kobayashi, Y., Merry, D.E., Yamamoto, M., Tanaka, F., Doyu, M., Hashizume, Y., Fischbeck, K.H. and Sobue, G. (1998) Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. *Ann. Neurol.*, **44**, 249–254.
22. Holmberg, M., Duyckaerts, C., Dürr, A., Cancel, G., Gourfinkel-An, I., Damier, P., Faucheux, B., Trottier, Y., Hirsch, E.C., Agid, Y. and Brice, A. (1998) Spinocerebellar ataxia type 7 (SCA7): a neurodegenerative disorder with neuronal intranuclear inclusions. *Hum. Mol. Genet.*, **7**, 913–918.
23. Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T. and Zoghbi, H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nature Genet.*, **19**, 148–154.
24. Sapp, E., Schwarz, C., Chase, K., Bhide, P.G., Young, A.B., Penney, J., Vonsattel, J.P., Aronin, N. and DiFiglia, M. (1997) Huntingtin localization in brains of normal and Huntington's disease patients. *Ann. Neurol.*, **42**, 604–612.
25. Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Broose, E., Hofman, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. and Numa, S. (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature*, **350**, 398–402.
26. Hillman, D., Chen, S., Aung, T.T., Cherksey, B., Sugimori, M. and Llinás, R.R. (1991) Localization of P-type calcium channels in the central nervous system. *Proc. Natl Acad. Sci. USA*, **88**, 7076–7080.
27. Westenbroek, R.E., Sakurai, T., Elliot, E.M., Hell, J.W., Starr, T.V.B., Snutch, T.P. and Catterall, W.A. (1995) Immunochemical identification and subcellular distribution of the alpha1A subunit of brain calcium channels. *J. Neurosci.*, **15**, 6403–6418.
28. Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M.G., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Hann, J., Lindhout, D., van Ommen, G.-J.B., Hofker, M.H., Ferrari, M.D. and Frants, R.R. (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca^{2+} channel gene CACNL1A4. *Cell*, **87**, 543–552.
29. Volsen, S.G., Day, N.C., McCormack, A.L., Smith, W., Craig, P.J., Beattie, R., Ince, P.G., Shaw, P.J., Ellis, S.B., Gillespie, A., Harpold, M.M. and Lodge, D. (1995) The expression of neuronal voltage-dependent calcium channels in human cerebellum. *Brain Res. Mol. Brain Res.*, **34**, 271–282.
30. Becher, M.W., Kotzuk, J.A., Sharp, A.H., Davies, S.W., Bates, G.P., Price, D.L. and Ross, C.A. (1998) Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. *Neurobiol. Dis.*, **4**, 387–397.
31. Lorenzon, N.M., Lutz, C.M., Frankel, W.N. and Beam, K.G. (1998) Altered calcium channel currents in Purkinje cells of the neurological mutant mouse *leaner*. *J. Neurosci.*, **18**, 4482–4489.
32. Hayashi, Y., Kakita, A., Yamada, M., Egawa, S., Oyanagi, S., Naito, H., Tsuji, S. and Takahashi, H. (1998) Hereditary dentatorubral-pallidoluysian atrophy: ubiquitinated filamentous inclusions in the cerebellar dentate nucleus neurons. *Acta Neuropathol.*, **95**, 479–482.
33. Matilla, A., Koshy, B.T., Cummings, C.J., Isobe, T., Orr, H.T. and Zoghbi, H.Y. (1997) The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1. *Nature*, **389**, 974–978.
34. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S.U., Bredesen, D.E., Tufaro, F. and Hayden, M.R. (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nature Genet.*, **18**, 150–154.
35. Klement, I.A., Skinner, P.J., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell*, **95**, 41–53.
36. Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell*, **95**, 55–66.
37. Brice, N.L., Berrow, N.S., Campbell, V., Page, K.M., Brickley, K., Tedder, I. and Dolphin, A.C. (1997) Importance of the different beta subunits in the membrane expression of the alpha1A and alpha2 calcium channel subunits: studies using a depolarization-sensitive alpha1A antibody. *Eur. J. Neurosci.*, **9**, 749–759.
38. Lau, F.C., Abbott, L.C., Rhyu, I.M., Kim, D.S. and Chin, H. (1998) Expression of calcium channel alpha1A mRNA and protein in the leaner mouse (tg^{la}/tg^{la}) cerebellum. *Mol. Brain Res.*, **59**, 93–99.
39. Fletcher, C.F., Lutz, C.M., O'Sullivan, N.T., Shaughnessy, J.D.Jr, Hawkes, R., Frankel, W.N., Copeland, N.G. and Jenkins, N.A. (1996) Absence epilepsy in tottering mutant mice is associated with calcium channel defects. *Cell*, **87**, 607–617.
40. Ishikawa, K. (1999) A clinical, neuropathological and molecular study in two families with spinocerebellar ataxia type 6 (SCA6). *J. Neurol. Neurosurg. Psychiatry*, in press.
41. Ishikawa, K., Mizusawa, H., Fujita, T., Ohkoshi, N., Doi, M., Komatsuzaki, Y., Iwamoto, H., Ogata, T. and Shoji, S. (1995) Calbindin-D28k immunoreactivity in the cerebellum of spinocerebellar degeneration. *J. Neurol. Sci.*, **129**, 179–185.
42. Mizusawa, H., Yoshizawa, K., Kanazawa, I., Mori, Y., Ogata, T. and Nakanishi, T. (1987) A family of cortical cerebellar atrophy (Holmes): a clinical and neuropathological study. *Neurol. Med.*, **26**, 257–264.