Acta Neuropathol (1999) 97 : 201–207

CASE REPORT

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Nuclear inclusions in spinocerebellar ataxia type 1

Received: 16 June 1998 / Accepted: 10 August 1998

Abstract Spinocerebellar ataxia type 1 is due to a CAG repeat expansion in the gene encoding ataxin-1. In a case with an expansion of 56 repeats, intranuclear inclusions were found only in neurons, both in severely affected regions (such as the pons) and in areas where the lesions were inconspicuous (such as the cortex or the striatum). The inclusions were labelled by a monoclonal antibody directed against long polyglutamine stretches (1C2); they were also detected by the anti-ubiquitin antibody. They were faintly eosinophilic, Congo red negative and were not stained by thioflavin S or by ethidium bromide.

Introduction

Expansion of CAG repeats, causing the synthesis of proteins exhibiting a large polyglutamine stretch, is the molecular basis of various neurodegenerative disorders including Huntington's disease, five types of autosomal dominant cerebellar ataxias [spinocerebellar ataxia (SCA) 1, 2, 3, 6 and 7], dentatorubropallidoluysian atrophy, and Kennedy (SBMA) disease (for review see [14]).

The monoclonal antibody 1C2, initially raised against the TATA binding protein (TBP), has been found to react with abnormal proteins containing a large polyglutamine tract [18]. This cross-reactivity is probably related to the large polyglutamine stretch epitope of normal TBP.

Nuclear inclusions have been identified in several of the disorders related to polyglutamine expansions: Huntington disease [6], SCA3 [13], SCA7 [8], and DRPLA [9]. The presence of nuclear inclusions, detected by the anti-ataxin-1 antibody was also observed in transgenic mice expressing the mutant protein and in SCA1 cases [3, 17]. Ataxin-1 aggregates were shown to colocalize with the proteasome and were reduced in number in HeLa cells overexpressing the molecular chaperone HDJ-2/HSDJ [3]. Here we show that the SCA1 nuclear inclusions are present in broad areas of the brains but only in a small subset of neurons. In most regions, less than 1% of the neuron nuclear profiles contain inclusions. The inclusions react with the 1C2 antibody, a fact that indicates that they contain the mutated protein with the enlarged polyglutamine stretch. They are often but probably not always ubiquitinated, the number of inclusions revealed by the 1C2 antibody being most often higher than after antiubiquitin.

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Materials and methods

Case (SAL 326-038 in [7])

The patient's father was affected as well as several members of his family (see pedigree Fig. 1). The patient had an unsteady gait and dysarthric speech at age 30. He requested a walking aid at 32, could not write any more at age 35 and was wheelchair-bound at age 38. Swallowing difficulties were prominent and caused repeated episodes of pneumopathy. Orthostatic hypotension was noticed after a course of 4 years. Three years later, cerebellar ataxia was severe, deep tendon reflexes were increased and plantar reflex was extensor. Extrapyramidal rigidity with blepharospasm and bradykinesia were present. There were fasciculations of the tongue and shoulders and deep sensory loss in the legs. The eye movements were slow without nystagmus. Some cognitive impairment was noticed. He died at the age of 42.

Molecular analysis revealed an expanded CAG repeat of 56 and a normal allele with 29 CAG repeats in the cerebral cortex.

Histopathology

Blocks from the spinal cord, medulla oblongata, pons, cerebellum and dentate nucleus, midbrain, striatum and cortex were available. Sections (8 μm thick) were obtained and stained by hematoxylin and eosin, Bodian silver technique coupled with Luxol-fast blue, Congo red and thioflavin S. To study the location of nucleic acid, we found that ethidium bromide (1 : 2000 of 10 mg/ml solution from Euromedex-Strasbourg, France for 15 min) gave the best results.

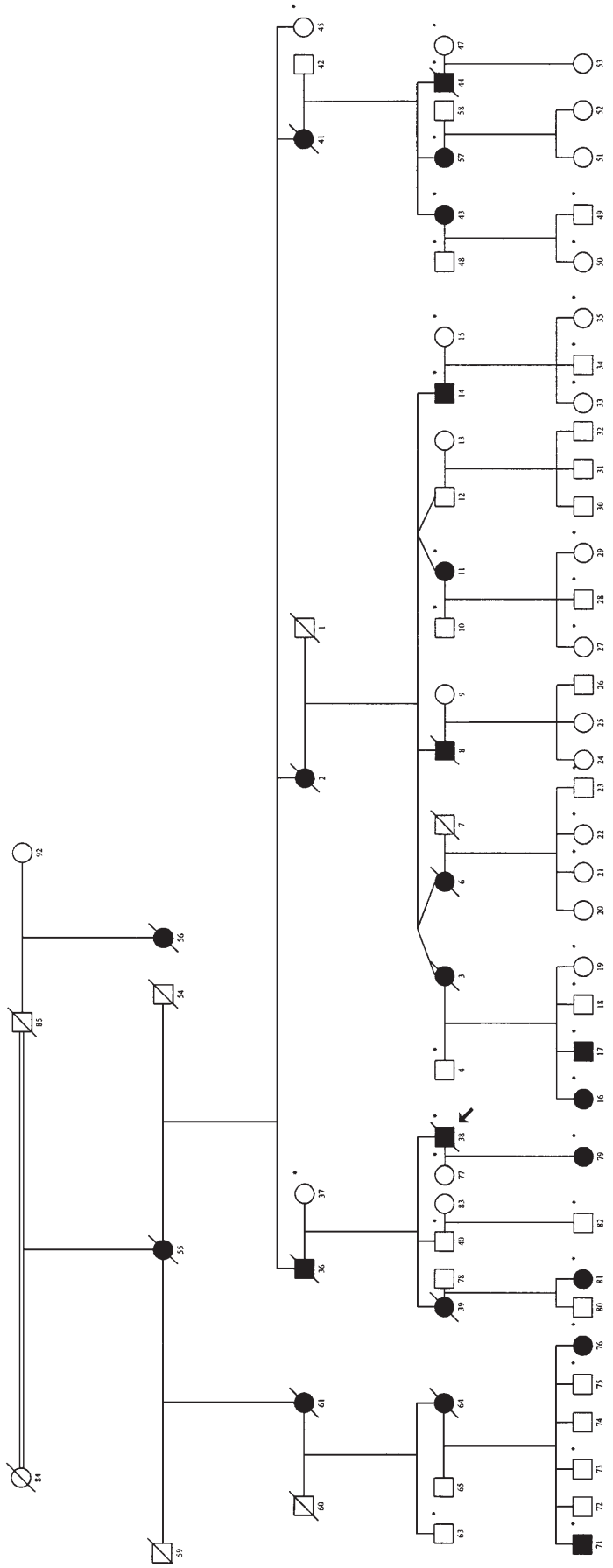

Fig. 1 Pedigree. *Square* Male, *circle* female, *black* affected, *crossed* deceased, *dot* examined

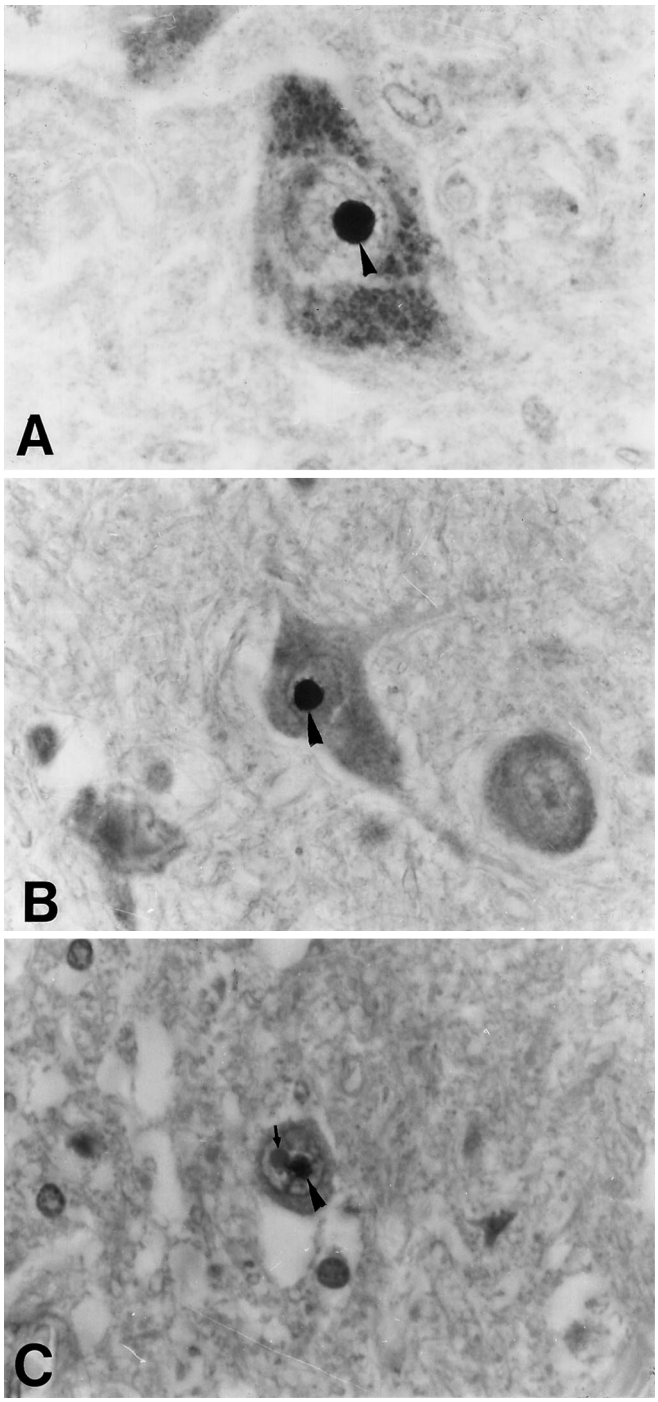

Fig. 2 A–C Labelling of nuclear inclusions by 1C2 antibody. Nuclear inclusions (*arrowheads*) were labelled by a monoclonal antibody detecting polyglutamine stretch (1C2, Euromedex® Soufflelweyersheim, France). *Small arrow* in **C** points to the nucleolus. **A** Substantia nigra; **B** pons; **C** spinal cord. **A–C** Original magnification × 330

Immunohistochemistry

Primary antibodies

A commercially available polyclonal antibody against ubiquitin (polyclonal, Dako) was used at a concentration of 1 : 1000 and incubated overnight at room temperature, after pretreatment of the section with 99% formic acid, for five min. The monoclonal antibody directed against the polyglutamine stretch (1C2: generous gift of Y. Trottier) has been previously characterized [18], and was used at a concentration of 1 : 4000, which avoids staining of the nuclear TBP. Incubation was carried out for 48 h at room temperature.

Labelling

For standard microscopy, the presence of the primary antibody was revealed by the streptavidin-biotin technique (Amersham), using diaminobenzidine as chromogen. For confocal microscopy, the primary antibody was labelled either by a secondary biotinylated antibody that was secondarily bound to a streptavidin-biotin complex containing CY2 (red fluorescence) or by a secondary antibody directly coupled with CY3 (green fluorescence).

Quantitation

In the anterior horn, pons, dentate nucleus and substantia nigra, the number of nuclear profiles containing an inclusion was directly evaluated. In the cerebral cortex and striatum (where the number of inclusions was low, while the density of neurons was high), the number of fields containing one nuclear profile with an inclusion was first assessed on a total of at least 150 microscopic fields examined with a x40 lens for the cortex and of at least 200 fields for the striatum. The number of neuronal profiles per field was secondarily assessed. The proportion of affected neurons was then calculated (mean number of neuronal profile per field x proportion of fields with an affected neuron). Chi^2 test or Fisher exact probability, when appropriate, was used to compare the numbers of inclusions labelled by the anti-ubiquitin and the 1C2 antibody.

Results

There was a severe neuronal loss in the dentate nucleus and in the inferior olivary complex, with a parallel atrophy of the superior and inferior cerebellar peduncles. The density of the Purkinje cell was markedly decreased. The pontine nuclei were atrophied and gliotic. Some gliosis was noticed in the external pallidum, subthalamic nucleus and red nucleus. The posterior columns and the spin-

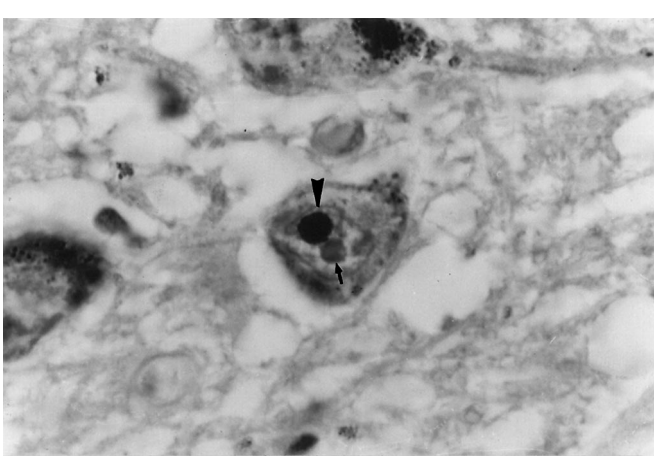

Fig. 3 Ubiquitin immunohistochemistry. After hematoxylin counterstaining, the nucleolus (*small arrow*) was stained blue; the inclusion (*arrowhead*) was strongly labelled by the anti-ubiquitin antibody (polyclonal, Dako). Original magnification × 330

Table 1 Percentage of neuronal nuclear profiles containing an inclusion labelled either by 1C2 (recognizing expanded polyglutamine stretches; Euromedex) or anti-ubiquitin (Dako)

Proportions (%) of neurons labelled by			
Region	1C2	Anti-ubiquitin	*P*-value
Cerebral cortex	0.5	0.3	NS
Striatum	0.6	0.2	$P < 0.01$
Substantia nigra	0.8	4.5	$P < 0.0001$
Pons	8.1	4.0	NS
Locus coeruleus	[a]	0	
Cerebellum	0	0	
Dentate nucleus	[a]	[a]	
Anterior horn	2.7	4.1	NS

[a] One neuronal profile containing a nuclear inclusion on the whole section

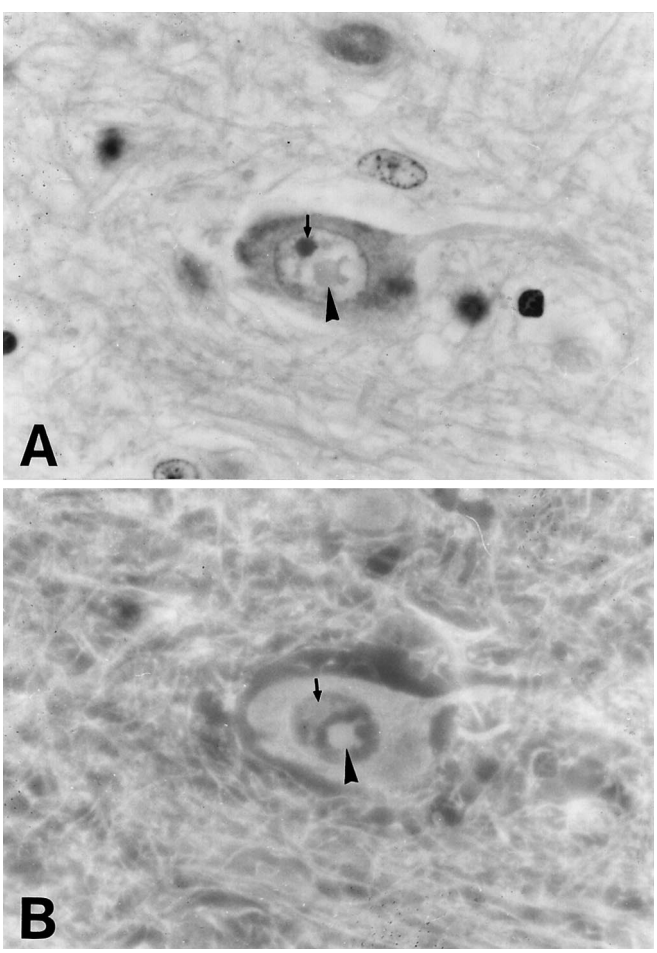

Fig. 4 A, B Hematoxylin-eosin and fluorescence microscopy. Same neuron located in the nuclei pontis. **A** The nucleolus (*small arrow*) appeared basophilic. The inclusion (*arrowhead*) was barely visible. **B** Under UV light the inclusion was faintly fluorescent. **A, B** Original magnification × 330

ocerebellar tracts were pale. There was some neuronal loss and gliosis in Clarke's column and in the anterior horn.

The 1C2 (Fig. 2) and the ubiquitin (Fig. 3) antibodies intensely labelled nuclear inclusions. Their colocalization

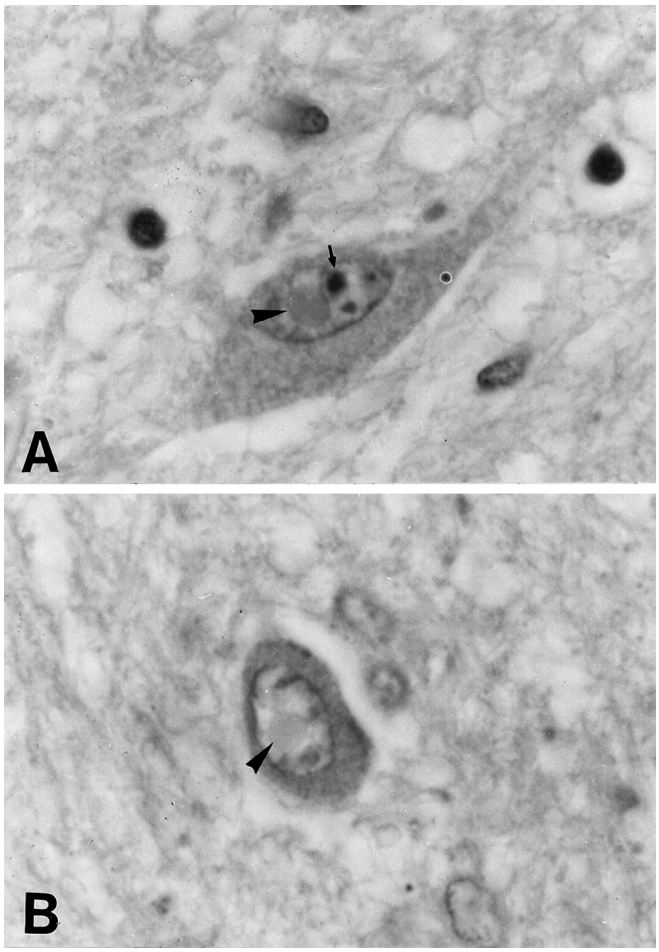

Fig. 5 A, B Hematoxylin-eosin and Congo red stain. **A** The inclusion (*arrowhead*) is faintly eosinophilic compared to the nucleolus (*arrow*). **B** The inclusion (*arrowhead*) is not stained by Congo red. **A, B** Original magnification × 330

was examined by confocal microscopy (see Fig. 6). They were spherical, 2–3 μm in diameter, and sometimes located next to the nucleolus. They were only seen in profiles identified as neuronal. Their prevalence is indicated in Table 1.

The percentage of neuronal profiles with nuclear inclusions reached its highest value (8.1%) in the pons as revealed by 1C2 antibody and in the substantia nigra (4.5%) as revealed by the anti-ubiquitin antibody. The percentage of nuclear profiles bearing inclusions was found to be higher with the 1C2 antibody than with anti-ubiquitin with two exceptions: the anterior horn of the spinal cord (not statistically significant) and the substantia nigra ($P < 0.0001$).

The nuclear inclusions were inconspicuous using hematoxylin-eosin staining, being unstained or faintly eosinophilic. Their pale color contrasted with the intense blue color of the nucleolus (Fig. 4). Examination of the slides under ultraviolet light with an FITC filter disclosed a light but definite spontaneous fluorescence (Fig. 4). They were not stained by Congo red (Fig. 5) or by thioflavin S. They were not stained by ethidium bromide, in contrast to the nucleolus which appeared brightly fluorescent (Fig. 6).

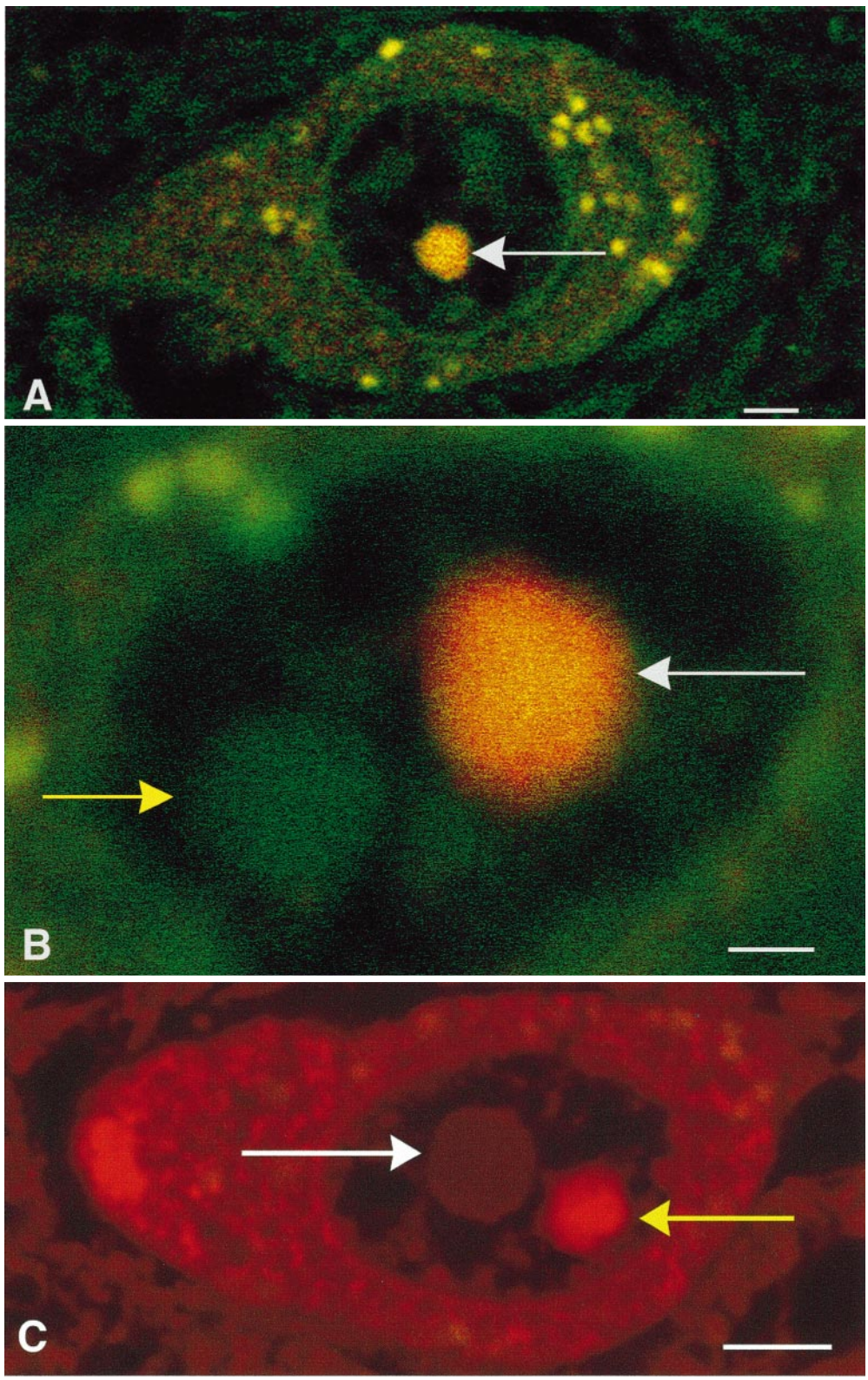

Fig. 6 A–C Confocal microscopy. **A** Doulbe labelling: ubiquitin antibody was revealed by an FITC-coupled secondary antibody. For the 1C2 antibody, a biotinylated secondary antibody was followed by streptavidin coupled with CY2 (*red*) fluorochrome. Colocalization is shown in yellow. The cytoplasmic *yellow* granules are lipofuscin. The inclusion is indicated by a *white arrow*. **B** Double labelling as in **A**. The nucleolus (*yellow arrow*) is spontaneously faintly visible. **C** Section stained by ethidium bromide (1 : 2000 of 10 mg/ml solution from Euromedex – for 15 min). The nucleolus appears bright red (*yellow arrow*). The inclusion is indicated by a *white arrow*. *Bars* **A** 2 µm, **B** 1 µm, **C** 5 µm

Discussion

The identification of five diseases in which ubiquitinated neuronal nuclear inclusions are associated with a translated CAG repeat expansion (SCA1, SCA3, SCA7, DRPLA, Huntington's disease) suggests that those inclusions are, indeed, reliable markers of that type of disorder. However, since they have not been described yet in SCA2, SCA6 and SBMA, they can not been definitely ac-

cepted as a necessary association. On the other hand, the nuclear inclusions have not been seen in other neurodegenerative disorders with an identified mutation. Up to now, their presence may thus be considered as highly suggestive of polyglutamine expansion.

The labelling of the inclusions by the 1C2 antibody may be taken as good evidence that they contain the mutated protein, or part of it including the polyglutamine stretch. The ubiquitination of the mutated protein suggests that it is recognized as abnormal. In two regions (substantia nigra and anterior horn of the spinal cord), ubiquitin-positive inclusions were more numerous than 1C2-positive ones. This might be due to sampling fluctuations. It might be related to the better sensitivity of the ubiquitin antibody, but the inclusions should then be always detected more easily. Finally, it could be related to different stages of the inclusions: the ubiquitination has indeed been shown to be a late phenomenon in transgenic mice with the Huntington gene [5]. Ubiquitination could hamper the 1C2 reactivity.

Only a few neurons contain inclusions. The distribution is presently badly understood. It could be conditioned by the level of expression of the mutated form of the protein ataxin 1. The level of expression of ataxin 1, although not quantitatively assessed, has indeed been shown to be different in various neuronal populations [16]. The immunohistochemical findings suggest that the protein is not expressed in glial cells, explaining the strictly neuronal topography of the inclusions [16]. Ataxin 1 is normally localized in the nucleus and interacts with the components of the nuclear matrix, including the promyelocytic leukemia protein [17] and the leucine-rich acidic nuclear protein [12]. The interaction between ataxin 1 and a partner could be necessary for formation of the inclusions. Their topography could then be restricted to neurons expressing both ataxin-1 and its partner.

The distribution might also be related to somatic mosaicism, the length of the CAG repeats expansion being variable from tissue to tissue and, within the brain, from region to region [19]. The abnormal allele usually contains a larger number of repeats in the cerebral than in the cerebellar cortex [1, 11]. It has been suggested that the expansion could sometimes enlarge during cell division and thus be more common in glia than in neurons [2]; this would not explain, however, why the inclusions are only neuronal. Finally, the mosaicism, being of a limited range, is probably insufficient to explain the topography of the changes.

It has been suggested that the presence of the nuclear inclusions is directly related to the pathogenetic mechanism of neurodegeneration in CAG repeat expansion [4, 10] by causing filamentous peri- and intranuclear aggregates [9]. Proteins aggregated in fibrillar structures (such as collagen, elastin or amyloid proteins) exhibit a spontaneous fluorescence that we also found in the nuclear inclusions. This finding is in agreement with ultrastructural data obtained in transgenic mice bearing the mutated Huntington gene [4] and in post-mortem material of DRPLA [9], showing that the inclusions were at least partly made of fibrils, as is amyloid [15]. However, we failed to find the congophilia and thioflavin affinity characteristic of amyloidosis.

The topography of the inclusions in disorders due to polyglutamine tract expansion does not parallel the severity of the neuronal loss: they were found in high numbers in regions which were considered to be little affected, such as the cortex in SCA7 [8], the inferior olive in SCA3 [13] or the substantia nigra in this SCA1 case. This finding is not incompatible, however, with their pathogenetic role. With the cross-sectional view that the pathology only permits, it is not possible to determine the course of neuronal death over time: the areas where inclusions are numerous in pathological examination could be those in which neuronal death would have taken place weeks, months or years later.

Conclusion

SCA1 is to be added to the list of the CAG repeat expansion disorders with nuclear inclusions. These inclusions are faintly eosinophilic, ubiquitin positive and labelled by an antibody directed against the polyglutamine stretch. They might play an important role in neuronal death.

Acknowledgements We are grateful to Lazlo Tora, Yves Lutz and Y. Trottier for advice in using the 1C2 antibody.

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Note added in proof The use of ethidium bromide to stain nuclei acids on section has been fully described in a reference of which we were not aware: Schmued LC, Sawchenko PE, Swanson LW (1982) Some fluorescent counterstains for neuroanatomical studies. J Histochem Cytochem 30 : 123–128

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