

# Expression and functional analysis of the SCA7 disease protein ataxin-7

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**Cover:**

Sequestering of CBP (red) into ataxin-7 inclusions (green) in the nucleus (blue) of co-transfected SK-N-SH neuroblastoma cells.

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The great tragedy of science:  
The slaying of a beautiful hypothesis by an ugly fact

Thomas H. Huxley

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## ABBREVIATIONS

Aa	amino acids
ADCA	autosomal dominant cerebellar ataxia
AR	androgen receptor
Bp	base pairs
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine 3', 5'-monophosphate
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
CRX	cone-rod homeobox protein
DNA	deoxyribonucleic acid
DRPLA	dentatorubral pallidoluysian atrophy
ER	endoplasmatic reticulum
GFP	green fluorescent protein
HAT	histone acetyltransferase
HD	Huntington's disease
HIP-1	huntingtin interacting protein 1
Hippi	HIP-1 protein interactor
HSP	heat shock protein
HPRT	hypoxanthine phosphoribosyltransferase
JNK	c-jun N-terminal kinase
kb	kilobase pairs
kD	kiloDalton(s)

LANP	leucine-rich acidic nuclear protein
mRNA	messenger ribonucleic acid
NES	nuclear export signal
NI	nuclear inclusion
NLS	nuclear localization signal
OPCA	olivoponto cerebellar atrophy
ORF	open reading frame
PDA	patent ductus arteriosus
PML	promyolytic leukemia
RNA	ribonucleic acid
SAGA	spt-ada-gcn5-acetyltransferase
SBMA	spinal and bulbar muscular atrophy
SCA	spinocerebellar ataxia
TAF	TBP-associated factors
TBP	TATA-binding protein
UTR	untranslated region

## **ABSTRACT**

Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disease characterized by cerebellar ataxia and visual problems due to a progressive and selective loss of neurons within the cerebellum, brainstem and retina.

The disease is caused by the expansion of a CAG repeat in the first coding exon of the SCA7 gene, resulting in an expanded polyglutamine domain in the N-terminal part of ataxin-7, a protein of unknown function.

To expand our knowledge of the ataxin-7 protein and the mechanism by which mutant ataxin-7 causes disease, we have studied the expression and function of both the normal and the mutated ataxin-7 protein.

Ataxin-7 expression was examined in brain and non-CNS tissues from SCA7 patients and age-matched controls. Expression was predominantly nuclear in neurons throughout the brain of both healthy and SCA7 individuals. We also observed aggregation of mutant ataxin-7 in the nuclei of neurons. No obvious difference in the expression level of ataxin-7 or the formation of aggregates could be observed between affected and non-affected brain regions in SCA7 patients. Based on these findings, we could conclude that the cell type specific neurodegeneration in SCA7 is not due to differences in expression levels or to the formation of ataxin-7 aggregates.

To widen our studies on ataxin-7 expression, we isolated and characterized the mouse SCA7 gene homolog. Cloning of the mouse SCA7 gene revealed two SCA7 mRNA isoforms that were highly homologous to their human counterparts. Immunohistochemical analysis also revealed a conserved expression pattern of ataxin-7 in adult mouse brain. In addition, ataxin-7 expression was observed during embryonic development in brain as well as in several non-neuronal tissues such as heart, liver and lung.

Besides SCA7, eight neurodegenerative disorders are known to be caused by expanded polyglutamine repeats, including SCA 1-3, 6 and 17, DRPLA, SBMA and Huntington's disease. The polyglutamine disorders have many features in common and a common pathological disease mechanism involving transcriptional dysregulation has been proposed. To investigate the possible involvement of transcriptional dysregulation in SCA7 pathology, we analyzed the effects of both wild-type and expanded ataxin-7 on transcription driven by the co-activator CBP, the Purkinje cell-expressed nuclear receptor ROR $\alpha$ 1 or a basic TATA promoter. As previously shown for other polyglutamine disease proteins, expansion of the polyglutamine domain in ataxin-7 leads to reduced transcription. Surprisingly, strong repression of CBP-mediated, ROR $\alpha$ 1-mediated and basal transcription was also observed with wild-type ataxin-7, suggesting that the normal ataxin-7 protein may have a role in transcriptional regulation.

## ORIGINAL PUBLICATIONS

This thesis is based on the following articles and manuscripts, which will be referred to by their Roman numerals:

I. Jonasson, J.\*, **Ström, A.\***, Hart, P., Brännström, T., Forsgren, L. and Holmberg, M. (2002). Expression of ataxin-7 in CNS and non-CNS tissue of normal and SCA7 individuals. *Acta Neuropath* 104:29-37.

II. **Ström, A.**, Jonasson, J., Hart, P., Brännström, T., Forsgren, L. and Holmberg, M. (2002). Cloning and expression analysis of the murine homolog of the spinocerebellar ataxia type 7 (SCA7) gene. *Gene* 285: 91-99.

III. **Ström, A.**, Forsgren, L. and Holmberg, M. (2003). Identification of spinocerebellar ataxia type 7 isoform SCA7b in mice (Manuscript).

IV **Ström, A.**, Forsgren, L. and Holmberg, M. (2003). A role for both expanded and wild-type ataxin-7 in transcriptional regulation (Manuscript).

\*These authors contributed equally to this work

## **AIM OF THIS THESIS**

The aim of the work for this thesis was to increase our knowledge of the ataxin-7 protein and the mechanism by which mutant ataxin-7 causes spinocerebellar ataxia type 7 (SCA7). Specific goals were to:

- Analyze the expression pattern of ataxin-7 in normal and SCA7 individuals
- To examine the distribution of nuclear inclusions in tissues of SCA7 patients
- To clone and analyze the murine ataxin-7 homolog
- To determine whether alternative SCA7 transcripts are expressed, and if so, to characterize them
- To investigate whether interference with transcriptional regulation by mutant ataxin-7 is involved in SCA7 pathology

# **INTRODUCTION**

## **Spinocerebellar ataxia type 7 (SCA7)**

Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disease characterized by cerebellar ataxia and visual problems due to a progressive and selective loss of neurons within the cerebellum, brainstem and retina (Konigsmark and Weiner, 1970; Martin et al., 1994). The disease is caused by the expansion of a CAG repeat in the first coding exon of the SCA7 gene, resulting in an expanded polyglutamine domain in the N-terminal part of the corresponding protein, ataxin-7 (David, 1997). The same type of mutation is known to cause eight other neurodegenerative disorders and a common disease mechanism has been proposed for these disorders (Koide et al., 1999; Evert et al., 2000). Even though these so called polyglutamine disorders have many features in common, several differences can also be seen, indicating that the host protein context influences the disease pathology. To learn more about polyglutamine disorders in general and also in what respects SCA7 is unique, we have studied both the wild-type and the expanded ataxin-7 protein. In this thesis, I start with a review of the current knowledge of SCA7 and other polyglutamine disorders.

## **Clinical manifestations and neuropathology of SCA7**

SCA7, previously known as OPCA III or ADCA type II, is inherited in an autosomal dominant fashion. Globally, families afflicted with the disease are relatively rare, with a reported prevalence of less than 1/100 000 (Gouw et al., 1998). In contrast to this, SCA7 is the major type of autosomal dominant cerebellar ataxia (ADCA) diagnosed in Sweden and Finland (Jonasson et al.,

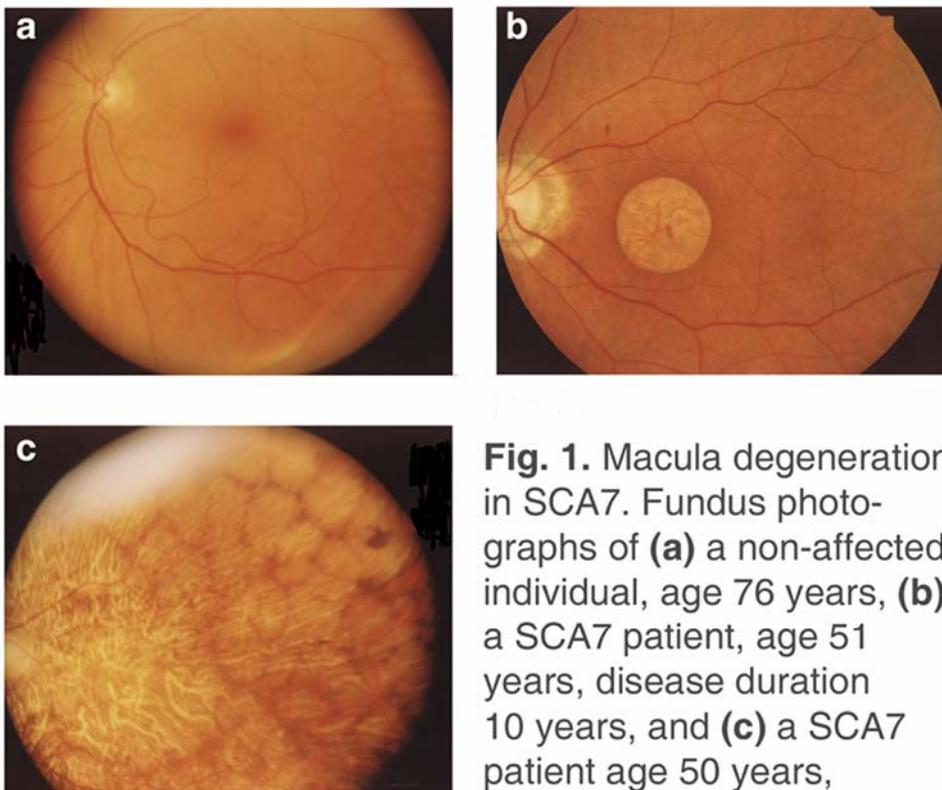
2000). Strong genetic anticipation presenting as a younger age of onset and a more severe progression of the disease in successive generations, is usually observed in families affected by SCA7. The age of onset of the disease can range from a few months to >75 years and the duration, i.e. time of onset until death, can be decades or as short as 3-4 months (Martin et al., 1994; Gouw et al., 1995; David et al., 1998; Johansson et al., 1998). The range of symptoms also varies greatly between SCA7 patients (Table 1).

**Table 1.** Symptoms of SCA7 patients

<b>Symptoms observed in:</b>		
<b>75-100% of patients</b>	<b>25-75% of patients</b>	<b>0-25% of patients</b>
Cerebellar gait ataxia	Decreased vibration sense	Decreased hearing
Dysarthria	Dysphagia	Postural tremor
Decreased visual acuity	Ophthalmoplegia	Parkinsonian symptoms
Hyperflexia	Extensor plantar reflexes	Facial myokymia
	Sphincter disturbances	Mental disorientation
	Spasticity	Scoliosis
	Muscle atrophy	Nystagmus
Symptoms and frequencies have been summarized from two publications: David et al. (1998) and Johansson et al. (1998)		

Cerebellar ataxia, manifesting itself as disturbed coordination of movements, wide-based gait (gait ataxia) and speech problems (dysarthria) is, however, observed in most patients (David et al., 1998; Johansson et al., 1998).

Another hallmark of SCA7 is loss of vision, which distinguishes the disease from other cerebellar ataxias (Enevoldson et al., 1994; David et al., 1998; Johansson et al., 1998). The visual problems usually start with defects in blue/yellow colour discrimination, followed by a progressive macular degeneration which results in decreased visual acuity and ultimately in blindness (Fig. 1) (Enevoldson et al., 1994; Johansson et al., 1998).



**Fig. 1.** Macula degeneration in SCA7. Fundus photographs of **(a)** a non-affected individual, age 76 years, **(b)** a SCA7 patient, age 51 years, disease duration 10 years, and **(c)** a SCA7 patient age 50 years, disease duration 31 years. Photographs, courtesy of Dr. Ola Sandgren, Department of Ophthalmology, University Hospital of Umeå, Sweden.

Besides gait ataxia, dysarthria and visual loss, SCA7 patients can show symptoms caused by pyramidal dysfunction such as increased reflexes and/or extensor plantar reflexes (Babinski's sign) and/or lower limb spasticity. Decreased vibration sense, dysphagia (difficulties in swallowing), sphincter disturbances and oculomotor symptoms such as paralysis of the eye muscle (ophthalmoplegia) are also seen in around 25-75% of the patients (David et al., 1998; Johansson et al., 1998). Impaired hearing, postural tremor, Parkinsonian symptoms, facial myokymia, mental deterioration, nystagmus and scoliosis have also been reported in a few patients (David et al., 1998; Johansson et al., 1998).

In cases with childhood onset, the symptoms and the progression of the disease are particularly severe. Furthermore, non-neurological symptoms such as muscle hypertonia and patent ductus arteriosus (PDA, an opening between the left lung artery and the aorta that usually closes at birth) have been reported in infantile SCA7 cases (Anttinen, 1986; Benton et al., 1998; Johansson et al., 1998). These children fail to thrive and the rapid disease progression leads to death within a few months.

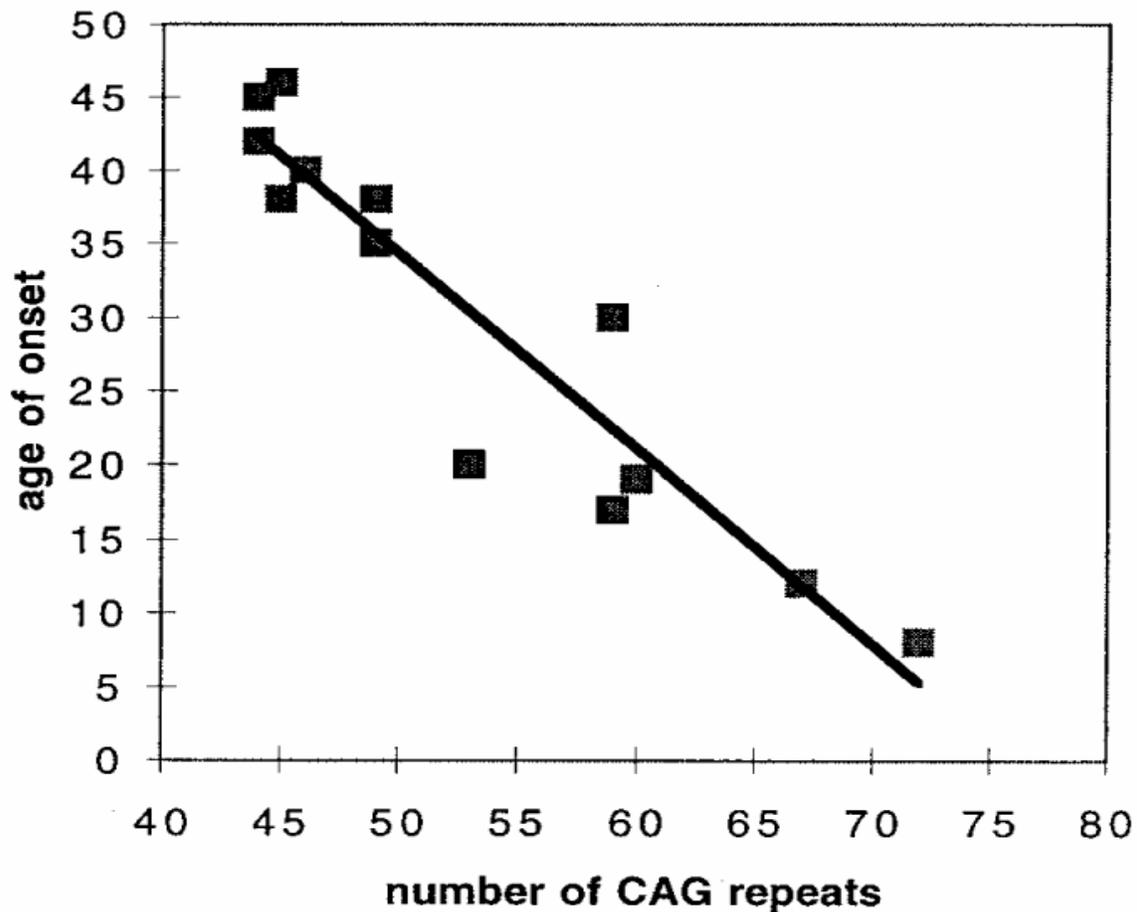
The neuropathological findings vary between different SCA7 patients. Atrophy of the retina, cerebellum, brainstem, spinal cord and spinocerebellar tracts is, however, commonly seen (Enevoldson et al., 1994; Gouw et al., 1994; Martin et al., 1994). Examination of the retina usually reveals atrophy of the choroids and degeneration of pigment epithelial cells, bipolar cells, ganglion cells and also both rod and cone photoreceptor cells (Gouw et al., 1994; Martin et al., 1994). Microscopic analysis of the brain usually discloses loss of neurons in cerebellar Purkinje and granule cell layers as well as in the dentate nucleus. In the medulla, the inferior olivary nucleus

often shows very prominent neuronal loss and the spinal cord neurons in the anterior horn are commonly affected (Enevoldson et al., 1994; Gouw et al., 1994; Martin et al., 1994). Mild atrophy of the cerebral cortex and substantia nigra has also been reported in a few cases, but the cerebral cortex and the basal ganglia are generally not considered to be primary sites of pathology (Enevoldson et al., 1994; Gouw et al., 1994; Martin et al., 1994).

### **The SCA7 mutation**

The gene causing SCA7 was mapped to chromosome 3p12-21.1 by three independent research groups in 1995 (Benomar et al., 1995; Gouw et al., 1995; Holmberg et al., 1995), and cloned in 1997 (David, 1997). An expansion of a CAG triplet repeat in the first coding exon of the gene, resulting in an expanded polyglutamine domain in a protein called ataxin-7, was found to be the cause of SCA7 (David, 1997; Del-Favero et al., 1998; Koob et al., 1998). SCA7 thus became the eighth neurodegenerative disorder shown to be caused by an expanded polyglutamine domain.

Genetic analysis of the SCA7 CAG repeat showed repeat lengths ranging from 7-17 in healthy individuals, while repeat lengths in affected individuals ranged from about 40 to over 200 (David, 1997; Benton et al., 1998; Johansson et al., 1998). These studies also revealed that there was a negative correlation between repeat length and age at onset of disease (Fig. 2). Furthermore, the repeat length was found to affect the symptoms displayed by the patients. Patients with longer repeats usually presented with visual problems, whereas patients with more moderate repeat lengths often presented with ataxia (David et al., 1998; Johansson et al., 1998; Giunti et al., 1999). Studies of parent to child transmissions of the CAG repeat



**Fig. 2.** Negative correlation between the number of CAG repeats and age at disease onset in 15 Swedish SCA7 patients (Johansson et al., 1998).

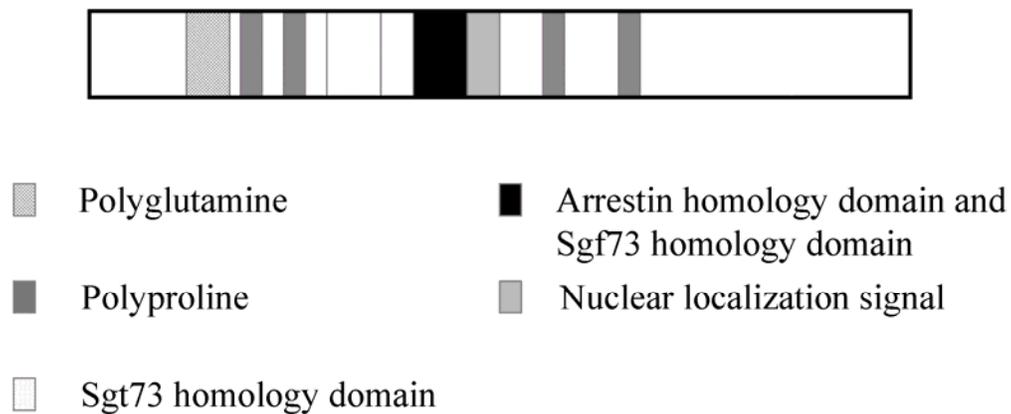
revealed instability of the repeat during germ line transmission. In most cases, the instability resulted in longer repeats in the offspring. Since longer repeats are associated with an earlier age of onset, this resulted in decreased age of onset in successive generations (David, 1997; Johansson et al., 1998). This molecular mechanism explains the genetic anticipation observed in SCA7 and most other polyglutamine diseases (La Spada et al., 1991; Group,

1993; Orr et al., 1993; Matilla et al., 1995). Earlier, it had been reported for other polyglutamine disorders that the gender of the transmitting parent influenced the stability of the CAG repeat (Jodice et al., 1994; Reddy and Housman, 1997). The same phenomenon could be observed for SCA7, where the repeat was shown to be especially unstable and prone to expansion during spermatogenesis. In agreement with this, the great majority of infant cases of SCA7, with extreme repeats, are the result of paternal transmissions (Anttinen, 1986; Enevoldson et al., 1994; David, 1997; Benton et al., 1998; David et al., 1998; Johansson et al., 1998).

### **The SCA7 gene and ataxin-7 protein**

Cloning of the SCA7 gene in 1997 revealed a 7.5-kb mRNA transcript with an open reading frame of 2727 bp, coding for a 892-kDa protein, ataxin-7, with a predicted molecular weight of 95 kDa (David, 1997). Besides the polyglutamine domain, which is located in the N-terminal part of the protein, several other regions showing homology to functional domains have been identified in ataxin-7, including four proline-rich regions predicted to be SH3-binding sites, a nuclear localization signal (aa 378-394), a motif homologous to the phosphorylation recognition site of arrestins and two regions homologous to a *bona fide* subunit (Sgf73) of the yeast histone acetyltransferase complex SAGA (Fig. 3) (David, 1997; Kaytor et al., 1999; Mushegian et al., 2000; Lebre et al., 2001; Einum et al., 2003; Scheel et al., 2003). Despite the identification of these regions, showing homology to known functional domains and also the identification of some proteins interacting with ataxin-7, the function of the protein is still largely unknown (La Spada et al., 2001; Lebre et al., 2001; Matilla et al., 2001).

Northern blot and immunohistochemical analysis have revealed expression of the 7.5-kb SCA7 mRNA/ ataxin-7 protein in various tissues including brain, heart, muscle, spleen, lung, liver, kidney and testis (David, 1997; Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001). In the



**Fig. 3.** Schematic drawing of the ataxin-7 protein.

central nervous system, expression of ataxin-7 has been observed in neurons of most areas including affected regions such as the cerebellar cortex and retina, as well as in regions considered to be unaffected in SCA7 such as the hippocampus (Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001). Wild-type ataxin-7 has been found in both the cytoplasm and nucleus of most neurons, however; in SCA7 patients expanded ataxin-7 was found to aggregate into nuclear inclusions. No correlation between degeneration and expression or inclusions could, however, be observed (Cancel et al., 2000; Einum et al., 2001).

Recently, an alternative human SCA7 transcript (SCA7b), containing not only the previously identified exons 1-13, but also a new 67-bp exon denoted exon 12b was identified (Michalik et al., 1999; Einum et al., 2003). Due to frame shifting caused by insertion of exon 12b into the ORF, the SCA7b transcript results in a slightly longer ataxin-7 protein (ataxin-7b, 945 aa) containing a novel 58-aa long C-terminus (Einum et al., 2003). The N-terminal 887 amino acids, containing the glutamine repeat, the nuclear localization signal, the arrestin homology domain and the Sgf73 homology domains were, however, found to be identical to the previously reported ataxin-7 protein (Fig. 3) (Einum et al., 2003).

Surprisingly, northern analysis using exon 12b as probe revealed a transcript of 4.4 kb as compared to the 7.5-kb transcript originally identified using a more 5' probe. Compared to ataxin-7, ataxin-7b expression was found to be more restricted to the brain. In neurons, ataxin-7b was exclusively observed in the cytoplasm and no inclusions could be detected with an ataxin-7b isoform-specific antibody in SCA7 patients (Einum et al., 2003).

## **Mechanisms of polyglutamine disorders**

### **A common disease mechanism**

To date, nine neurodegenerative disorders are known to be caused by expanded polyglutamine domains (Table 2). Besides SCA7, the group also includes several other spinocerebellar ataxias, SCA1, SCA2, SCA3, SCA6 and SCA17, as well as spinal and bulbar muscular atrophy (SBMA), Huntington's disease (HD) and dentatorubral-pallidoluysian atrophy (DRPLA) (La Spada et al., 1991; Group, 1993; Orr et al., 1993; Kawaguchi et al., 1994; Koide et al., 1994; Nagafuchi et al., 1994; Matilla et al., 1995;

Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996; David, 1997; Zhuchenko et al., 1997; Nakamura et al., 2001).

**Table 2.** The polyglutamine disorders

<b>Disease</b>	<b>Normal (CAG)<sub>n</sub></b>	<b>Expanded (CAG)<sub>n</sub></b>	<b>Protein</b>	<b>Subcellular localization</b>	<b>Brain regions most affected</b>
SCA1	6-39	40-88	Ataxin-1	Predominantly nuclear	Cerebellar Purkinje cells, dentate nucleus, brainstem
SCA2	14-32	33->200	Ataxin-2	Predominantly cytoplasmic	Cerebellar Purkinje cells, brainstem
SCA3	12-40	55-86	Ataxin-3	Predominantly cytoplasmic	Dentate nucleus, basal ganglia, brainstem, spinal cord
SCA6	4-18	21-31	P/Q Ca <sup>2+</sup> channel subunit	Cell membrane	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	7-39	40->200	Ataxin-7	Predominantly nuclear	Cerebellum, inferior olive, retina
SCA17	29-42	44-63	TBP	Nuclear	Striatum, cerebral cortex, cerebellum
HD	6-35	38-68	Huntingtin	Predominantly cytoplasmic	Striatum, cerebral cortex
SBMA	9-36	36-62	Androgen receptor	Nuclear and cytoplasmic	Anterior horn and bulbar cells, dorsal root ganglia
DRPLA	3-36	49-88	Atrophin-1	Predominantly cytoplasmic	Cerebellum, basal ganglia

The polyglutamine disorders have several features in common (i-iv): (i) An autosomal dominant inheritance pattern is seen in all polyglutamine disorders, except for the x-linked recessive SBMA. (ii) All polyglutamine disorders, with the exception of SCA6, share the phenomenon of genetic anticipation caused by instability of the CAG repeat during meiosis, resulting in longer repeats and lower age of onset in the offspring. (iii) In most polyglutamine disorders, there appears to be a threshold around 35-40 repeat-units which the CAG repeat must exceed in order to cause disease (Table 2). (iv) The expanded polyglutamine proteins aggregate in the cytoplasm or in the nucleus of neurons. The similarities observed between the polyglutamine disorders have led to the hypothesis of a common disease-causing mechanism for all of these disorders.

### **Evidence for a gain-of-function mechanism**

Early on, the polyglutamine disorders were suggested to be caused by a toxic gain-of-function mechanism and this hypothesis is supported by a number of observations: (i) Despite the genetic similarities observed between the different polyglutamine disorders, no significant sequence similarity apart from the CAG repeat can be found between the different polyglutamine disease genes. (ii) The subcellular distributions of the different polyglutamine proteins differ (Table 2) (Servadio et al., 1995; Yazawa et al., 1995; De Rooij et al., 1996; Paulson et al., 1997a; Merry et al., 1998; Koyano et al., 1999). (iii) Patients with deletions in the Huntington's disease gene do not show HD phenotypes (Gusella et al., 1985; Quigley et al., 1992). Loss of the huntingtin gene instead results in aberrant brain development, including developmental closure defects in both humans and

mice (Gusella et al., 1985; Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995; White, 1997).

Several animal models have further supported the gain-of-function mechanism. In 1999, a transgenic mouse expressing an expanded polyglutamine domain in the hypoxanthine phosphoribosyl transferase (HPRT) protein, a protein normally not carrying this motif, was created (Ordway et al., 1999). These mice developed a neurological phenotype, including handling induced seizures, clamping of hind legs when held by the tail, tremor, mild ataxia and reduced lifespan, supporting the hypothesis that expanded polyglutamine domains are toxic. Several of the polyglutamine disorders have also been mimicked in transgenic mice expressing the different human polyglutamine disease genes with expanded repeats or in knock-in animals with expanded CAG repeats in the mouse gene homologs. In both transgenic and knock-in animal, expression of expanded ataxin-7 has, for instance, been shown to cause degeneration of the cerebellum and retina, resulting in deficiencies in motor coordination and vision similar to those observed in SCA7 patients (Yvert et al., 2000; La Spada et al., 2001; Yoo et al., 2003). While mice expressing different forms of expanded huntingtin develop Huntington's disease-like symptoms including chorea-like movements, stereotypic involuntary movements, loss of body weight and seizures (Mangiarini et al., 1996; Reddy et al., 1998; Shelbourne et al., 1999; Lin et al., 2001).

The expression of expanded polyglutamine domains has been shown to not only cause toxicity in humans and mice, but also in a number of invertebrates such as *aplysia*, *drosophila* and *C. elegans*, suggesting that the proposed gain-of-function mechanism is highly conserved (Warrick et al., 1998; Faber et al., 1999; Lee et al., 2003).

## **Contribution of loss-of-function to disease pathology**

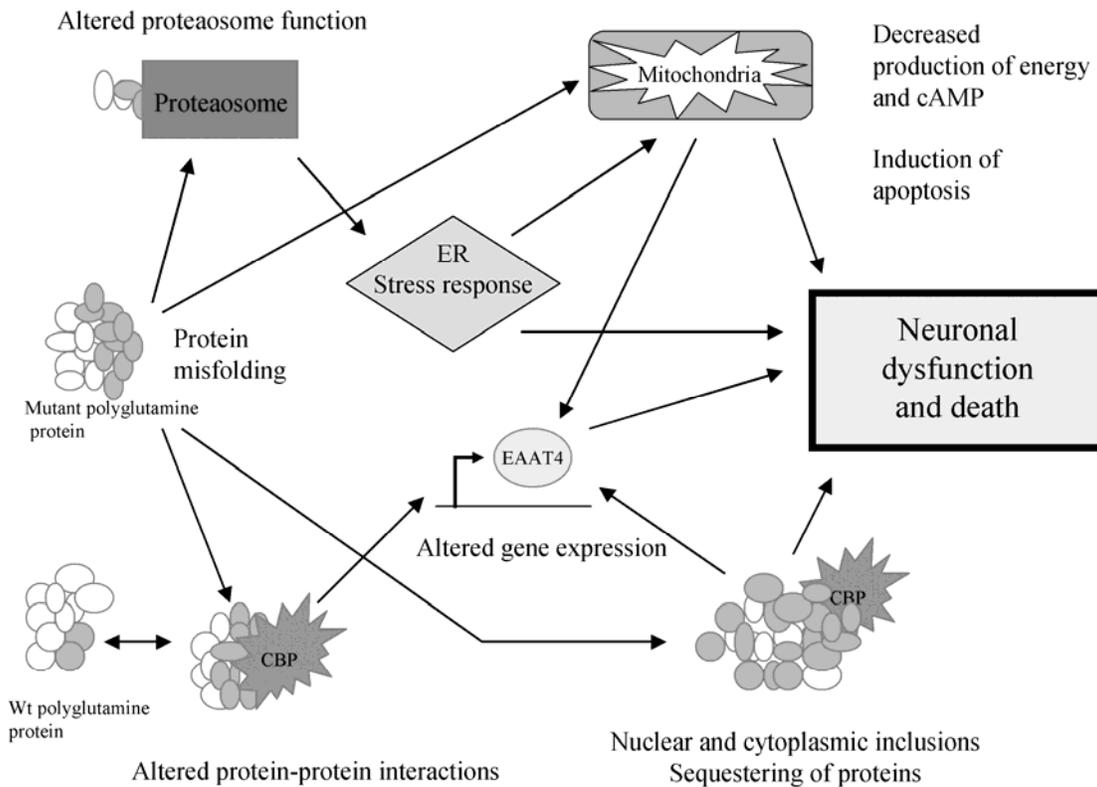
The large number of observations supporting the idea that polyglutamine diseases are caused by a gain-of-function mechanism, together with little information about the wild-type function of the polyglutamine proteins, originally meant that there were few studies carried out to investigate whether inhibition of the normal function by the expanded repeat could also contribute to disease pathology. In 1999 and 2000, however, two studies showed that reduced levels of huntingtin in adult mouse brain result in a progressive neuronal loss showing similarities to the neurodegeneration observed in HD transgenic mice (O'kusky et al., 1999; Dragatsis et al., 2000). Based on these studies, the huntingtin protein was proposed to be important for ensuring neuronal survival in adult brain and loss of this function was suggested to contribute to the neurodegeneration in HD. Studies in cell and animal models have later shown that the huntingtin protein has multiple neuroprotective properties. One mechanism by which huntingtin has been shown to prevent cell death is by binding and sequestering of the pro-apoptotic HIP-1 protein (Gervais et al., 2002). The huntingtin protein has also been shown to upregulate the expression of the neurotrophic factor BDNF, required for the survival of striatal neurons which are highly sensitive in HD (Zuccato et al., 2001). Both of the abilities of huntingtin to bind HIP-1 and upregulate BDNF have been shown to be blocked by expansion of the polyglutamine domain in huntingtin, further strengthening the hypothesis that loss of huntingtin's pro-survival functions could affect HD pathology (Zuccato et al., 2001; Gervais et al., 2002). Contribution of loss-of-function to disease pathology has also been reported in SBMA patients, who carry an expanded polyglutamine domain in the androgen receptor. In these patients, testicular feminization due to partial

androgen insensitivity is observed, showing that the expanded polyglutamine domain interferes with androgen receptor function (Quigley et al., 1992). Whether the partial loss of androgen receptor function also contributes to the loss of neurons in SBMA patients still remains to be determined. Also in SCA7, loss of ataxin-7 function has been suggested to contribute to disease pathology. In addition to the neurological symptoms, infantile cases of SCA7 also display muscle hypertonia and heart problems. These additional symptoms have been suggested to be caused by loss of ataxin-7 function, due to the extreme repeat sizes in these children (Benton et al., 1998; Johansson et al., 1998).

Despite the recent progress in understanding the function of some of the polyglutamine proteins, the possible role of loss-of-function is still unknown in the majority of polyglutamine disorders. Several events suggested to underlie the common gain-of-function mechanism shared by the polyglutamine diseases have, however, been identified (Fig. 4). These events include aggregation of the expanded protein into inclusions, inhibition of the proteasome machinery, altered protein-protein interactions and transcriptional repression.

### **Protein aggregation and pathology**

In 1997, expanded huntingtin was found to aggregate and form intracellular inclusions in the neurons of both HD transgenic mice and patients (Davies et al., 1997; DiFiglia et al., 1997). Shortly after that, the same kind of aggregates - called neuronal inclusions (NIs) - were reported in the brain of patients with other polyglutamine disorders, including SCA1-3, SCA7,



**Fig. 4.** Potential mechanisms in polyglutamine disease. The expanded polyglutamine domain is believed to cause misfolding of the polyglutamine protein, resulting in a sticky protein that is resistant to proteasome degradation and which aggregates into inclusions. The resistance of the expanded polyglutamine proteins to degradation has been suggested to alter proteasome function and thereby induce stress responses, which ultimately results in cell death. The misfolded polyglutamine protein has also been suggested to cause neuronal dysfunction by altering gene expression, due to altered interactions with - or sequestering of - certain transcription factors.

DRPLA and SBMA (Paulson et al., 1997b; Skinner et al., 1997; Becher and Ross, 1998; Holmberg et al., 1998; Li et al., 1998; Koyano et al., 1999). Based on several observations, the nuclear inclusions were suggested to be the common cause of polyglutamine-induced neuronal death. In HD and SCA3 brain, for instance, the inclusions were reported to be most frequent in the regions affected in the respective disease and inclusions were found at higher frequency in juvenile onset cases than late onset cases (DiFiglia et al., 1997; Paulson et al., 1997b). In HD transgenic mice, inclusion formation was shown to precede the appearance of neurological abnormalities and onset of motor dysfunctions (Davies et al., 1997). Downregulation of expanded huntingtin in transgenic mice was also shown to first lead to the disappearance of inclusions, after which there was a marked improvement in motor performance (Yamamoto et al., 2000).

Several different mechanisms by which the inclusions could cause toxicity to neurons have been suggested. Multiple studies have shown that apart from the expanded polyglutamine protein, ubiquitin and a number of other cellular proteins can be found in the inclusions (Davies et al., 1997; Paulson et al., 1997b; Skinner et al., 1997; Becher and Ross, 1998; Holmberg et al., 1998; Chai et al., 1999; McCampbell, 2000; Steffan, 2000). Sequestering of important cellular proteins into the aggregates has therefore been suggested to be a possible mechanism by which the inclusions alter cellular functions and cause neuronal death (Fig. 4). These studies have also shown that the wild-type form of the polyglutamine protein is sequestered into the inclusions, suggesting a possible dominant negative mechanism by which the expanded polyglutamine domain could cause loss-of-function symptoms (Paulson et al., 1997b; Kazantsev et al., 1999; Uchihara et al., 2001; Zander et al., 2001). Recently, cytoplasmic aggregates in Huntington's disease

neurons were also shown to block axonal transport, suggesting yet another way in which inclusions could inhibit neuronal function and cause disease (Gunawardena and Goldstein, 2001; Szebenyi et al., 2003).

The involvement of inclusions in the pathology of polyglutamine disease has, however, been challenged by several studies in the last few years. In SCA7 and DRPLA disease brain, nuclear inclusions were found throughout the CNS and not only in affected brain regions (Hayashi et al., 1998; Holmberg et al., 1998). The presence of inclusions in non-neuronal tissues such as skin, pancreas, kidney and testis have been reported in SCA7 and SBMA patients as well as in HD transgenic mice (Holmberg et al., 1998; Li et al., 1998; Sathasivam, 1999). Inclusions and pathology have also been disassociated in a number of disease models. In cultured striatal neurons, expanded huntingtin was observed to induce apoptosis, but no correlation between the presence of inclusions and toxicity could be found (Saudou et al., 1998). Instead, the authors showed that blocking the aggregation of expanded huntingtin using a dominant-negative mutant of the ubiquitin-conjugating enzyme resulted in increased cell death of the transfected neurons (Saudou et al., 1998). Likewise, deletion of the self-associating region of ataxin-1, leading to inability of expanded ataxin-1 to form inclusions, did not prevent the development of an ataxic phenotype in an animal model of SCA1 (Klement et al., 1998). Finally, inclusions have been shown to be dynamic structures that do not irreversibly trap sequestered proteins and prevent them from performing their function (Kim et al., 2002; Stenoien et al., 2002).

In most of the studies showing disassociation between inclusions and pathology, however, the presence of minute aggregates undetectable with the

methods of today cannot be excluded. Thus, the question of whether inclusions are deleterious or harmless continues to be one of the most disputed issues in the study of polyglutamine disease.

### **Protein misfolding, altered proteasome function and apoptosis**

Accumulation of misfolded or damaged proteins is highly harmful to cells. Protein synthesis, protein maturation and protein degradation are thus highly controlled cellular functions (Goldberg, 2003). Aberrations of protein degradation have been implicated in the pathology of several neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Ciechanover and Brundin, 2003). Dysregulation of protein folding and degradation in polyglutamine disease was first suggested when expanded polyglutamine proteins were found to misfold and subsequently aggregate into nuclear inclusions also containing various chaperones (Cummings et al., 1998; Stenoien et al., 1999; Zander et al., 2001). Chaperones are used to guide the folding of newly synthesized eukaryotic proteins and to restore the fold of misfolded or otherwise damaged proteins (Goldberg, 2003; Sitia and Braakman, 2003). Misfolded and damaged proteins which cannot be refolded to their proper conformation are rapidly targeted for degradation by ubiquitination, and subsequently digested by the proteasome (Goldberg, 2003; Sitia and Braakman, 2003). The fact that ubiquitin and several components of the proteasome were found in inclusions together with the chaperones has led to the suggestion that folding of expanded polyglutamine proteins is unsuccessful and the cell instead tries to degrade the mutant proteins (Chai et al., 1999; Stenoien et al., 1999; Zander et al., 2001). Several polyglutamine disease models have, however, shown that the expanded polyglutamine proteins are resistant to degradation and that there

is a negative correlation between the rate of degradation and the length of the polyglutamine domain (Cummings et al., 1999; Jana et al., 2001). The involvement of protein misfolding and decreased protein degradation in polyglutamine disease is further strengthened by the observation that overexpression of several chaperones including Hsp 40, Hsp 70 and MRJ in various cell cultures, and transgenic fly and mouse models of polyglutamine disease, reduces cell toxicity to different degrees (Cummings et al., 1998; Warrick et al., 1999; Chan et al., 2000; Cummings et al., 2001; Zhou et al., 2001; Bailey et al., 2002; Chuang et al., 2002; Adachi et al., 2003; Hansson et al., 2003).

Expanded polyglutamine domains have been shown to not only affect the degradation of the polyglutamine protein itself, but also the degradation of other proteins, such as p53, by inhibiting overall proteasome function (Bence et al., 2001; Jana et al., 2001). The mechanism behind the proteasome inhibition is not fully understood; it has, however, been suggested that the expanded polyglutamine proteins, by sequestering ubiquitin, chaperones and proteasome components, saturate the degradation system (Jana and Nukina, 2003). Chemical inhibition of proteasome function, leading to accumulation of unsuccessfully folded or damaged proteins, has previously been shown to induce a stress response in order to restore protein folding (Dobson, 2003; Goldberg, 2003; Sitia and Braakman, 2003). If the stress response is unable to restore homeostasis, responses that are additionally activated will ultimately result in apoptosis (Dobson, 2003; Goldberg, 2003; Sitia and Braakman, 2003). As with chemical inhibition, inhibition of proteasome function by expanded polyglutamine proteins has been shown to induce a stress response (Fig. 4). Activation of the JNK stress

pathway has been reported in cells expressing expanded huntingtin, expanded androgen receptor, and an expanded polyglutamine domain fused to GFP (Kouroku et al., 2002; Nishitoh et al., 2002; Cowan et al., 2003; Merienne et al., 2003). Evidence of apoptosis induced by proteasome inhibition has also been reported in these cell models. Proteosomal inhibition by expanded huntingtin has, for example, been reported to be associated with disruption of the mitochondrial membrane, release of cytochrome c from mitochondria, and also activation of caspase-9- and caspase-3-like proteases (Jana et al., 2001). Activation of apoptosis via the ER by caspase-12 has also been reported (Kouroku et al., 2002).

### **Nuclear localization and alterations in transcription**

Nuclear localization of the expanded polyglutamine proteins has been shown to be important for disease pathology in several disease models. Addition of a nuclear export signal (NES) to expanded huntingtin, thus retaining the mutant protein in the cytoplasm, prevented polyglutamine-induced cell death in a cell culture model of Huntington's disease (Saudou et al., 1998).

Likewise, in a mouse model of SCA1, targeting of the mutant ataxin-1 protein to the cytoplasm prevented the animals from developing an ataxic phenotype (Klement et al., 1998). Recently, the translocation of mutant androgen receptor into the nucleus, after binding of the male hormone testosterone, was reported to contribute to the more severe phenotype observed in male (as opposed to female) SBMA transgenic mice (Katsuno et al., 2002).

In the nucleus, several expanded polyglutamine proteins have been found to be associated with the nuclear matrix and PML bodies (Skinner et al., 1997; Tait et al., 1998; Kaytor et al., 1999). Such expanded polyglutamine proteins

were suggested to cause toxicity by disrupting these nuclear structures, thereby causing alterations in gene expression. To evaluate the effect of expanded polyglutamine proteins on transcription, gene expression profiling has been performed in various polyglutamine disease models (Lin et al., 2000; Luthi-Carter et al., 2000; Wyttenbach et al., 2001; Lieberman et al., 2002; Luthi-Carter et al., 2002a; Luthi-Carter et al., 2002b). The results have shown that expanded polyglutamine proteins can indeed alter gene expression, and that it is a progressive process which starts before the onset of any disease symptoms (Cha et al., 1998; Lin et al., 2000). These studies also revealed that the altered expression levels were limited to a subset of genes involved in important neuronal functions such as neurotransmission, intracellular signaling and calcium homeostasis (Lin et al., 2000; Luthi-Carter et al., 2000).

Analysis of the genes with altered expression levels in the polyglutamine models revealed that many of their promoters contained CRE (cyclic 3'5'-adenosine monophosphate response element) sites, to which the CREB protein and its co-activators bind and activate transcription (Wyttenbach et al., 2001). CREB-mediated transcription has previously been reported to be important for survival signaling in neurons (Bonni et al., 1999; Riccio et al., 1999). Furthermore, two CREB co-activators, CBP and TAFII130 have been found sequestered in polyglutamine inclusions (Shimohata, 2000; Steffan, 2000; Walton and Dragunow, 2000). Expanded polyglutamine proteins were therefore suggested to cause neuronal death by inhibiting CRE-mediated transcription. This hypothesis was further supported by the observation of decreased CREB signaling in the presence of various expanded

polyglutamine proteins in transfected cells (Shimohata, 2000; Steffan, 2000; Nucifora et al., 2001; Wyttenbach et al., 2001).

Expanded polyglutamine proteins have been proposed to interfere with CRE signaling in two different ways (Fig. 4). By impairing the mitochondrial respiratory chain, expanded huntingtin has been shown to reduce the production of energy and cAMP, which is a second messenger required for CREB activation (Gines et al., 2003). Depletion or reduction of TAF130II and CBP co-activator levels in the nucleus, by sequestering into inclusions, has also been suggested to cause the transcriptional repression (McC Campbell, 2000; Nucifora et al., 2001; Jiang et al., 2003). TAF130II is not only involved in CREB-mediated transcription; the protein can also interact with various other transcriptional activators such as Sp1 and Sp1/TAF130II-mediated transcription has also been reported to be repressed by expanded huntingtin (Dunah et al., 2002; Li et al., 2002). The CREB co-activator CBP is a histone acetyltransferase (HAT) (McManus and Hendzel, 2001). The role of histone acetylation is not fully understood, but it is believed that acetylation weakens the interaction between DNA and histones and thereby facilitates the access of RNA polymerase II and its associated factors to DNA templates (Giordano and Avantaggiati, 1999; Carrozza et al., 2003; Legube and Trouche, 2003). Expanded polyglutamine proteins have been found to interact with several HATs besides CBP and reduce the level of histone acetylation, which suggests yet another mechanism by which expanded polyglutamine proteins may inhibit transcription (McC Campbell et al., 2001; Steffan et al., 2001; Hockly et al., 2003; Katsuno et al., 2003).

### **Polyglutamine disease specificity**

Most of the polyglutamine disease proteins have widespread expression in both CNS and non-CNS tissues (Clancy et al., 1992; Li et al., 1993; Strong et al., 1993; Nagafuchi et al., 1994; Jou and Myers, 1995; Servadio et al., 1995; Sharp et al., 1995; Trottier et al., 1995; Pulst et al., 1996; Paulson et al., 1997a; Li et al., 1998; Nakamura et al., 2001). Despite this, symptoms outside the nervous system have rarely been reported and the neurons that malfunction and die are distinct, or only partially overlap in the different polyglutamine disorders. Several non-mutually exclusive mechanisms, including variations in expression levels, tissue variations in inclusion formation, increased somatic instability of the CAG repeat in affected neurons, as well as proteolytic processing resulting in shorter more toxic polyglutamine peptides in affected neurons, have been suggested to cause the selective neurotoxicity.

Recently, the hypothesis that aberrant interactions between the respective disease proteins and other proteins account for the disease specificity has been studied most extensively (La Spada and Taylor, 2003). Expansion of the polyglutamine domain has been suggested to both inhibit wild-type interactions and lead to interactions with new proteins. Both of these mechanisms could lead to interference with cell-specific functions or functions that are particularly important for specific neurons, thereby leading to selective cell death.

Expanded ataxin-7 has, for instance, been shown to bind and inhibit the function of the transactivator cone-rod homeobox protein CRX (La Spada et al., 2001). CRX is expressed predominantly in retinal photoreceptor cells, and regulates the expression levels of several photoreceptor-specific genes such as rhodopsin and color opsins. Thus, interference with CRX has been

proposed to cause the retinal sensitivity in SCA7 (La Spada et al., 2001). Another example is ataxin-1, which has been shown to interact in a polyglutamine repeat length-dependent fashion with the LANP protein (Matilla et al., 1997; Waragai et al., 1999). LANP (leucine-rich-acidic nuclear protein) is particularly abundant in Purkinje cells, the main cell type affected in SCA1 pathology, and has been implicated in several cellular functions such as inhibition of histone acetylation, apoptosis, RNA stability and RNA transport. Recently, the LANP protein has also been suggested to modulate neuritogenesis, and loss of LANP function was proposed to contribute to the loss of neurites and the cytoarchitectural disarray seen in SCA1 pathology (Opal et al., 2003). Furthermore, interaction between ataxin-1 and the 14-3-3 protein has also been shown recently to influence SCA1 pathology (Chen et al., 2003; Emamian et al., 2003). 14-3-3 is a multifunctional molecule, which has been implicated in signal transduction, cell cycle control and apoptosis. The protein is expressed in brain, and several isoforms are particularly abundant in Purkinje cells. Binding of 14-3-3 to ataxin-1 has been shown to require phosphorylation of ataxin-1 by the Akt kinase and inhibition of this phosphorylation, preventing the ataxin-1-14-3-3 interaction, delayed SCA1 pathology in transgenic mice expressing expanded ataxin-1 (Chen et al., 2003; Emamian et al., 2003). In cellular models and transgenic flies, 14-3-3 was shown to increase the stability and slow the degradation of ataxin-1. The neurotoxic effect of expanded ataxin-1 has therefore been suggested to be more pronounced in cells expressing high levels of 14-3-3, resulting in increased accumulation of mutant ataxin-1 (Chen et al., 2003; Emamian et al., 2003).

Despite much research, the mechanism(s) behind the selective neuronal death are still largely unknown in most of the polyglutamine disorders. It is likely that a combination of different pathways, specific for each disease, causes the cell type-specific degeneration. Additional studies are clearly required to learn more about the expression, proteolytic processing and interactions of all the polyglutamine proteins. To truly understand the differences between the different polyglutamine disorders, greater efforts will also have to be made in elucidating the wild-type functions of these proteins and studying the influence of the sequence surrounding the polyglutamine domain on disease pathology.

## **RESULTS AND DISCUSSION**

### **Expression of ataxin-7 in normal and SCA7 tissue (paper I)**

Three recent studies have described the expression pattern of normal ataxin-7 in adult human brain (Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001). Expression of the mutant ataxin-7 has, however, only been examined in five SCA7 patients (Mauger et al., 1999; Cancel et al., 2000; Einum et al., 2001). Genetic instability of the disease mutation, a CAG repeat, causes great variation in the age of onset, clinical symptoms and neurodegeneration between different SCA7 patients. To gain further insight into the role of mutant ataxin-7 expression in SCA7 pathology, we analyzed the expression pattern of ataxin-7 in a number of CNS and non-CNS tissues from three Swedish SCA7 patients with different ages of onset as well as in age-matched controls (paper I). The first SCA7 patient presented with symptoms at age 19 (case 1), the second patient presented with ataxia at age 4 (case 2) and the third patient presented with symptoms at age 1 (case 3). To get a more complete picture of ataxin-7 expression in SCA7 individuals of different ages, we also compiled our data with studies that had been published earlier.

To study the expression of ataxin-7, two polyclonal antibodies, SCA7 (1-15) and SCA7 (1-135) were generated, both of which were directed against the N-terminus of ataxin-7. The specificities of the two anti-ataxin-7 antibodies were determined by western blot analysis performed on extracts from cells overexpressing ataxin-7, and by preincubating the antibodies with their antigens (Figs. 1 and 2, paper I). Tissue analysis was performed on cryo-preserved or paraffin-embedded material, including brain areas considered to

be major sites of pathology in SCA7 (cerebellum, olivary nucleus, pons and retina), brain areas considered to be unaffected (hippocampus and four different regions of cerebral cortex) and also non-CNS tissues (heart, lung, kidney, adrenal gland, liver, spleen, thyroid and pancreas).

### **Expression of ataxin-7 in the cerebellum, olivary nucleus and retina**

Primary signs of pathology in SCA7 patients include atrophy of the cerebellum and the inferior olivary nucleus (ION), as well as marked neurodegeneration of the retina.

As expected, sections of the cerebellum from two Swedish patients (cases 1 and 2 respectively) revealed massive loss of Purkinje cells and neurons in the dentate nucleus (Figs. 3A and 3C, paper I). Remaining Purkinje cells in affected individuals appeared shrunken (Figs. 3A and 3C, paper I).

Degeneration and a general disorganisation of the granular and molecular layers, more pronounced in the young-onset case, were also observed (data not shown). In both patients and controls, ataxin-7 immunoreactivity was predominantly nuclear in neurons of the cerebellar granular and molecular layers (Figs. 3A-D, paper I). Ataxin-7 staining was also predominantly nuclear in the few Purkinje cells identified in the young-onset SCA7 patient, and also in the young control (Figs. 3A and 3B, paper I). However, in the Purkinje cells of the adolescent-onset patient, only cytoplasmic staining was observed (Fig. 3C, paper I), while the staining in the adolescent control varied between primarily nuclear, primarily cytoplasmic, or a combination of both (Fig. 3D, paper I).

When we assembled our data with those previously reported, we could define three different patient groups based on the distribution of ataxin-7 in Purkinje cells of the cerebellum: (1) patients with late onset, long disease

duration and moderate-sized mutations (<45 CAG), displaying a predominant nuclear staining, (2) patients with adolescent onset, somewhat shorter disease duration and larger mutations (>60 CAG), with more pronounced cytoplasmic staining and (3) patients with young onset, rapid disease progression and extreme mutations (>85 CAG), showing a predominant nuclear staining (Table 1, paper I). A similar age-dependent pattern, largely consistent with that of SCA7 patients, can also be seen when comparing controls of different ages (Table 1, paper I). Cancel et al. reported that 32% of the Purkinje cells in a young-onset patient showed nuclear ataxin-7 staining and proposed that translocation of the mutant protein to the nucleus was induced by the polyglutamine stretch (Cancel et al., 2000). Our data suggest that the differences in subcellular localisation between the young-onset SCA7 patient and the adult control observed by Cancel et al. could reflect normal differences in subcellular localization between different age groups.

In the inferior olivary nucleus (ION), ataxin-7 staining was detected in the nuclei of neurons in patients and controls of all ages. The ataxin-7 staining was, however, more defined in the adolescent-onset patient and the age-matched control than in the young-onset patient (case 3) (Figs. 3E and 3F, paper I, and data not shown). This is in agreement with two late-onset patients and one patient with adolescent onset reported by Einum et al., although cytoplasmic staining has also been described in one young-onset patient (Cancel et al., 2000) as well as in adult controls (Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001).

The retina obtained from the adolescent SCA7 patient (case I) had degenerated severely (Fig. 3G, paper I), which made the analysis of cellular distribution of ataxin-7 in this affected tissue impossible. As previously reported, however, staining of adolescent control retina showed nuclear staining in the majority of ganglion cells and cells in the bipolar cell layer, whilst little staining was observed in the photoreceptors (Fig. 3H, paper I) (Mauger et al., 1999; Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001).

### **Expression of ataxin-7 in brain areas not considered to be primary sites of pathology**

Ataxin-7 expression was also examined in regions of the CNS not considered to be primary sites of pathology, such as the cerebral cortex and the hippocampus. Various regions of the cerebral cortex, including frontal, parietal, temporal and occipital lobes, displayed intense nuclear immunoreactivity in neurons throughout layers III-VI in both patients and controls (Figs. 4A and 4B, paper I, and data not shown). Intense nuclear staining was also detected in both the dentate gyrus (Figs. 4C and 4D, paper I) and the CA3 region (Figs. 4E and 4F, paper I) of the hippocampus in both SCA7 patients and controls. Patients with cytoplasmic staining in neurons of the hippocampus, and controls with predominant cytoplasmic staining in neurons of both structures have, however, also been described (Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001). As previously shown, no obvious difference in the expression level of ataxin-7 could be observed between affected and non-affected brain regions, supporting the hypothesis that the cell type-specific neurodegeneration in SCA7 is not due to

differences in expression levels of ataxin-7 (Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001).

### **Presence of nuclear inclusions in affected and spared brain regions**

Nuclear inclusions were initially reported to be restricted to sites of pathology (DiFiglia et al., 1997; Paulson et al., 1997b), but have since been shown to be frequent in brain regions not involved in the pathology of patients with SCA7 and DRPLA (Hayashi et al., 1998; Holmberg et al., 1998). These findings are in agreement with our present data, in which nuclear inclusions were found throughout the brain without correlation to pathology (data not shown). We observed inclusions most frequently in cerebral cortex, a region not severely affected in SCA7 (Fig. 5, paper I). The frequency of inclusion formation was, however, ten times higher (20% of the neurons) in the young-onset case (case 2) than in the adolescent-onset case (2% of the neurons) (Figs. 5A and 5B, paper I). In neurons containing NIs, we observed less intense ataxin-7 staining in the rest of the nucleus (Fig. 4A, arrow, paper I) as compared to those neurons without NIs (data not shown), suggesting that both normal and mutant ataxin-7 is recruited into the aggregates. In agreement with this hypothesis, recruitment of other wild-type polyglutamine proteins into inclusions has also been reported in cellular polyglutamine disease models, as well as in brain from SCA1, SCA2, SCA3 and DRPLA patients (Perez et al., 1998; Uchihara et al., 2001).

### **Expression of ataxin-7 in non-neuronal tissues**

In previous studies, ataxin-7 expression has been demonstrated in non-CNS tissue of control individuals, including heart, lung, spleen, liver, kidney, testis, thyroid, pancreas, skeletal muscle, lymphoid cells and intestine

(Cancel et al., 2000; Lindenberg, 2000; Einum et al., 2001). We analyzed ataxin-7 expression in non-CNS tissues of both patients and controls, but could not detect staining in the heart, adrenal gland, liver, lung, thyroid or spleen of either patients or controls (data not shown). Ataxin-7 positive, non-ubiquitinated nuclear inclusions were, however, observed at low frequency in kidney, and more frequently in pancreas of SCA7 patients (Figs. 6A and 6B, paper I). To our knowledge, this is the first demonstration of aggregated ataxin-7 in non-CNS tissue of SCA7 patients.

## **Cloning and expression analysis of the murine SCA7 gene homolog (paper II)**

### **Characterization of the murine SCA7 gene**

To expand our studies on ataxin-7 expression, and to compare the human and murine ataxin-7 proteins functionally and from an evolutionary standpoint, we isolated and characterized the mouse SCA7 gene homolog. Murine SCA7 cDNA was isolated by screening a mouse brain cDNA library using a genomic SCA7 fragment as probe, and by RT-PCR on adult mouse brain mRNA. Using this approach, a 3039-bp contig containing an open reading frame of 2604 bp was isolated (Fig. 1A, paper II). The mouse SCA7 transcript was predicted to encode an 867-amino acid protein with a molecular weight of approximately 93 kDa (Fig. 1B, paper II). Comparison of the mouse and the human SCA7 coding sequences revealed high homology, 88.2 % and 88.7 % identity at the nucleotide and amino acid levels respectively (Fig. 1B, paper II, and data not shown).

Analysis of the murine CAG repeat in three different mouse strains revealed that the murine repeat is smaller (5 CAGs) compared to the human

counterpart (7-17 CAGs), and not polymorphic. Smaller, more stable repeats have also been observed in mouse homologs of other polyglutamine disease genes, including SCA1, SCA2 and huntingtin, suggesting that CAG repeats are more stable in mice than in humans (Lin et al., 1994; Banfi et al., 1996; Nechiporuk et al., 1998). Sequence analysis also revealed high conservation of several functional domains in murine ataxin-7, including the nuclear localization signal (NLS) and the arrestin homology domain (Fig. 1B, paper II). The complete conservation of the arrestin homology domain in mouse ataxin-7 supports the hypothesis proposed by Mushegian et al. (2000) that ataxin-7 may interact with one or more yet unidentified protein(s) in a phosphorylation-dependent way.

### **Expression analysis of murine ataxin-7**

#### *Expression of ataxin-7 in adult mouse tissues*

Northern blot analysis using a radiolabeled probe directed against the 5'-end of the SCA7 ORF revealed a murine SCA7 mRNA transcript of 7.5 kb (Fig. 2A, paper II), similar in size to the human transcript originally identified (David, 1997). As in humans, expression of the murine SCA7 transcript was found in all tissues examined, including heart, brain, liver, kidney, testis, spleen and skeletal muscle. In murine testis as in human testis, an additional smaller transcript of 3.0 kb was observed (Fig. 2A, paper II and data not shown).

In agreement with data from the northern blot, western blot and immunohistochemical analysis revealed expression of the ataxin-7 protein in most adult mouse tissues examined (Table 1, Figs. 2-4, paper II, and data not shown). As in humans, a strong expression of ataxin-7 was observed in testis, where primarily spermatogonia stained positive (Fig. 4A, paper II)

(Cancel et al., 2000; Lindenberg et al., 2000). In agreement with our human expression data showing nuclear ataxin-7 aggregates in pancreas of SCA7 patients, strong expression of ataxin-7 was also observed in islets of Langerhans (Fig. 4B, paper II). The conserved expression of ataxin-7 in various tissues suggests that although high expression of ataxin-7 is mainly observed in neurons, the function of ataxin-7 may not be restricted to neuronal cells.

In adult mouse brain, ataxin-7 expression was observed in most regions (Table 1 and Fig. 3, paper II, and data not shown). Comparisons with human expression studies revealed a highly conserved expression pattern; however, ataxin-7 expression was also observed in areas not previously examined in humans.

In brainstem, several nuclei including cranial nerve nuclei, colliculi, hypothalamus and substantia nigra showed varying levels of nuclear and cytoplasmic ataxin-7 expression (Table 1 and Fig. 3F, paper II, and data not shown). As in humans, an extensive ataxin-7 staining could also be observed in several regions of the forebrain - including in neurons throughout layers I-VI of the cerebral cortex (Fig. 3G, paper II) and also in hippocampal neurons in the dentate gyrus, the CA1-CA4 and the entorhinal cortex (Fig. 3H, paper II, and data not shown) (Lindenberg et al., 2000; Cancel et al., 2000; Einum et al., 2001; paper I). A conserved expression pattern of murine ataxin-7 was also observed in the retina, with strong nuclear and cytoplasmic ataxin-7 expression in ganglion cells and cell bodies of the inner nuclear layer (Fig. 3I, paper II).

In the cerebellum, strong nuclear and weaker cytoplasmic ataxin-7 expression was observed in deep cerebellar nuclei such as the dentate

nucleus, as well as in the majority of Purkinje cells of the cerebellar cortex (Figs. 3D and 3E, paper II). In contrast to the situation in humans, expression of ataxin-7 could not be detected in granule cells of the murine cerebellar cortex (Lindenberg et al., 2000; Cancel et al., 2000; Einum et al., 2001).

#### *Embryonic and postnatal expression of ataxin-7*

Ataxin-7 expression in embryonic tissues has not been analyzed previously. Northern blot analysis revealed gradually increasing levels of the 7.5-kb transcript in RNA extracted from whole mouse embryos at days 7, 11, 15 and 17 (data not shown). To determine the tissue distribution of ataxin-7 during mouse embryonic development, sections from embryonic days 11.5, 13.5, 15.5 and 17.5 were analyzed. In agreement with the mRNA analysis, ataxin-7 expression was detected in most embryonic tissues including lung, limbs, liver, kidney, gut and heart throughout development (Fig. 5, paper II, and data not shown). Most parts of the CNS, including retina, spinal cord and dorsal root ganglia showed ataxin-7 expression from embryonic day 11.5 (E11.5) and onwards (Fig. 5, paper II, and data not shown). The ataxin-7 staining became more defined in neurons as the embryos developed (data not shown).

In postnatal brain, most regions showed an ataxin-7 expression similar to that in adult brain; however, in the cerebellar cortex no ataxin-7 expression was observed during the first two postnatal weeks, a time when the cortex is still undergoing major growth and development (Fig. 3B, paper II). From postnatal day 15 (P15) and onwards, however, Purkinje cells in the cerebellar cortex showed gradually increasing expression of ataxin-7 (Figs. 3C and 3D, paper II, and data not shown). The gradually increasing

expression of ataxin-7 in both embryonic tissues and in the postnatal cerebellar cortex suggests that ataxin-7 may play a role in mature cells.

## **Isolation of an alternative murine SCA7 transcript (paper III)**

### **Cloning of the murine SCA7b transcript**

To investigate whether alternative SCA7 transcripts exist in mice, we performed RT-PCR on adult mouse RNA from various tissues. The screen revealed one transcript (3R1) isolated from pancreatic RNA that contained a novel 67-bp insert between exons 12 and 13, with 89.6% identity to the newly identified human exon 12b (Fig. 1A, paper III) (Einum et al., 2003). Alignment of the 3R1 clone with genomic DNA (accession number AC116479) revealed that the new 67 bp is located in intron 12, 699 bp downstream of exon 12 and 2938 bp upstream of exon 13 (Fig 1A, paper III).

In agreement with the newly identified human alternative SCA7 transcript, the insertion of the 12b exon into the murine ORF resulted in a frameshift which gave rise to an ataxin-7 protein with a novel 58-amino acid C-terminus (Fig. 1B, paper III). The alternative murine ataxin-7 protein (denoted ataxin-7b) consists of 920 amino acids, showing 83.8% homology to the human counterpart (Fig. 1B, paper III). The N-terminal 862 amino acids, containing the glutamine repeat, the nuclear localization signal and the arrestin homology domain are identical to those of the previously cloned murine ataxin-7 protein (paper II).

### **Expression analysis of SCA7b/ataxin-7b**

Northern blot analysis using a radiolabeled oligonucleotide corresponding to the murine exon 12b as probe revealed a 7.5-kb transcript and a 3.0-kb

transcript (Fig 2, paper III) similar in size to previously identified murine SCA7 transcripts (paper II). In addition, a transcript of approximately 4.4 kb was observed, similar in size to the exon 12b containing SCA7b isoform identified in humans (Fig. 2, paper III). In contrast to humans, where the 4.4-kb transcript was mostly expressed in brain (Einum et al., 2003), only weak expression of the 4.4-kb transcript was seen in murine brain. Instead, high expression was observed in heart, liver, kidney and testis (Fig. 2, paper III). Based on the northern blot observations, we suggest that at least three SCA7 isoforms exist in mice.

In humans, the ataxin-7b protein was found exclusively in the cytoplasm of neurons, and no intranuclear inclusions could be detected in SCA7 patient tissues stained with the Ab161 antibody directed against the human ataxin-7b C-terminal (Einum et al., 2003). To evaluate the effect of the new murine C-terminus on ataxin-7 expression, we replaced the C-terminus of normal human ataxin-7 with the C-terminus of murine ataxin-7b in expression constructs coding for human full-length ataxin-7 with 10 or 65 repeats. Staining with anti-ataxin-7 antibodies revealed that the subcellular localization of the hybrid constructs called FL-Q10B and FL-Q65B in transfected neuroblastoma cells, was more cytoplasmic than that of the original FL-Q10 and FL-Q65 constructs (Fig. 3, paper III and data not shown). The murine ataxin-7b C-terminus could not, however, exclude ataxin-7 completely from the nucleus and the expanded hybrid protein (FL-Q65b) still formed nuclear inclusions, in contrast to the completely cytoplasmic human ataxin-7b. This suggests that a nuclear milieu may be important for inclusion formation by ataxin-7.

## **Functional analysis of wild-type and mutant ataxin-7 (papers III and IV)**

The mechanism of polyglutamine pathology is still largely unknown, but it has been shown that several of the polyglutamine proteins can sequester transcription factors into inclusions and inhibit transcription. Transcriptional repression has therefore been suggested to play a role in the disease pathology, with repression of cell type-specific transcription factors accounting for the disease specificity. For instance, repression of CRX-mediated transcription by expanded ataxin-7 has been suggested to cause the retinal degeneration in SCA7 (La Spada et al., 2001).

Not only expanded ataxin-7, but also the wild-type protein has been implicated in transcription, since two domains in ataxin-7 were recently found to have homology to the yeast protein Sgf73, a *bona fide* subunit of the histone remodeling complex SAGA.

To study the possible roles of wild-type and expanded ataxin-7 in transcriptional regulation, we examined the effect of both wild-type and expanded ataxin-7 on transcription mediated by two co-activators/transcription factors: CBP and ROR $\alpha$ . CBP is a major mediator of survival signaling in mature neurons and the protein has been found to be sequestered into ataxin-7 inclusions (Zander et al., 2001). Repression of CBP-mediated transcription has also been reported in models of both Huntington's disease and DRPLA (Nucifora et al., 2001; Wyttenbach et al., 2001). ROR $\alpha$  is a nuclear receptor and two of its isoforms, 1 and 4, are highly expressed in Purkinje cells (Giguere et al., 1994; Matsui et al., 1995; Matysiak-Scholze and Nehls, 1997; Steinmayr et al., 1998). Mice lacking

one copy of the ROR $\alpha$  gene show progressive loss of Purkinje cells and inferior olivary nucleus neurons (Zanjani et al., 1992; Giguere et al., 1994; Steinmayr et al., 1998). Both of these cell types are affected in SCA7, making ROR $\alpha$  an interesting candidate target for expanded ataxin-7.

### **Expression, inclusion formation and sequestration by expanded ataxin-7**

To study the effect of wild-type and expanded ataxin-7 on inclusion formation and sequestering of CBP and ROR $\alpha$ 1, human neuroblastoma cells, SK-N-SH, were transfected with constructs expressing flag and c-myc tagged truncated or full-length ataxin-7 containing 10 or 65 polyglutamines (Fig. 1A, paper IV). The cellular localization of the ataxin-7 proteins was determined by anti-ataxin-7, anti-flag and anti-c-myc immunofluorescence (Fig. 2, paper IV, and data not shown). As previously reported, truncated ataxin-7 constructs (Tr-Q10 and Tr-Q65) lacking the nuclear localization signal (NLS) showed both nuclear and cytoplasmic expression (Fig. 2, rows 1-2, paper IV), while the full-length constructs (FL-Q10 and FL-Q65), had a predominantly nuclear localization (Fig. 2, rows 3-4, paper IV) (Kaytor et al., 1999; Zander et al., 2001). As expected, both truncated and full-length mutant ataxin-7 aggregated in cells, while no aggregates were observed in cells expressing ataxin-7 with normal glutamine repeat lengths. Expression of Tr-Q65 resulted in nuclear and/or large cytoplasmic inclusions, often localizing to the perinuclear area, in 40.2% ( $\pm$  5.9%) of the transfected cells, while expression of FL-Q65 resulted in only nuclear inclusions in 8.3% ( $\pm$  1.3%) of the transfected cells. A higher degree of inclusion formation by truncated ataxin-7 has also been reported previously (Zander et al., 2001). As previously shown, co-immunostaining revealed sequestering of both endogenous and co-transfected CBP into ataxin-7 inclusions formed by Tr-

Q65 and FL-Q65 (Fig. 3B, rows 1-2 and Fig. 3A, rows 3-4, paper IV, and data not shown) (Zander et al., 2001). However, no sequestering of either endogenous or co-transfected ROR $\alpha$ 1 into ataxin-7 inclusions could be observed (Fig. 3A, rows 1-2, paper IV, and data not shown). Altogether, 60.5% ( $\pm$  14.2%) of the Tr-Q65 nuclear inclusions and 20.2% ( $\pm$ 13.2%) of the FL-Q65 nuclear inclusions stained positive for CBP in co-transfected cells (Fig. 3A, rows 3- 4, paper IV). However, no perinuclear or cytoplasmic ataxin-7 aggregates formed by Tr-Q65 stained positive for CBP (Fig. 3A, row 3, paper IV). In comparison, 70.0 % ( $\pm$  5.2%) of both nuclear and cytoplasmic inclusions stained positive for CBP if truncated expanded huntingtin (Htt-Q65) was co-expressed with CBP (Fig. 3A, rows 5, paper IV). Redistribution of CBP from the nucleus to the cytoplasm by mutant huntingtin has previously been demonstrated, and has been suggested to cause the interference of CBP-mediated transcription seen in cells expressing mutant huntingtin (Nucifora et al., 2001).

## **Repression of transcription by both wild-type and expanded ataxin-7**

### *Effect of ataxin-7 on CBP-mediated transcription*

To establish whether by sequestering CBP, expanded ataxin-7 might interfere with CBP-mediated transcription, we used a reporter construct (pCRE-Luc) containing three cyclic 3'5'-adenosine monophosphate response elements (CRE sites) to drive CBP/CREB mediated luciferase activity in neuroblastoma SK-N-SH or non-neuronal DG75 cells. Cells were co-transfected with the pCRE-Luc reporter construct and the different ataxin-7 expression constructs (Fig. 1A, paper IV), or constructs expressing truncated wild-type (Htt-Q10) or expanded (Htt-Q65) huntingtin for comparisons. As previously shown, expanded huntingtin reduced CBP-

mediated transcription (Figs. 4A and 4B, paper IV) (Nucifora et al., 2001; Wyttenbach et al., 2001). A similar level of reduction was observed with truncated expanded ataxin-7, while truncated ataxin-7 with a normal glutamine repeat (Tr-Q10) had no effect on CBP-mediated transcription (Figs. 4A and 4B, paper IV).

Surprisingly, wild-type full-length ataxin-7 (FL-Q10) repressed transcription more effectively than Tr-Q65 and Htt-Q65, while a somewhat stronger inhibition of transcription was observed with full-length expanded ataxin-7 (FL-Q65) (Figs. 4A and 4B, paper IV).

To investigate whether the repression of CBP-mediated transcription by expanded ataxin-7 was due to sequestering and depletion of CBP, we overexpressed CBP in the SK-N-SH cells. No significant difference could be observed between cells transfected with or without a CBP expression vector, indicating that reduced CBP levels due to sequestering is not the cause of the transcriptional repression by expanded ataxin-7 (Fig. 4C, paper IV).

#### *Ataxin-7 and ROR $\alpha$ 1- and TATA-mediated transcription*

The effect of wild-type and expanded ataxin-7 on transcription driven by the nuclear receptor ROR $\alpha$ 1 was analyzed using a reporter construct, RORE-Luc, with a ROR $\alpha$ 1 binding site (RORE) in front of the luciferase gene (Fig. 4D, paper IV). An inhibitory effect on ROR $\alpha$ 1-mediated transcription, even stronger than that observed on CBP-mediated transcription, was observed by both truncated and full-length expanded ataxin-7 (Fig. 4D, paper IV).

Since ROR $\alpha$ 1 was not sequestered into ataxin-7 inclusions, the repression of ROR $\alpha$ 1-mediated transcription further strengthens the hypothesis that repression of transcription by expanded ataxin-7 does not involve sequestering.

Interestingly, again we observed a strong repression of transcription by full-length, but not truncated, ataxin-7 with a normal repeat length on ROR $\alpha$ 1-mediated transcription (Fig. 4D, paper IV). The repression of both CBP- and ROR $\alpha$ 1-mediated transcription by full-length ataxin-7 with 10 glutamines, suggests that wild-type ataxin-7 may have a role in transcriptional regulation. The similarity in repression observed on both CBP- and ROR $\alpha$ 1-mediated transcription further suggests that the repression may not be a specific effect on CBP or ROR $\alpha$ 1, but may be due in whole or part to inhibition of the basal transcription machinery. We therefore analyzed the effect of wild-type and expanded ataxin-7 on expression driven by a basal TATA promoter. As expected, the luciferase expression was much lower (~30 times) than for the CBP- and ROR $\alpha$ 1-driven transcription; but still a repression by both full-length wild-type and expanded ataxin-7 was observed (Fig. 4E, paper IV), suggesting that wild-type ataxin-7 may interact directly or indirectly with the basal transcription machinery and thereby regulate transcription.

#### *Decreased transcriptional repression by ataxin-7b*

To investigate whether the alternative ataxin-7 C-terminus influences ataxin-7 mediated transcriptional repression, neuroblastoma cells were co-transfected with the pCRE-Luc or the TATA-Luc luciferase reporter and the FL-Q10B and FL-Q65B constructs coding for full-length human ataxin-7 (10 or 65 glutamines) containing the alternative murine C-terminus. FL-Q10B and FL-Q65B repress both CBP/CREB-mediated and basal transcription (Fig. 4, paper III). FL-Q10B and FL-Q65B did, however, not repress CBP/CREB mediated transcription to the same level as their ataxin-7 counterparts containing the normal C-terminal (Fig. 4, paper III). This

weaker inhibition may be due to the more cytoplasmic localization of the ataxin-7 proteins containing the alternative C-terminus (Fig. 3, paper III) and indicates that there may be functional differences between ataxin-7 and ataxin-7b.

#### *The role of ataxin-7 in transcription*

Our suggestion that wild-type ataxin-7 may have a function in regulating basal transcription is supported by the recent identification of homology between ataxin-7 and the yeast protein Sgf73, a *bona fide* subunit of the histone acetyltransferase complex SAGA (Scheel et al., 2003). In yeast, the SAGA complex has been shown to regulate transcription by remodelling of chromatin at specific promoters, and by recruitment of the TATA-box binding protein (TBP) to a subset of RNA polymerase II-dependent genes (Carrozza et al., 2003). SAGA consists of several transcription-related factors such as the histone acetyltransferase Gcn5, spt proteins and TBP-associated factors (TAFs) (Lee et al., 2000; Bhaumik and Green, 2002). The complex is believed to be dynamic *in vivo*, where different subunits of SAGA are required to activate transcription of different SAGA-dependent promoters. The presence of inhibitory subunits that can dissociate under certain conditions in order to activate transcription has also been suggested (Belotserkovskaya et al., 2000; Bhaumik and Green, 2002). In humans, there are at least three SAGA-like complexes PCAF, TFTC and STAGA, but the specific function of each complex is poorly understood. The STAGA complex has, however, been implicated in histone acetylation, transcription, splicing and DNA repair, suggesting that ataxin-7 may also be involved in these functions (Martinez et al., 2001).

If ataxin-7 is a component of a SAGA-like complex, it is possible that these functions are disturbed in SCA7 patients carrying expanded polyglutamine domains in ataxin-7. In fact, expanded polyglutamine domains have previously been suggested to disturb SAGA function. Expression of an expanded polyglutamine domain in yeast has been found to repress gene transcription in a similar pattern to that seen in three SAGA mutant strains, and the toxicity caused by deletion of the SAGA component, SPT3, was found to be enhanced in the presence of an expanded polyglutamine domain (Hughes et al., 2001).

## CONCLUSIONS

- The expression pattern, the subcellular localization and the inclusion formation of expanded ataxin-7 are not the main determinants of cell-specific neuronal degeneration in SCA7 disease.
- The SCA7 gene is highly conserved between mouse and humans, both structurally and at the level of expression.
- An alternative SCA7 transcript homologous to the human isoform SCA7b is present in mice
- As previously reported for other expanded polyglutamine proteins, both truncated and full-length forms of expanded ataxin-7 repress transcription.
- Wild-type full-length ataxin-7, but not truncated wild-type ataxin-7, also had an inhibitory effect on transcription mediated by CBP, ROR $\alpha$ 1 or a TATA-box, suggesting that ataxin-7 may have a role in regulating the basal transcription machinery.

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