Modelling and treating Huntington’s disease: 
Generation of high-capacity adenovirus vectors to express 
normal and mutant huntingtin and to block huntingtin 
expression by short hairpin interference RNA 

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I. Abstract

Huntington’s disease (HD) is an inherited autosomal dominant, neurodegenerative disease caused by the expansion of a CAG repeat in exon 1 of the huntingtin (Htt) gene. Presently there is no cure for HD and patients normally die 15-20 years after the appearance of the first symptom. The exact mechanisms underlying HD are still unknown. However, it is generally believed that HD is caused by deleterious functions conferred by the CAG expansion. The progression of HD depends on continuous expression of mutant Htt. Therefore; blocking expression of mutant Htt might be a promising therapeutic strategy to treat HD. Since short hairpin interference RNA (shRNA) is able to inhibit specific gene expression in vitro and in vivo, silencing Htt expression by vector-mediated expression of an shRNA could form the basis of a new treatment for HD. We identified one anti-Htt shRNA (ie-1-1) targeting Htt exon-1, which efficiently inhibited expression of both an Htt exon 1-EGFP fusion protein and the endogenous Htt gene. An E1-deleted first-generation adenoviral (Ad) vector Adie-1-1 was constructed to express this shRNA from the U6 promoter. Adie-1-1 efficiently silenced Htt exon 1 expression and prevented HD aggregate formation in A549 cells expressing mutant Htt exon-1. Compared to first-generation Ad vectors, high-capacity adenoviral (HC-Ad) vectors allow for long-term expression in the brain in vivo due to decreased toxicity and immunogenicity. Therefore; we generated an HC-Ad vector (HC-AdHB04) expressing the anti-Htt shRNA ie-1-1. In vitro, HC-AdHB04 not only efficiently inhibited Htt expression, but also the formation of cellular Htt aggregates. Importantly, the attenuation of aggregate formation by shRNA was also observed in vivo after stereotaxic injection into the striatum of HD mouse models. We tested this vector in two HD models: a mouse model based on the local injection of an Ad vector expressing a truncated version of mutant Htt and the R6/2 transgenic HD mouse model. In both models the formation of Htt aggregates was efficiently reduced. Our results prove the concept that shRNAs can be used to inhibit Htt aggregates in vivo and support the further development of shRNA for HD therapy.

To study the function of Htt, understand the pathogenesis of HD and test potential therapeutic strategies, cDNAs coding for truncated or full-length Htt have been used to generate HD models in vitro in different cell lines and in vivo in transgenic mice,
rats, flies and C. elegans. Vector-mediated delivery of truncated mutant Htt gene has also been used to generate HD models \textit{in vitro} and \textit{in vivo}. However, due to the large size of the Htt cDNA and low transgene capacity of vectors used, vector-mediated delivery of full-length Htt has not been achieved so far. Since HC-Ad vectors have a large transgene capacity of up to 38 kb, feature low toxicity and allow for long term transgene expression in the brain, we generated a series of HC-Ad vectors expressing from the neuron-specific SYN1-WPRE promoter truncated or full-length Htt with a normal or increased number of glutamines. \textit{In vitro} these vectors were used to transduce primary neuronal cells. Expression of mutant Htt resulted in the formation of Htt aggregates. However kinetics of generation and localization of Htt aggregates differed between truncated and full-length mutant Htt. When injected into the mouse striatum, expression of truncated mutant Htt led to prominent accumulation of Htt aggregates in cell nuclei, while aggregates formed from expression of full-length mutant Htt mainly localized into the cytoplasm. These results indicated that HC-Ad vectors could be used to generate HD models by expressing truncated and full-length mutant Htt in primary neuronal cells \textit{in vitro} and in the mouse brain \textit{in vivo}. These vectors will be useful tools for studying HD and for generating large animal HD models in the future.
II. Papers in this thesis

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-III).


III. Abbreviations

3-NP 3-nitropropionic acid
AAV Adeno-associated virus
Ad Adenovirus
BDNF Brain-derived neurotropic factor
c-AMP Cyclic adenosine monophosphate
CAR Coxsackie and Adenovirus Receptor
CBP cAMP-response-element-binding protein
cDNA Complementary DNA
CMV Cytomegalovirus
CNS Central nervous system
CNTF Ciliary Neurotrophic Factor
CRE cAMP-responsive elements
DRPLA Dentatorubal-pallidoluysian atrophy
dsRNA Double-stranded RNA
FIV Feline immunodeficiency virus
GDNF Glial derived neurotrophic factor
HC-Ad High capacity adenovirus
HD Huntington’s disease
HIV Human immunodeficiency virus
HPRT Hypoxanthine phosphoribosyltransferase
HSV Herpes simplex virus
Htt Huntingtin
ITR Inverted terminal repeat
LTR Long-terminal repeat
Mo-MuLv Moloney murine leukemia virus
NGF Nerve growth factor
NMAD N -Methyl-D-Aspartic acid
NRSE Neuron-restrictive silencer elements
NT Neurotrophin
ORF Open reading frames
PD Parkinson’s disease
PolyQ Poly glutamine
REST-NRSF Repressor-element-1 transcription factor-neuron restrictive silencer factor
SBMA Spinobulbar muscular atrophy
SCA Spinocerebellar ataxias
shRNA Short hairpin interference RNA
siRNA Short interference RNA
SNpC Substantia nigra pars compacta
Sp1 Specificity protein 1
SYN1-WPRE Human synapsin-1 promoter with the woodchuck hepatitisvirus posttranscriptional regulatory element
TAFII130 TATA-binding protein associated factor
TBP TATA binding protein
IV. Introduction

Huntington’s disease (HD) is an inherited autosomal dominant neurodegenerative disorder, which was first described by an American physician, George Huntington, in 1872 (Huntington, 1872). HD is characterized by selective loss of neuronal cells, primary in the striatum, leading to progressive chorea, cognitive impairment and neuropsychiatric symptoms (Bates et al., 2002). The onset of HD is usually in the middle age (35-45 years) but can also occur less commonly in juveniles. The disease is progressive and patients normally die 15-20 years after the appearance of the first symptom. To date HD is still fatal and incurable.

It has been 14 years since the HD gene was discovered in 1993, however, the exact mechanisms underlying HD are still unknown (Huntington’s Disease Collaborative Research Group, 1993). The HD gene encodes a 350-kDa protein called huntingtin (Htt). It has been clear that the unusual expansion of the polyglutamine (PolyQ) tract in Htt is responsible for the disease. With the progress of our understanding of molecular details of HD since the discovery of the HD gene, several possible mechanisms of HD have been suggested. The leading postulated mechanisms for the pathogenesis of the disease involve toxic aggregates, the cleavage of the mutant htt, excitotoxicity, impaired energy production, cell death and transcriptional dysfunctions (Ho et al., 2001; Young, 2003; Landles and Bates, 2004). Many agents that act through the mechanisms described above have been tested in animal models of HD and even in human clinical trials (Feigin and Zgaljardic, 2002; Leegwater-Kim and Cha, 2004). Compounds have shown promise in animal models, including creatine, coenzyme Q10, remacemide, interleukin-6, minocycline, dichloroacetate and others. However, clinical trials with coenzyme Q10, remacemide and minocycline have failed to show any benefit in HD patients, highlighting the complexity of HD and the difficulty in translating positive results from animal models to humans.

Because HD is fatal and cannot be cured with current drugs, novel therapeutic strategies including cell therapy and somatic gene therapy have been intensively studied (Hsich et al., 2002; Dunnett and Rosser, 2004; Handley et al., 2006). Somatic gene therapy is defined as treatment of disease by the introduction of nucleic acids
into somatic cells. The modified cells express the introduced gene and their new phenotype provides some advantages to the patient. Gene transfers with therapeutic genes such as CNTF (ciliary neurotrophic factor) and GDNF (glial derived neurotrophic factor) have been neuroprotective in HD mouse models (de Almeida et al., 2001; Mittoux et al., 2002; Mcbride et al., 2003; Zala et al., 2004; Mcbride et al., 2006). Recently, vector-mediated expression of short interference RNA (siRNA) specifically targeted to HD gene transcript has been shown to inhibit Htt aggregate formation and improve the behaviour in HD mouse models (Harper et al., 2005; Wang et al., 2005; Rodriguez-Lebron et al, 2005; Machida et al., 2006). All these results encourage to further study gene therapy for HD.

IV.1 Overview of HD

IV.1.1 Clinical features

HD is diagnosed clinically in the presence of progressive motor disability involving both involuntary and voluntary movements, mental disturbances including a cognitive decline and/or changes in personality, family history consistent with autosomal dominant inheritance (Bird 1978; Folstein et al., 1986; Kremer et al., 1994; Macmillan et al., 1995). The frequency of HD in populations of Western countries is 4 to 10 affected per 100,000 individuals. Initially patients have subtle changes in coordination, minor involuntary movements, difficulty in mental planning, and often a depressed or irritable mood (Hayden 1981; Harper 1996). With the progress of the disease, chorea becomes more prominent with increasing difficulties in voluntary movements and worsening dysarthria and dysphagia. At this stage although patients can still maintain their personal independence they normally have to give up their employment. At later stages of HD, behavioural problems gradually decrease; motor disability becomes severe and patients are often totally dependent, mute and incontinent (Penney et al., 1990). Patients normally die 15-18 years after onset of the disease (Farrer et al., 1984).

Chorea, an involuntary movement disorder consisting of non-repetitive, non-periodic jerking of limbs, face or trunk, is the typical movement pattern in HD. Except chorea,
other involuntary movements such as bradykinesia, rigidity, dystonia and myoclonus, are also seen (Hayden 1981; Folstein 1989). A global decline in cognitive capabilities occurs in all HD patients (Bruyn 1968). Cognitive changes include forgetfulness, slowness of thought process, impaired visuospatial abilities and impaired ability to manipulate acquired knowledge (Mayeux et al., 1986; Morris 1995; Wilson et al., 1987). HD patients also develop significant personality changes, affective or schizophrenic psychosis (Mayeux et al 1986; Shiwach 1994; Mendez 1994; Cummings 1995). Suicide occurs in up to 12% of individuals. Other abnormalities include weight loss, sleep disturbances and incontinence, which are features of late HD (Bruyn 1968; Hayden 1981; Hansotia et al 1985; Wheeler et al., 1985).

IV.1.2 Pathology

HD is characterized by a primary cellular pathology with atrophy of the caudate nucleus and putamen (neostriatum) (Vonsattel et al., 1985; Gutekunst et al., 2001; Leegwater-Kim and Cha, 2004; Handley et al., 2006). Pathology in the caudate is usually more severe and occurs earlier than in the putamen. As neurons are lost, the tissue collapses, resulting in a secondary characteristic enlargement of the lateral ventricles. Degenerative changes also occur in other brain regions connected to the striatum, particularly in the neocortex but also including the thalamus, subthalamic nucleus, globus pallidus, substantia nigra pars compacta (SNpC) and hypothalamus.

Within the striatum the most vulnerable cells in HD are the medium-spiny projection neurons, which constitute 90-95% of all neurons within the striatum in rodents. The interneuronal cells are relatively spared in HD (Ferrante et al., 1985). The reason that causes the selective striatal projection neuron to degenerate is unknown.

A neuropathological hallmark of HD is Htt aggregates, in which the mutant Htt protein aggregates to form large dense protein inclusions particularly in the nuclei of affected cells, but also within the cytoplasm, neurites and terminals (Menalled and Chesselet, 2002; Bates, 2003).
IV.1.3 Huntington’s disease is a dominant gain-of-function polyglutamine disorder

The HD gene is located on the short arm of chromosome 4 (4p16.3). Exon 1 of the wild-type gene contains a stretch of uninterrupted CAG trinucleotide repeats which is translated into a series of consecutive glutamine residues (polyQ tract). Healthy individuals have 26 or fewer CAG repeats. An individual carrying an allele of 27-35 CAG repeats will not develop symptoms of HD but may be at risk to have a child with an abnormal CAG expansion (Goldberg et al., 1993). Thus alleles in this range have also been described as “mutable normal alleles” (Potter et al., 2004). HD patients have alleles with 36 or more CAG repeats (Rubinszttein et al., 1996). There is an inverse relationship between CAG repeat number and the age of onset of symptoms: the larger the number of CAG repeats, the earlier the age of onset (Ross and Hayden, 1998).

Htt is essential for normal development, as knockout mice that are deficient of endogenous Htt expression die at an embryonic stage (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Htt has also been implicated in different cellular processes such as apoptosis, vesicular transport and the regulation of transcription (Cattaneo et al., 2005). However, the exact function of Htt is still unclear.

HD is a member of a class of neurondegenerative diseases, which are referred to as CAG trinucleotide repeat disorders or polyglutamine disorders (Rubinszttein and Hayden, 1998; Vonsattel and DiFiglia, 1998; Ho et al., 2002). Other members include Dentatorubal-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA or Kennedy’s disease) and several spinocerebellar ataxias (SCA-1, 2, 3, 6, 7, 12 and 17). They have in common to be caused by CAG expansions in a particular gene. Although the different members of polyglutamine disorders have a unique neuropathology character, they share some remarkable features: they are all autosomal dominant or sex-linked dominant diseases, characterized by progressive neurodegeneration of selected neuronal cell populations; generally, age at onset of the disease is inversely correlated with the CAG repeat number; polyglutamine protein-containing aggregates are detected in all cases.
HD is an autosomal dominant disease and the presence of a mutation in one allele is sufficient to cause the disease. In humans, losing one of the two HD alleles does not cause an HD phenotype. Also, mice that have only one functioning HD gene do not show features of HD (Duyao et al., 1995; Nasir et al., 1995; White et al., 1997; Zeitlin et al., 1995). On the other hand, transgenic HD mouse models (generated by adding a mutant HD gene or HD gene fragment to the normal mouse genome with two wild-type mouse HD genes) and knock-in HD mouse models (generated by directly engineering one of the two endogenous HD alleles to express a mutant polyQ tract) develop a HD phenotype supporting the concept of a gain-of-function mutation mechanism (Shelbourne et al., 1999; Levine et al., 1999; Ishiguro et al., 2001; Lin et al., 2001).

This gain-of-function mechanism has been elegantly demonstrated in a mouse model where a 146 CAG repeat was inserted into the hypoxanthine phosphoribosyltransferase (HPRT) gene, which is not involved in any polyglutamine disorder. Previous work had demonstrated that inactivation of the HPRT gene in mice does not cause any deleterious effects. However, mice with an expanded CAG repeat in the HPRT gene developed a late-onset neurological phenotype (Ordway et al., 1997). This data strongly suggest that HD is caused by novel deleterious functions conferred by the CAG mutation.

It has also been suggested that by the HD mutation htt normal function could be affected (Zuccato et al., 2001). Therefore a loss of function effect could play an additional part in the pathogenesis of HD.

**IV.2 Potential pathogenic mechanisms of Huntington’s disease**

**IV.2.1 Htt aggregates**

Htt aggregates were first found in the first HD mouse model (R6/2 mice), a transgenic mouse expressing exon 1 of the human HD gene containing 113-156 CAG repeats
(Mangiarini et al., 1996; Davies et al., 1997). These mice developed aggregates in neuronal nuclei and processes. Similar aggregates have been identified in post-mortem human HD brains and in all transgenic or knock-in HD mouse models (Difiglia et al., 1997; Sapp et al., 1999). A causal role of aggregates for the pathogenesis of HD has been suggested, since they appeared before signs of the disease in the transgenic HD models (Davies et al., 1998). Aggregate formation in cultured cells correlated with susceptibility to cell death and also with axonal pathology in a knock-in mouse model and in a C. elegans model of HD (Cooper et al., 1998; Martindale et al., 1998; Igarashi et al., 1998; Wyttenbach et al., 2000; Li et al., 2001; Parker et al., 2001). Reduction of aggregates correlates with a decrease in cell death in vitro in cell culture and with an improved phenotype in the transgenic mouse model of HD (Cummings et al., 1998; Carmichael et al., 2000). However, other studies have dissociated aggregate formation and cell death (Kim et al., 1999; Martin-Aparicio et al., 2001) or even suggested that aggregate formation may serve as a protective mechanism for affected cells (Saudou et al., 1998; Arrasate et al., 2004). Whether there is a causal relationship between aggregate formation and disease is still debated. Aggregate formation may not be simply linked to cell death.

Aggregates have also been shown to have important indirect effects on cells. Several transcription factors including CBP, SP1 and TBP are sequestered into aggregates, which can lead to transcriptional dysfunction (Cha, 2000; Sugars and Rubinsztain, 2003). Aggregates of HD are normally ubiquitinated and also associated with several proteasome subunits, which strongly suggests a failure of the ubiquitin-proteasome degradative machinery of the cell (Ciechanover and Brundin, 2003; Valera et al., 2005). Thus aggregate formation after all might affect HD progression.

**IV.2.2 Cleavage of full-length mutant huntingtin**

Htt can be cleaved by some proteases including caspases and calpain (Goldberg et al., 1996; Wellington et al., 1998; Sanchez et al., 1999; Bizat et al., 2003). Mutant Htt is more susceptible to proteolysis than normal Htt. The N-terminal fragment containing the polyQ tract, which is released by the cleavage of the mutant Htt, is much more toxic and prone to aggregation compared with the full-length Htt. Modifications in the
activity of caspases and calpain reduce the proteolysis and toxicity of the mutant protein and delay disease progression (Kim et al., 1999; Ona et al., 1999; Wang et al., 1999; Chen et al., 2000). Graham reported recently that mice expressing mutant htt, resistant to cleavage by caspase-6 but not caspase-3, maintain normal neuronal function and do not develop striatal neurodegeneration, indicating that proteolysis of Htt at the caspase-6 cleavage site might be an important event in mediating neuronal dysfunction and neurodegeneration highlighting the significant role of htt proteolysis in HD (Graham et al., 2006). In HD patients and transgenic mice aggregates in the nucleus are mainly composed of N-terminally truncated Htt and caspase cleaved mutant Htt, which precedes neurodegeneration in HD (Davies et al., 1997; Difiglia et al., 1997; Scherzinger et al., 1997; Sieradzan et al., 1999; Wellington et al., 2000). Thus the cleavage of Htt and the release of the toxic N-terminal Htt fragment might be an important mechanism in the pathogenesis of HD.

**IV.2.3 Excitotoxicity and impaired energy production**

Excitotoxicity refers to the death of neurons as a result of chronic exposure to excitatory amino acids such as glutamate. Excitotoxicity could be an important pathogenic mechanism in HD. Injection of excitatory toxins such as kainite and quinolinic acid into the striatum of rodents and primates resulted in striatum and behavioural pathology similar to that seen in HD, and has been used to create animal models of HD (Coyle and Schwarcz, 1976; McGeer et al., 1978; Beal et al., 1989; Huang et al., 1995). Glutamate receptors have been shown to be reduced in human HD brains, which could result in a reduced threshold for glutamate toxicity so that normal levels of glutamate might become toxic (Young et al., 1988). Expressing the mutant Htt in neuronal cell lines enhanced the excitotoxic cell death induced by glutamate (Zeron et al., 2001).

A relation of impaired energy production and mitochondrial dysfunction with HD is also suggested by the effects of the toxin 3-nitropropionic acid (3-NP) that irreversibly inhibits succinate dehydrogenase (an enzyme involved in the tricarboxylic acid cycle and the electron transport chain during ATP synthesis) (Brouiller et al., 1995; Palfi et al., 1996). Administering 3-NP chronically in low doses to animals
reproduces the neuropathological and neurological outcomes of human HD (Brouillet and Hantraye, 1995; Borlangan et al., 1997). Humans surviving 3-NP toxicity develop choreiform movements and dystonia like HD patients (Ludolph et al., 1991; He et al., 1995). Defects in energy metabolism have also been well documented in HD: Glucose metabolism is decreased in brains of HD patients (Antonini et al., 1996) and lactate levels are increased in areas of HD brain (Jenkins et al., 1993). In addition reduced activities of enzymes involved in oxidative phosphorylation have been observed in HD brain (Butterworth et al., 1985; Gu et al., 1996; Browne et al., 1997). Panov and colleagues have demonstrated that mitochondria isolated from HD patients’ lymphoblasts have reduced calcium-buffering capacity and were depolarised (Panov et al., 2002). The mitochondria membrane potential is directly related to ATP production and reduced membrane potential likely results in energetic failure in HD. Indeed in an HD knock-in mouse model, there was a progressive reduction in the amount of c-AMP, strongly indicating an energy defect (Gines et al., 2003).

The mechanism by which mitochondrial dysfunction ultimately leads to neuronal death has been proposed to be linked to excitotoxicity, because NMDA receptor antagonists can block the effect of mitochondrial inhibitors and 3-NP treatment induces long-term NMDA-mediated excitation in medium spiny neurons (Henshaw et al., 1994; Calabresi et al., 2001).

It is not clear whether mitochondrial dysfunction is a primary event or a consequence of the early neuropathological change in HD. However, mitochondrial dysfunction, even as a secondary event, might affect the pathological processes.

**IV.2.4 Cell death or cell dysfunction**

Apoptosis, known as programmed cell death, is a feature of a number of chronic neurodegenerative diseases such as Alzheimer’s disease, amyotrophic lateral sclerosis and HIV dementia. A role of apoptosis and caspases, critical cysteine proteases in apoptosis, has been proposed in the pathogenesis of HD (Ona et al., 1999; Dragunow et al., 1995; Portera-Cailliau et al., 1995). Expression of mutant htt can induce apoptosis in HD cell models (Kim et al., 1999; Saudou et al., 1998; Li et al., 2000).
Htt is the substrate for some caspases including caspase-1, 3 and 6 (Wellington et al., 2000). The cleavage of the mutant Htt releases N-terminal fragments of Htt, which is a stimulus for upregulation of caspases. Inhibition of caspase-1 appears to slow disease progression in HD transgenic mice and there is increased activation of caspase-1 in pre-symptomatic and early symptomatic HD transgenic mice (Ona et al., 1999). It was reported recently that mice expressing mutant htt, resistant to cleavage by caspase-6 but not caspase-3, maintained normal neuronal function and did not develop striatal neurodegeneration (Graham et al., 2006). In addition the activation of caspases has also been shown in human HD striatal tissue (Ona et al., 1999; Sanchez et al., 1999; Kiechle et al., 2002). At the same time cell dysfunction could also significantly contribute to Huntington’s disease. Guidetti demonstrated morphologic abnormalities that included a significant decrease in the number of dendritic spines and a thickening of proximal dendrites in striatal and cortical neurons in a HD transgenic mouse before any detectable cell loss, which was found also in a HD cell model (Guidetti et al., 2001; Wyttenbach et al., 2001).

IV.2.5 Transcriptional dysfunction

Htt contains a polyglutamine stretch, which is a feature of several transcription factors (e.g., Sp1, TBP and CBP), and there is increasing evidence that Htt has an important role in transcription. Both the wild type and mutant Htt have been shown to interact with numerous transcription factors (Harjes and Wanker, 2003). Furthermore microarray analyses established that a number of transcriptional pathways are affected in HD (Iannicola et al., 2000; Luthi-Carter et al., 2000).

Among many different genes affected, the cAMP-responsive elements (CRE) and SP1-mediated pathways are the most extensively studied because of their involvement in the expression of genes needed for neuronal survival. The ablation of CREB leads to an HD-like phenotype in mice with progressive neurodegeneration in the hippocampus and striatum (Mantamadiotis et al., 2002) and reduced levels of products of CRE-response genes (proenkephalin, substance P and others) are seen in HD patients’ brains (Wyttenbach et al., 2001). The mechanism of the down-regulation of CRE-response transcription may occur as a result of the sequestration of
coactivators such as CREB-binding protein (CBP) and TAFII130 by the mutant htt into aggregates, as these coactivators are important positive regulators of CREB-mediated transcription (Li and Li, 2004; Shimohata et al., 2000). In a similar way, mutant Htt has been shown to disrupt SP1-mediated transcription such as the dopamine-D2-receptor by sequestering SP1 into aggregates. Furthermore, the enhanced association of mutant htt with SP1 can also block the binding of SP1 to its promoter, impairing SP1-mediated transcription (Dunah et al., 2002).

Recently Zuccato and colleagues demonstrated a new mechanism of Htt to affect transcription by regulating the transcription of genes that contained neuron-restrictive silencer elements (NRSE) (Zuccatto et al., 2003). In this study, wild-type Htt was shown to interact with REST-NRSF (repressor-element-1 transcription factor-neuron-restrictive silencer factor) in the cytoplasm, reducing its availability for binding to nuclear NRSE-binding sites and ultimately promoting the transcription of neuronal genes containing NRSE, including the brain-derived neurotropic factor (BDNF) gene coding for an important pro-survival factor for striatal neurons in the brain. However, mutant Htt loses the ability to interact with REST-NRSF and to retain it in the cytoplasm, resulting in increased levels of REST-NRSF in the nucleus and the suppression of transcription of NRSE-regulated genes including the BDNF gene. The loss of BDNF production can cause the death of striatal neurons, since they no longer receive BDNF supply from cortex neurons.

Mutant Htt can also affect transcription by regulating histone acetylation, a process involved in the regulation of transcription through covalent modification of chromatin. The mutant Htt protein binds to acetyltransferase domains of histone acetylases such as CBP and p300 (McCampbell et al., 2001; Steffan et al., 2001), impairing histone acetylation. Histone deacetylase inhibitors slow the progression of the disease in a Drosophila HD model (Steffan et al., 2001).

**IV.3 Huntington’s disease cell models and animal models**

To understand the disease process and test HD therapeutic strategies, different HD disease models *in vitro* and *in vivo* have been generated. Prior to the cloning of the
Huntington’s disease gene, the only HD models available were those generated by the intrastriatal or systemic administration of toxins, quinolinic acid or 3-NP (Beal et al., 1986; Beal et al., 1993). These models aimed to replicate the cell death observed in HD brains. However, models generated by the administration of toxins are inadequate representations of progressive neurodegeneration in HD. Since the cloning of HD gene, the ability to manipulate and mutate the HD gene has made it possible to create HD models in vitro in cells and in vivo in a wide variety of species including invertebrates (C. elegans and D. melanogaster) and mammalians including mice and rats (Bates and Hockly, 2003).

Several HD cell models are available now. Mouse neurons have been fused with mouse teratoma cells and the resulting hybrids have been stably transfected with various polyQ-containing htt fragments (Kim et al., 1999). Another model is based on stably transfected, temperature-sensitive, immortalized mouse striatal neurons (Rigamonti et al., 2000). The mammalian cell-based HD models can be developed into high-throughput assays capable of screening libraries of up to several million compounds in the search for potential drugs to treat HD. HD models in C. elegans (Parker et al., 2001) and Drosophila (Jackson et al., 1998; Marsh et al., 2000) have also been developed. Since these invertebrates have short generation times and are without the complication of a blood-brain barrier, they are not only useful in studying the process of HD but also provide the means to screen large numbers of potential HD drugs in a whole organism. More than ten research groups have independently generated mouse models of HD (Bates and Murphy, 2002). Of these, six are transgenic models and express either a fragment of the human HD gene or the entire human HD gene under a variety of promoters. Four further mouse models are knock-in models. In these mice, a long CAG repeat has been inserted into the relevant place in one of the murine HD genes, which in principle are models most precisely mimicking HD.

In addition, viral vectors based on AAV, lentivirus and E1-deleted adenovirus have been used to deliver N-terminal fragments of mutant htt into cultured cells and also in vivo in rats (Senut et al., 2000; de Almeida et al., 2002; Regulier et al., 2003; Tagawa et al., 2004; Zala et al., 2005). Vector-mediated HD models complement research performed in transgenic animal models based on several advantages. Localized
expression of htt is possible and high levels or regulated htt expression can be achieved. In addition, the parallel generation and testing of a large number of different htt expression constructs is possible in a relatively short period of time. A further advantage may lie in the possibility to use viral vectors for htt delivery in large animal models such as in non-human primates.

IV.4 Gene therapy of Huntington’s disease

Although there has been considerable progress in understanding the pathogenesis of HD, it still remains an untreatable disease until now. Gene therapy might be a new approach with significant potential for successful treatment of HD.

IV.4.1 Gene therapy of Huntington’s disease by vector-mediated expression of neurotrophic factors

Vector-mediated expression of neurotrophic factors in the striatum to protect neurons from degeneration has played a major role in current studies to treat HD by gene therapy (Handley et al., 2006). Neurotrophic factors refer to a group of growth factors that regulate the growth, differentiation, and survival of certain neurons in the peripheral and central nervous systems. NGF (nerve growth factor), BDNF (Brain derived neurotrophic factor), GDNF (glial derived neurotrophic factor), CNTF (ciliary neurotrophic factor), NT (neurotrophin)-3 and NT-4/5 have been shown to increase the survival of striatal neurons in cell culture and in vivo (Anderson et al., 1996; Perez-Navarro et al., 2000). In addition, there is evidence that some trophic factors may be affected in HD; in particular, BDNF has been found to be downregulated by mutant Htt expression (Zuccato et al., 2001). For neurotrophic factors to be effective in the brain of patients with HD, especially in the striatum, they have to be delivered into brains of HD patients, where they are needed (Emerich and Winn, 2004). Unfortunately, these molecules do not cross the blood-brain barrier and systemic delivery can cause significant undesirable side effects, such as cough, weakness, fever, pain and weight loss (Barinaga, 1994). Thus, direct expression of these factors in the brain by vector-mediated gene delivery is an obvious choice and sustained delivery can be relatively easily achieved by gene therapy.
CNTF, BDNF and GDNF have shown to be effective in HD animal models. CNTF was discovered through its ability to sustain parasympathetic neurons of the ciliary ganglia. It exhibits an extremely broad range of activity, acting on a number of neuronal populations. In the CNS it promotes the survival of neurons in the hippocampus, as well as both cholinergic and GABAergic neurons in the basal forebrain. CNTF has been found to be effective in protecting against striatal lesion in both rodents (de Almeide et al., 2001; Mittoux et al., 2002; Emerich and Winn, 2004) and primates (Mittoux et al., 2000). There have been several studies conducted using viral vector-delivered neuroprotective CNTF therapy in HD animal models that showed protective and reparative effects on skilled motor and cognitive tasks in addition to preserving the functional neuroanatomy of the striatum (de Almeida et al., 2001; Mittoux et al., 2002; Regulier et al., 2002; Zala et al., 2004). BDNF was identified on the basis of its ability to stimulate process outgrowth from peripheral sensory neurons. In the CNS BDNF supports the survival and process outgrowth of basal forebrain cholinergic neurons, dopaminergic neurons in the striatum, retinal ganglion cells and some motor neurons. Vector-mediated gene delivery of BDNF in the striatum can significantly reduce the loss of striatal neurons and provide neuronal protection in rodent models of HD (Perez-Navarro et al., 2000; Kells et al., 2004). GDNF is synthesized by glial cells, in the CNS by astrocytes and in the PNS by Schwann cells. GDNF was first characterized as a trophic factor that supported the survival and differentiation of dopaminergic cells of the midbrain and striatum and therefore it has been suggested as a therapeutic agent in Parkinson’s disease and HD (Lin et al., 1993; Choi-Lundberg et al., 1997). In addition GDNF has also been shown to protect striatal, medium-sized spiny GABA projection neurons, which are most vulnerable in HD (Perez-Navarro et al., 1996). Vector-mediated delivery of GDNF improves behavior and protects striatal neurons not only in neurotoxic rat models of HD but also in a transgenic mouse model of HD (Gratacos et al., 2001; Mcbride et al., 2003; Mcbride et al., 2006).

**IV.4.2 Ex vivo gene therapy of Huntington’s disease by neurotrophic factors**
Another approach to express neurotropic factors in the brain is to genetically engineer cells \textit{in vitro} to express therapeutic molecules and transplant these cells into the brain, a procedure which has been called \textit{ex vivo} gene therapy (Dunnett and Rosser, 2004). Cells for \textit{ex vivo} gene therapy are transfected with vectors \textit{in vitro} to express therapeutic transgenes and then placed into biocompatible polymer capsules before implantation in the brain. The capsule membrane contains pores in order to release neurotrophins, allow nutrients and oxygen to enter for maintaining cells, but prevent the host immune system from penetrating and destroying cells. The capsule can be easily removed if necessary to prevent unexpected side effects. This \textit{ex vivo} gene therapy can protect against the neural damage and behavioural deficits in the neurotoxic insult HD models (Emerich et al., 1997; Mittoux et al., 2000). In one study, monkeys had severe motor and cognitive deficits before capsule implantation with cells expressing CNTF. This implantation gradually improved task performance and behaviour of animals, suggesting it to be a viable treatment for HD. Recently a Phase I clinical trial on the effects of CNTF from implantation in HD patients has been completed (Bachould-Levi et al., 2000; Bloch et al., 2004). Six patients received a unilateral ventricular implant of a semi-permeable polymer capsule with CNTF-producing BHK cells. There were no adverse side effects, suggesting that it is safe to pursue this treatment method. A significant functional improvement of striatal circuitry was demonstrated in three patients, with the return of normal electrophysiological traces, indicating some beneficial effects from this therapy.

**IV.4.3 Gene therapy of Huntington’s disease by vector-mediated expression of shRNA**

Since HD is autosomal dominant gain of function disease caused by mutant Htt, one potential straightforward therapeutic strategy for gene therapy is to silence the mutant Htt gene. Indeed the therapeutic promise of this strategy was demonstrated in an inducible mouse model of HD (Yamamotto et al., 2000). When mutant Htt was inducibly expressed, mice developed pathological and behavioural features of HD, including neuronal aggregates and abnormal motor behaviour. After repression of the mutant gene expression in affected mice, mice fully recovered from the pathological and abnormal behaviour. There have been some studies by exploring inhibition of
mutant htt with antisense RNAs (Dykxhoorn et al., 2003). However, while anti-sense RNAs can be effective *in vitro*, they failed to inhibit mutant htt expression *in vivo* (Haque and Isacson, 1997).

Recently, RNA interference (RNAi) emerged to be a powerful tool to efficiently and specifically silence gene expression. RNAi has been used to knock down the specific gene expression for the analysis of gene function in invertebrates and plants for a long time (Hutvagner and Zamore, 2002; Mello and Conte, 2004; Meister and Tuschl, 2004). Introduction of double-stranded RNA (dsRNA) into the cells of these organisms leads to the sequence-specific destruction of endogenous RNAs that match the dsRNA. During RNAi, long dsRNA molecules are processed into 19-23 nt RNAs known as short-interfering RNAs (siRNAs) that serve as guides for enzymatic cleavage of complementary RNAs. In addition, in plants, fungi and C. elegans siRNAs can function as primers for an RNA-dependent RNA polymerase that synthesizes additional dsRNA, which in turn is processed into siRNAs, amplifying the effects of the original siRNAs. However, RNAi with long dsRNA had not been successful in mammalian cells because of non-specific response elicited by dsRNA molecules longer than 30nt, until Tuschl and his colleagues observed that transfection of synthetic 21-nt siRNA duplexes into mammalian cells effectively inhibits endogenous genes in a sequence-specific manner (Elbashir et al., 2001a,b). From then on siRNAs have quickly been developed into a powerful tool to knock down specific gene expression in mammalian cells and in animals *in vivo* (Hommel et al., 2003). SiRNAs can be introduced into mammalian cells and experimental animals either directly as synthetic RNA duplexes or indirectly by DNA constructs expressing siRNAs or short hairpin RNA (shRNA) from RNA polymerase III promoter. ShRNA expressing from DNA constructs is processed to form siRNA by the RNAi pathway in cells. Using siRNAs as therapeutic molecules to treat hereditary diseases like HD and viral-infection diseases like chronic hepatitis B infection is explored by many groups (Grimm and Kay, 2006; Raoul et al., 2006). Several groups including ours have demonstrated that vector-mediated expression of shRNA can efficiently inhibit the expression of Htt in cell cultures and in HD mice and improve the pathology in HD mice (Huang and Kochanek, 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005; Machida et al., 2006). In addition, Harper and his colleagues showed that RNAi not only improved neuropathological abnormalities but also motor deficits in an HD
mouse model (Harper et al., 2005).

However, several issues need to be considered before introducing siRNA technology into the clinic. One question concerns the depletion of the wild-type Htt. Since it will be difficult, if not impossible, to develop a siRNA strategy selectively targeting the expanded CAG repeat in the mutant Htt without negatively interfering with the expression of other CAG repeat-containing genes including the normal Htt, all shRNAs have been designed to target the HD transcript region outside of the CAG repeat, which would reduce the expression of both the mutant and normal htt alleles. Htt is essential in embryogenesis and postnatal neurogenesis. However, the effect of partial reduction of the normal htt expression in adult postmitotic neurons in vivo is unknown. Thus, it is an important question whether partial reduction of normal Htt in the brain would be tolerable in HD patients. If not, disease allele-specific silencing might be required. Another concern relates to the efficient delivery of siRNA to the brain. The brain is not accessible to systemically delivered siRNA. Viral vectors expressing shRNAs do not cross the brain barrier. Therefore direct stereotaxic injection was used to deliver siRNAs and viral vectors to the striatum. The localized injection of siRNA in the striatum normally results in a localized reduction of Htt expression only in an area around the injection site. In addition to the striatum, degenerative changes in HD also occur in other brain regions connected to the striatum, including the neocortex, the thalamus, subthalamic nucleus, globus pallidus, substantia nigra pars compacta (SNPc) and hyperthalamus. Localized injection of AAV vectors expressing shRNA in the striatum improved the behaviour deficit but failed to stop weight loss and to prolong the life in an HD transgenic mouse model (Harper et al., 2005). Therefore, an efficient siRNA-based treatment of HD might depend on delivering siRNA to most areas of the brain, a tremendous challenge that has not been achieved up to now. A last concern relates to side effects of RNAi. RNAi depends on overexpression of siRNA or shRNA and the use of the endogenous RNAi machinery, which might disturb the biological events regulated by endogenous RNAi and lead to toxicity. High-level expression of shRNA in livers resulted in liver injury and even in the death of mice (Grim et al., 2006). RNAi may also inhibit the expression of genes that are not considered targets, so-called off target effects (Saxena et al., 2003; Scherr and Eder, 2004), and even cause non-specific immune stimulation such as interferon or IL-12 response (Sledz et al., 2003; Kariko et al., 2004).
Fortunately all these side effects might be minimized by optimising siRNA dose and sequence.

IV.5 Vector systems in CNS gene therapy

Vector-mediated gene therapy of HD is performed by gene delivery into the brain, normally the striatum, using viral vectors. Viral vectors are directly injected into the striatum of the brain of HD models by stereotaxic injection. An “ideal” vector for gene therapy of the brain would be easily distributed in the brain, efficiently transduce cells in the brain, sustain long term and regulatable transgene expression, have no toxicity to cells in the brain and have little immunogenicity. Different vectors have been used for gene delivery in CNS.

IV.5.1 Retroviral vectors

Retroviruses are enveloped single-strand RNA viruses that can integrate their genomes into the chromosome DNA of host cells, thus facilitating long-term transgene expression. Retrovirus genomes are composed of four gene regions termed gag, pro, pol and env, which encode for structural capsid proteins, viral protease, integrase, viral reverse transcriptase and envelope glycoproteins. The retroviral genome also has a packaging signal and cis-acting sequences, termed long-terminal repeats (LTRs), at each end, involved in transcriptional control and integration. Recombinant retroviral vectors are deleted of all retroviral genes, which are replaced with therapeutic genes. LTRs and packaging signals are the only sequence left in vectors (Sena-Esteves et al., 1996; Weber et al., 2001). They are generated in packaging cell lines, which express viral genes necessary for the vector growth. The most commonly used retrovirus vector is derived from Moloney murine leukaemia virus (Mo-MuLv) and can only deliver genes to dividing cells. Thus, it is not suitable for gene delivery to neurons, except for cancer gene therapy in the brain or ex vivo gene therapy in CNS. Lentivirus vectors, derived from feline immunodeficiency virus (FIV) or HIV, have been developed to overcome this shortage of Mo-MuLv vectors. Lentiviral vector are able to transduce and integrate into the genome of nondividing cells and long-term in vivo expression has been reported in neurons (Naldini et al.,
Lentiviral vectors have great potential for treatment of CNS diseases; however, issues about the safety of lentiviral vectors need to be resolved before their clinical application.

**IV.5.2 Herpes simplex virus (HSV) vectors**

HSV is an enveloped, double stranded DNA virus with a genome of 152 kb encoding more than 80 genes. HSV infects cells either lytically or can establish latency. There are two types of HSV vectors: recombinant HSV vectors and amplicon vectors (Wilkinson et al., 1994; Laquerre et al., 1999). Recombinant HSV vectors are generated by the insertion of transgene expression cassette directly into the HSV genome by homologous recombination. The amplicon vectors are based on plasmids bearing the expression cassette of transgenes, an origin of replication and a packaging signal. The plasmid is transfected into a cell line, which is subsequently infected with a helper virus. The helper virus provides replication and packaging functions in trans, enabling the amplicon to be packaged into infectious HSV virions. HSV vectors were the first vectors to be used for gene delivery to CNS and are among the most efficient vectors of gene delivery to the brain (Palella et al., 1989; Chiocca et al., 1990). Although HSV vectors have the advantage of a large capacity for the insertion of foreign genes, the capacity to establish latency in neurons and the ability to confer long-term expression of transgenes in the CNS for over 18 months (Carpenter and Stevens, 1996) the problem of vector toxicity has limited its application in gene therapy in CNS, except for gene therapy of brain tumours (Lowenstein et al., 1994; Laquerre et al., 1999).

**IV.5.3 Adeno-associated virus (AAV) vectors**

AAV belong to the family of Parvoviridae, Genus Dependovirus. They are single-stranded, non-enveloped DNA viruses, whose genomes are composed of two open reading frames (ORFs), rep (coding for replication proteins) and cap (coding for structure proteins), and two small (145bp) ITRs (Srivastava et al., 1983). AAVs transduce a wide spectrum of mammalian cells, however, they do not replicate without functions provided in trans by a helper virus, such as an adenovirus or HSV.
Recombinant AAV (rAAV) vectors are generated by co-transfection of two plasmids into produce cells (Samulski et al., 1989). The first contains the transcription unit of the transgene flanked by ITRs, and the second contains the rep and cap ORFs. In order to propagate rAAVs, infection with a helper virus (normally an adenovirus) is necessary. The vector preparation may contain helper virus contamination by this approach. A further developed approach is to provide helper virus functions by co-transfection with a plasmid coding for the required adenoviral helper functions, thus enabling to prevent helper virus contamination (Xiao et al., 1998).

AAV vectors have several advantages, thus making them the currently most popular vectors used for gene therapy in the CNS (Okada et al., 2002; Ruitenbergh et al., 2002; Grieger and Samulski, 2005). AAV vectors are relatively well distributed in the CNS after injection, better than other viral vectors. AAV vectors support long-term stable expression in CNS and cause less toxicity and immunogenicity than other vectors in CNS (Lo et al., 1999). After *in vivo* gene transfer of AAV vectors, no evidence of a cellular immune response has been detected. However, specific circulating antibodies to AAV vectors have been detected, which may limit their potential re-administration (Xiao et al., 1996). This could possibly be solved by administrating different AAV serotypes. The cellular tropism and vector-diffusion of AAV-mediated gene transfer in CNS varies, depending on the AAV serotype used (Davidson et al., 2000). AAV2 vectors preferentially transduce neurons whereas AAV5 and AAV1 transduce both neurons and glial cells. AAV5 diffuses farther and more uniformly than AAV2. AAV have been used in pioneering work of gene therapy to several CNS diseases (McCown, 2005). These include enzyme replacement in lysosomal storage diseases, Canavan disease and Parkinson’s disease, delivery of neuroprotective factors in Parkinson’s disease, Huntington’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, ischemia and spinal cord injury, as well as modulation of neurotransmission in epilepsy and Parkinson’s disease. Several of these experiments have demonstrated promising results in relevant animal models.
AAV vectors have a major drawback of limited packaging capacity for the transgene (4.7kb). Recently, a new approach has been developed to overcome this shortage by exploiting the unique heterodimerization ability of AAV DNA (Wright et al., 2003). AAV genomes often form head-to-tail concatemers through intermolecular recombination. Therefore by splitting a gene and its regulatory elements into two separate AAV vectors, head-to-tail heterodimers of the two AAV vectors will be formed after co-delivering two vectors into target cells. The presence of an appropriate intron or splicing signal sequence then allows rejoining of an intact expression cassette following post-transcriptional processing. This so-called split-gene or trans-splicing strategy has effectively increased the packaging capacity of AAV vectors to 10 kb.

IV.5.4 Adenovirus (Ad) vectors

Ad vectors have several advantages such as the ability to transduce a wide variety of cell types including both dividing and non-dividing cells, a high efficiency of gene transfer, no integration into host genomes, relatively large transgene capacity, easy manipulation and production at high titers resulting in their extensive use in gene therapy strategies including for the CNS. Since they are the vectors we applied in our own experiments, Ad vectors are described here in detail.

IV.5.4.1 Adenovirus structure and characteristics

Adenoviruses form a large group of DNA viruses, constituting the Adenoviridae family (Shenk, 1996). Adenoviridae include four genera: Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus. The Mastadenovirus genus includes human, simian, bovine, equine, porcine, ovine, canine and opossum viruses. So far over 50 human adenovirus serotypes have been distinguished on the basis of their resistance to neutralization by antisera to other known adenovirus serotypes. These various serotypes of human adenoviruses are classified into six species (A-F) based on their ability to agglutinate red blood cells. Most adenoviral vectors are derived from adenovirus serotype 5 (Ad5) and adenovirus serotype 2 (Ad2), which belong to the species C of human adenoviruses. All adenoviruses contain a linear, double-strand
DNA genome encapsidated in an icosahedral protein shell with 70-100 nm in diameter (Horne et al., 1959). The protein shell is composed of 252 subunits (capsomers), of which 240 are hexons and 12 are pentons (Ginsberg et al., 1966). Each penton contains a base, which forms part of the surface of the capsid in the vertex, and a projecting fiber. The virus genome has a size of about 36 kb with a terminal protein covalently attached to the 5 termini. It has ITRs at both ends, where the replication origins of adenoviruses are located, and a cis acting sequence required for packaging at the left end, which is responsible for the polar encapsidation of viral DNA. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2), and one late unit (major late), which is processed to generate five families of late mRNAs (L1 to L5) (Pettersson and Roberts, 1986).

The process of transducing cells by adenoviruses is initiated by attachment of the fiber knob protein to a high-affinity receptor called the Coxsackie and Adenovirus Receptor (CAR) at the cell surface (Bergelson et al., 1997). Entry of the virus into cells is then further mediated by an interaction between cell-surface integrins \( \alpha_v\beta_3 \) and \( \alpha_v\beta_5 \) with the capsid penton base (Wickham et al., 1993). After these initial extracellular interactions the adenovirus virion is endocytosed via a clathrin-coated pit into an endosome (Wang et al., 1998). A subsequent decrease in pH within the endosome triggers a conformational change to the virus capsid, resulting in the release of the viral capsid into the cytoplasm. From there, the virus is then transported to the nuclear pore, where the viral genome is released into the nucleus and undergoes replication and transcription (Greber et al., 1993). After nuclear entry, E1A is the first gene to be expressed and has a key role as transactivator of all other adenoviral genes (Shenk and Flint, 1991). E1A also plays a role in modulating cellular metabolism to make the cell more susceptible to virus replication. E1B gene products are involved in prolonging cell survival by inhibiting apoptosis. The E2 gene products provide the machinery for replication of virus DNA and the ensuing transcription of late genes. The E3 genes produce a compendium of proteins that subvert the host defence mechanisms. One of these E3 gene products has been termed the adenovirus death protein (ADP), since it facilitates late cytolysis of the infected cell to release the progeny virus more efficiently. The gene products derived from the E4 region mainly facilitate virus messenger RNA metabolism, promote virus DNA replication and shut
off host protein synthesis. They are also associated with resistance to lysis by CTLs. Adenoviruses also transcribe a set of RNAs that are not translated, termed the VA RNAs, and these VA RNAs play a role in combating cellular defence mechanisms. Replication of the viral DNA occurs after accumulation of E2 gene products and the infected cell enters the S phase of the cell cycle. Following DNA replication the major late promoter is activated to transcribe the major late unit, resulting in the production of the viral structural proteins. At last viruses are assembled and released from the cell (Russell, 2000).

IV.5.4.2 First and second generation adenovirus vectors

Adenoviral vectors are created by partial deletion or even fully deletion of viral genes except the ITRs and the packaging signal. According to the part of viral genes deleted, adenoviral vectors can be classified into three different types of vectors: first generation, second generation and third generation adenoviral vectors (Hitt et al., 1997; Tashiro et al., 1999; Zhang, 1999; Ruessell, 2000; Volpers and Kochanek, 2004).

In first generation adenoviral vectors, the E1 and/or E3 gene cassette are removed, allowing the introduction of up to 8 kb of foreign DNA. Because an adenoviral vector without the E1 region cannot replicate, first generation adenoviral vectors need to be propagated in complementing cell lines like the 293 or N52E6 cell lines, which provide the E1 gene products in trans (Graham et al., 1977). Frequently, first generation Ad vectors are generated by *in vivo* recombination in complementing cell lines between shuttle plasmids containing the gene of interest flanked by the Ad E1 or E3 sequence and a second plasmid containing essentially the entire Ad genome in a circular form. The generated Ad vector is then purified by plaque purification. First generation Ad vectors can also be generated by directly transfecting complementing cells with the linearized Ad genome without the E1 and/or E3 gene cassette but with the gene of interest.

A major drawback of first generation Ad vectors *in vivo* is an immune response elicited against viral proteins, either against proteins of the incoming viral capsid, or against vector-encoded proteins expressed in tansduced cells (Muruve 2004). These immune responses can be separated into two phases, comprising an early innate
inflammatory response and a later acquired immune response. The initial inflammatory response is directed against the viral capsid proteins. It is characterised by up-regulation of proinflammatory cytokines and activation of innate inflammatory cell types, including macrophages, neutrophils and natural killer cells (Worgall et al., 1997; Cartmell et al., 1999). The acquired immune response, consisting of cellular and humoral components, directed against adenoviral gene products, which are expressed at low levels in infected cells even in the absence of the E1A gene product (Yang et al., 1994; Yang et al., 1995), or the transgene (Yang et al., 1996; van Ginkel et al., 1997; Michou et al., 1997). As a consequence of this immune response transgene expression from first generation Ad vectors has been shown to be relatively short in the periphery, being optimal at 3-4 days and disappearing around 1-2 weeks, although within the CNS, elimination of Ad-infected cells is much slower with gene expression still present after at least 3 months (Byrnes et al., 1995; Geddes et al., 1997, Dewey et al., 1999). Thus first generation Ad vectors are mainly used in applications where transgene expression for a short duration may be enough for the desired effects, e.g., for developing recombinant vaccines for humans and for cancer gene therapy.

Second generation adenoviral vector shave been generated by a further deletion of the E2 and/or E4 regions to increase the capacity for gene transfer and reduce the immunogenicity. However, the advantage of second-generation Ad vectors versus the first-generation Ad vectors is somewhat controversial.

**IV.5.4.3 High capacity adenoviral (HC-Ad) vectors**

In an attempt to address both the capacity and immunogenicity problem of first- and second-generation adenoviral vectors, a third-generation of adenoviral vectors called high capacity adenoviral (HC-Ad) vectors has been developed (Mitani et al., 1995; Kochanek et al., 1996; Parks et al., 1996; Alemany et al., 1997; Hardy et al., 1997). This vector type has also been called gutless, gutted, mini, deleted, delta or helper-dependent adenoviral vector. In HC-Ad vectors all viral coding sequences are deleted and are replaced with foreign DNA. In addition to the therapeutic genes, frequently “stuffer” DNA is included to bring the vector genome to a size of at least 27kb in order to decrease instability during production (Parks and Graham, 1997). Thus HC-AD vectors contain only the ITRs and the packaging signal from adenovirus and can
accommodate up to 37 kb of foreign DNA. The production of HC-Ad vectors requires three components: a helper virus, a permissive cell line and a linear vector genome. Helper viruses and the permission cell line provide all genes needed for vector production in trans. The helper virus is a first-generation Ad vector, which is deleted for the E1/E3 regions and that has been engineered to contain two LoxP sites flanking the packaging signal. When the HC-Ad vector is propagated in permissive cells like 293 cells expressing the enzyme Cre-recombinase (293 Cre66 cells), the packaging signal of the helper virus is excised by Cre recombinase and the resulting helper virus genome cannot be packaged, avoiding helper virus contamination. To rescue and produce HC-Ad vectors, the linearized HC-Ad DNA and helper virus are introduced into Cre66 cells. The vectors are amplified by serial propagation in Cre66 cells. In the vast majority of helper virus genomes the packaging signal is excised and helper virus genomes are not packaged. In contrast, the vector genome is efficiently packaged. At last the lysate containing vectors is subjected to CsCl equilibrium centrifugation to purify the HC-Ad vector and further reduce the contamination of helper virus.

By removing all coding sequences of the Ad genome, HC-Ad vectors eliminate the problem of residual viral gene expression associated with first-generation Ad vectors. Compared to first and second generation Ad vectors, HC-Ad vectors have reduced the host adaptive immune response, have a high cargo capacity, and result in long-term gene expression in both small laboratory animals and nonhuman primates without causing significant toxicity (Kochanek et al., 2001; Imperiale and Kochanek, 2004). Thus HC-Ad vectors have been used in preclinical models of liver diseases, hemophilia and DMD. In an example, an HC-Ad vector containing the complete locus for the α-antitrypsin gene expressed the protein in immuno-competent mice for more than one year with negligible toxicity (Morral et al., 1998). HC-Ad vectors have also been successfully used for gene transfer to the CNS (Thomas et al., 2000; Thomas et al., 2001; Lowenstein et al., 2003; Xiong et al., 2006). While transgene expression from first-generation Ad vector was completely eliminated following peripheral immune priming, HC-Ad vectors produced sustained transgene expression in the rat brain (Thomas et al., 2001). In another example, even in the presence of anti-HC immunity, an HC-Ad system resulted in sustained and regulatable transgene expression in the brain (Xiong et al., 2006). With the highly efficient delivery of
foreign genes to the CNS with low toxicity and immunogenicity and the ability to sustain long term and regulatable transgene expression in the CNS, HC-Ad vectors might become interesting vectors for CNS gene therapy.
V. Results and Discussion

V.1 Inhibiting the expression of huntingtin by shRNAs directed to exon 1 of the huntingtin RNA

Because HD is a dominantly inherited gain of function neurodegenerative disease caused by mutant huntingtin, inhibition of mutant Htt expression would be a direct approach to treat HD. In recent years, gene silencing through RNAi has emerged as a powerful method to specifically inhibit gene expression in cells in vitro and in animals in vivo (Hutvagner and Zamore, 2002; Mello and Conte, 2004; Meister and Tuschl, 2004). To prove the concept that RNAi could silence htt expression and prevent HD pathology in an animal model, as a first step we tried to identify an shRNA molecule that was able to efficiently and inhibit Htt expression in cell cultures. We designed two shRNAs (ie-1-1 and ie-1-2) targeting the human Htt cDNA sequence around the start codon in exon 1, which could be validated in the R6/2 HD mouse model, a mutant human Htt exon 1 transgenic model, in the future (Paper I, Fig. 1A). Expression plasmids pU6-ie-1-1 and pU6-ie-1-2 were generated to express the shRNAs from the murine U6 promoter. These two shRNAs inhibited Htt exon 1 expression when they were cotransfected into 293 cells with a second plasmid qp25 expressing a normal htt exon 1 EGFP fusion protein with 25 polyglutamine repeats (Paper I, Fig. 1B). Particularly shRNA ie-1-1 efficiently inhibited Htt exon 1 expression by up to 80%. A similar result was achieved when a plasmid qp103 expressing a mutant Htt exon 1-EGFP fusion protein with 103 polyglutamine repeats was cotransfected (Paper I, Fig. 1B, lane 6,7). The control shRNA ie-2, targeting to htt exon 2 (not present in qp25), did not inhibit htt exon 1-EGFP fusion protein expression, indicating the specificity of the exon 1-targeted shRNAs (Paper I, Fig.1B, lane 3). To test whether shRNA ie-1-1 could inhibit the endogenous full-length human htt expression, human 293 cells were transfected with plasmid pU6-ie-1-1-1 expressing shRNA ie-1-1. The expression of endogenous htt was significantly reduced by shRNA ie-1-1 (Paper I, Fig. 1C,D).
V.2 Adenovirus vector-mediated inhibition of huntingtin aggregate formation in vitro by shRNA

To efficiently deliver anti-htt shRNA ie-1-1 into cells in vitro and in vivo, we generated an E1-deleted Ad vector (AdU6ie-1-1) expressing the shRNA ie-1-1 (Paper I, Fig. 2A). AdU6ie-1-1 inhibited expressing of the normal or mutant Htt exon 1-EGFP fusion protein in A549 cells transduced with a second Ad vector Adqp25 expressing normal htt exon 1 or Adqp103 expressing mutant htt exon 1 (Paper I, Fig2B, C). The mechanism for this decrease in htt exon 1 expression was by mRNA degradation, not by translational inhibition as demonstrated by Northern blot analysis (Paper I, Fig. 2D, E). To test whether AdU6ie-1-1 could inhibit the endogenous Htt expression, we transduced with AdU6ie-1-1 different cell lines, including HeLa cells, A549 cells and the neuronal cell line SH-SY5Y. The expression of endogenous Htt was significantly reduced in all these cell lines, and an up to 20-fold reduction of Htt protein levels occurred 5 days after transduction (Paper I, Fig. 4).

HD-associated aggregates are a major pathology marker of HD in HD patients and in HD mouse models (Davis et al., 1997; DiFiglia et al., 1997). The formation of aggregates was initially suggested to be an important cause of HD. Later studies dissociated aggregate formation and cell death and even suggested that aggregate formation may protect cells from death. The exact role of htt aggregate formation in HD is still unclear and debated. However, it is clear that reduction of aggregates can decrease cell death in cell culture and improves the phenotype of the HD mouse model (Bates, 2003). Thus Htt aggregates are also used as a biomarker to test the potential of HD treatment. We investigated whether AdU6ie-1-1 inhibited the formation of Htt aggregates in cell cultures. Adqp103 is an E1-deleted Ad vectors expressing mutant htt exon 1-EGFP fusion protein with 103 polyglutamine repeats. Transducing A549 cells with Adqp103 resulted in the formation of numerous Htt aggregates in cells (Paper I, Fig. 2B). After cotransducing A549 cells with Adqp103 and AdU6ie-1-1, htt aggregate formation was almost completely prevented (Fig. 3A, Lane 1, 2). To test whether Ad vector-mediated expression of shRNA could decrease the number of aggregates after they had formed similar to what would be required in HD patients, we at first transduced A549 cells with Adqp103 and waited for 48 hours.
to let aggregates form. Cells were then transduced with Adie-1-1. After additional 72 hours, there was a significant reduction in the number of aggregates in the presence of shRNA expressed from AdU6ie-1-1 (Paper I, Fig. 3). It can thus be concluded that Ad vector-mediated expression of shRNA efficiently inhibited the formation of htt aggregates in cell cultures.

V.3 HC-Ad vector-mediated inhibition of huntingin aggregate formation in vitro and in vivo by shRNA

Although E1-deleted first generation Ad vectors have been used to deliver transgenes into the brain, they could trigger an anti-viral immune responses after their injection, resulting in short-term transgene expression and toxicity (Bangari and Mittal, 2006). The toxicity of first generation Ad vectors has limited their use in gene transfer to the brain, except for the treatment of brain tumours (Hsich et al., 2002). HC-Ad vectors, with all viral genes deleted, have been proven to be highly efficient for long-term gene expression in the brain with low toxicity and immunogenicity (Imperiale and Kochanek, 2004). Therefore, we generated HC-Ad vectors expressing shRNAs, to allow for long-term expression in vivo.

The U6-ie-1-1 shRNA expression cassette was inserted into the Ad shuttle vector pSTK129 (an HC adenoviral shuttle plasmid with an HC adenoviral genome of 28 kb), which has been used to generate HC-Ad vectors in several studies, resulting in HC-Ad shuttle plasmid pHB02. pHB02 was linearized by PmeI digestion and transfected into 293cre66 cells to generate HC-Ad vector HC-AdHB02 expressing shRNA ie-1-1. However, there was always one unexpected extra band appearing upon HindIII digestion of HC-AdHB02 genome after propagation in cells compared with the restriction pattern of the original plasmid pHB02, indicating that the vector genome was unstable during propagation. We suspected that the instability of the vector was due to its small genomic size. Adenoviral vectors with genome sizes of below 28kb tend to be unstable. Because the size of the U6-shRNA expression cassette is small with about 400 bp, the size of the viral vector genome (HC-AdHB02) after inserting the U6-shRNA expression cassette into pSTK129 was about 28 kb, which is at the lower size limit of stable adenoviral vectors probably explaining the instability of the
HC adenoviral vector during production in producer cells. To overcome this shortage, we inserted an 1.2 kb stuffer DNA from the human lamin B2 locus into pSTK129, resulting in a new HC adenoviral shuttle vector pHBO3 with an increased size of stuffer DNA of 29.5 kb. Based on pHBO3 an HC adenoviral vector with U6-ie-1-1 expression cassette, HC-AdHB04, was successfully generated and produced. The integrity of the vector genome was confirmed by restriction digestion indicating preventing of instability of the vector during production (Fig. 1). The HC-Ad shuttle vector pHBO3 can be also used to generate HC-Ad vectors with other small transgene expression cassettes.

Fig 1. Generation of the HC adenoviral vector HC-AdHB04 with the U6-shHtt expression cassette to inhibit htt expression. (A) Maps of the HC adenoviral vectors HC-AdHB02 and HC-AdHB04. HC-AdHB02 is an HC-Ad vector with U6-ie-1-1 shRNA expression cassette inserted into STK129. HC-AdHB04 is an HC adenoviral vector with the U6-ie-1-1 shHtt expression cassette inserted into HB03. They all contain 20kb stuffer DNA derived from the human HPRT locus, 6.5 kb stuffer DNA
fragment from C346, the left and right terminus of Ad5 (ITR) and the packaging signal from Ad5 (Ψ). HC-AdHB04 contains an extra 1.2kb stuffer DNA from the human lamin B2 locus (LB) to increase the stability. (B) HindIII restriction analysis of vector DNA. Lane 1, 4: DNA marker; Lane 2: the viral shuttle plasmid pHB02; Lane 3: the HC-AdHB02 genomic DNA; Lane 5: the viral shuttle plasmid pHB04; Lane 6: the HC-AdHB04 genomic DNA. An extra band (marked with a triangle) after HindIII digestion of HC-AdHB02 genomic DNA is not present at the HindIII digestion of the original shuttle plasmid pHB02, indicating rearrangement of the vector genome.

HC-AdHB04 efficiently inhibited not only expression of the Htt exon 1-EGFP fusion protein, but also of endogenous Htt expression in different cells including A549, Hela and SHSY-5Y cells (Paper II, Fig. 2).

We then tested whether HC-AdHB04 could inhibit htt aggregate formation in cell culture in vitro. On co-transduction of A549 cells with Adqp103 and HC-AdHB04, the formation of htt aggregates was almost completely inhibited (Paper II, Fig. 3A). HC-AdHB04 prevented Htt aggregate formation even when the Htt aggregates had already formed in cells (Paper II, Fig 3, B, C).

At last we tested whether HC-AdHB04 could inhibit htt aggregate formation in the mouse striatum in vivo. Htt aggregates are mainly formed in neuronal cells in HD patients. To mimic htt aggregate formation in neurons in vivo, we generated a HC-Ad vector (HC-AdHB07) expressing N-terminally truncated mutant Htt under control of the neuron-specific SYN1-WPRE promoter (Paper II, Fig. 1). Injection of HC-AdHB07 into the mouse striatum resulted in htt aggregate formation in neuronal cells around the injection site. However, four weeks after co-injection of HC-AdHB07 and HC-AdHB04 into the mouse striatum, no Htt aggregates were observed (Paper II, Fig. 4), indicating that the HC-ADHB04 vector efficiently attenuated the formation of Htt aggregates in neurons in vivo. Next we tested the HC-AdHB04 vector in the R6/2 HD mouse model. R6/2 transgenic mice express the mutant human Htt exon 1 with a large glutamine repeat and have been used to evaluate the potential of HD treatment. Htt aggregates begin to be detectable in neurons of 4 week-old R6/2 mice and are the most striking neuropathological alteration in this model. We injected HC-AdHB04 vector into the striatum of 5-week-old R6/2 mice. Four weeks after injection the formation of Htt aggregates was significantly reduced at the injection site (Paper II, Fig. 5).
Several groups have successfully used AAV-mediated expression of shRNA or direct siRNA injection to inhibit htt expression and neuropathological abnormalities in HD mouse models were found to be reduced (Huang and Kochanek, 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005; Machida et al., 2006). Harper even reported that AAV-mediated shRNA improved the motor abnormality in the N171-82Q HD mouse model (Harper et al., 2005). Together, our data and published data with AAV vector systems support the concept of treating patients with HD or other dominant neurodegenerative disorders with shRNA based gene therapy.

V.4 Allele-specific expression of normal huntingtin by combining anti-Htt shRNA expression and expression of an Htt cDNA with neutral nucleotide exchanges in the shRNA target sequence

A major challenge in devising an shRNA based approach to treat HD is the difficulty to selectively target the mutant but not the wild-type Htt allele. An shRNA or siRNA against CAG repeats would affect both wild-type and mutant Htt alleles, as well as other RNAs containing CAG repeats. Therefore, all shRNA against Htt are only Htt-specific until now, inhibiting expression of both normal and mutant Htt without discrimination. Htt is essential in embryogenesis and postnatal neurogenesis. But the effect of partial reduction of the normal Htt expression in adult postmitotic neurons in vivo is unknown. No obvious side effects were observed after a partial reduction of wildtype htt in the striatum of mice or adult rhesus monkeys, indicating that partial reduction of normal Htt may be tolerable (Chicurel, 2006). However, the possibility of subtle changes cannot be excluded. To avoid this potential problem, one strategy capitalizes on the exquisite sequence specificity of RNAi and disease-linked single nucleotide polymorphism (SNP) to design siRNA or shRNA specifically targeted to the mutant allele (Rodriguez-Lebron and Paulson, 2006). Selectively reduced expression of the mutant allele with siRNAs in the HD-like polyglutamine disorder Spinocerebellar Ataxia Type 3 (SCA3) has been successful taking advantage of a SNP (G to C) that exists in tight linkage disequilibrium with the CAG repeat expansion (Li et al., 2004). Nevertheless, SNP-based siRNA allele specificity can be difficult to
achieve. All the safety concerns of each newly designed siRNA will have to be addressed before being translated into a clinical situation and the cost of developing individual therapeutics for different SNPs would be prohibitive. An alternative strategy would be to express a wild-type Htt that is refractory to RNAi silencing, which would replace the endogenous normal and mutant Htt inhibited by siRNA (Raoul et al., 2005). As a proof-of-concept experiment, we generated plasmids qp25(c1) and qp25(c2) carrying, compared to the parental qp25 control, neutral nucleotide exchanges in the shRNA target sequence at the 5’ part of the Htt exon1-EGFP fusion constructs (Fig 2.A). A549 cells were transfected with these plasmids followed by transduction with the shRNA-expressing adenoviral vector HC-AdHB04 or HC-Adbgal as a control. Htt exon 1-EGFP expression from vector qp25 was significantly inhibited by HC-AdHB04, while htt exon 1-EGFP expression from qp25 (c1) or qp25 (c2) was unaffected by co-expression of the shRNA directed to htt (Fig 2.B). In the future an Htt construct with this neutral nucleotide exchange, refractory to RNAi inhibition, could be used to prevent the side effect of reduced expression of the endogenous normal Htt, when delivered with anti-htt shRNA at the same time.
Fig. 2. Htt exon1-EGFP construct with neutral nucleotide exchanges in the shRNA target sequences resists inhibition by anti-Htt shRNA. (A) The plasmids qp25(c1) and qp25(c2) were constructed to express two different versions of the Htt exon1-EGFP fusion RNAs, which had been engineered to contain neutral nucleotide exchanges (bold) not effecting the translational code. (B) A549 cells were transfected with qp25 (lane 1 and 2), qp25(c1) (lane 3,4) and qp25(c2) (lane 5,6) following transduction with HC adenoviral vector HC-Ad β gal (lane 1, 3 and 5) as the control and HC-AdHBO4 (lane 2, 4 and 6). After 72 hours fluorescence was determined by flow cytometry. Each value represents the mean from duplicate experiments.

V.5 HC-Ad vector-mediated neuron-specific transgene expression in vitro and in vivo in the mouse striatum by the SYN1-WPRE promoter

High-level, long-term and neuronal cell specific expression of transgenes is a prerequisite to study gene function in neuronal cell cultures in vitro and in central nervous system (CNS) gene therapy. HC-Ad vectors are devoid of all viral coding sequence, have significantly reduced toxicity and immunogenicity of vectors and mediate long-term gene expression in the brain. However, since the coxsackievirus
and adenovirus receptor (CAR) is expressed in many cell types, adenoviral vectors do not target transgene expression to neuronal cells (Volpers and Kochanek, 2004). Efforts have been made to target adenovirus-mediated gene expression to specific cell types, which can be achieved by genetic and chemical modification of the capsid protein or by changing the viral binding properties with the help of bispecific adapters to redirect Ad vectors to certain cell surface receptors (Biermann et al., 2001; Nicklin et al., 2001; Kreppel et al., 2005). Another strategy to achieve neuron-specific transgene expression is to use neuron-specific promoters (Navarro et al., 1999). Compared to other neuron-specific promoters, the human synapsin 1 promoter with the woodchuck hepatitis element (SYN1-WPRE) has been shown to direct high-level and neuron-specific transgene expression in vitro and in vivo (Glover et al., 2002; Kugler et al., 2003). To target transgene expression to neuronal cells with HC-Ad vectors, we generated an HC-Ad vector HC-AdHB01 expressing EGFP under the control of the SYN1-WPRE promoter. Primary cortical cells, which consisted mainly of neuronal cells with a small mixture of glia cells, were transduced with HC-AdHB01 or with FK-7, a control vector expressing EGFP from the standard CMV promoter. Transduction with HC-AdHB01 vectors resulted in EGFP expression exclusively in neuronal cells, as defined by positivity for the neuron-specific marker Beta III tubulin (Paper III, Fig 1B a-c). In contrast, transduction of FK7 resulted in EGFP expression only in glia cells, as defined by positivity for the glia marker GFAP (Paper III, Fig. 1B d-f). We then injected HC-AdHB01 and FK7 into the mouse striatum. Injection of HC-AdHB01 led to EGFP expression in neuronal cells, which were positive for the neuronal marker NeuN, while injection of FK7 resulted in EGFP expression only in glia cells, which were positive for the glia marker GFAP (Paper III, Fig. 2A, B). Neuron-specific EGFP expression from HC-AdHB01 was maintained for at least 2 months (Paper III, Fig. 2C). Therefore, by using SYN1-WPRE promoter, HC-Ad vectors deliver neuron-specific transgene expression in vitro and in vivo. Long-term stable transgene expression can be maintained in neuronal or glial cells by the choice of different promoters delivered with HC adenoviral vectors.

Adenoviral vectors can be retrogradely transported in neuron cells, which means that injection of vectors into one part of brain may also lead to transgene expression in neuron cells projecting to the injection site (Barkats et al., 1998; Berry et al., 2001). This character gives adenoviral vectors the advantage to deliver genes to a special part
of the brain like the substantia nigra pars compacta (SNpC), which is difficult to reach by direct injection. SNpC is mainly composed of the dopaminergic neurons. The degeneration of these neurons causes Parkinson’s disease (PD). Therefore, they are a potential target for gene therapy to PD. Since dopaminergic neurons from SNpC project to the striatum, striatal vector application should not only lead to gene expression in the striatum itself but also in dopaminergic neurons of the substantia nigra. Indeed we detected significant EGFP expression in the substantia nigra after striatal injection with HC-AdHB01 and FK7. Interestingly, although in the striatum HC-AdHB01 and FK7 mediated EGFP expression in different cell types, they all mediated EGFP expression in dopaminergic neurons following their retrograde transport to the SNpC, as defined by positivity for the dopaminergic neuronal marker tyrosine hydroxylase (TH) (Fig. 3).
Fig. 3. HC-AdHB01 and FK7 vectors were retrogradely transported to the SNpC and mediated EGFP expression in dopaminergic neurons. (A) EGFP fluorescence in SNpC 7 days after HC-AdHB01-mediated transduction (left image) or FK7 transduction (right image). (B) Brain sections were stained by an antibody against the dopaminergic neuron marker TH. (a, d): EGFP fluorescence; (b, e): Immunofluorescence stained by an anti-TH antibody from the same slide as a or d; (c, f): Merged fluorescence from a+b or d+e.

EGFP expression in SNpC was maintained for at least 2 months after striatal injection of vectors (Fig. 4).

![Fig. 3. HC-AdHB01 and FK7 vectors were retrogradely transported to the SNpC and mediated EGFP expression in dopaminergic neurons.](image)

Fig. 4. HC-AdHB01 and FK7 vectors mediated stable EGFP expression in SNpC for at least 2 month: EGFP fluorescence in SNpC 2 month after HB01 transduction (c) or FK7 transduction (d).

Together these data show that HC adenoviral vectors may confer long-term and neuron-specific expression in SNpC making them interesting for gene therapy of Parkinson’s disease. The different cell-type specificity of the CMV promoter in the striatum and SNpC also highlighted the complication of the activity of the CMV promoter, which is strongly affected by the vector systems use and by types and activity of neurons (Fritschy et al., 1996; von den Pol and Ghosh, 1998; Wheeler and Cooper, 2001). The CMV promoter, as a strong unspecific promoter, has been used in many studies of gene transfer to the brain. However, our observations of its glia-specificity question its future application for gene transfer into neurons.
V.6 Inducing huntingtin inclusion formation in primary neuronal cell culture and in vivo by HC-Ad vectors expressing truncated and full-length mutant huntingtin

Efficient delivery of the HD gene into neurons could facilitate studying the function of Htt and the pathogenesis of HD, but with traditional transfection reagents gene transfer to postmitotic neurons is inefficient, unreliable, and cytotoxic (Washbourne and McAllister, 2002). Due to their high efficiency of gene transfer to neurons and their low toxicity, viral vectors based on AAV, lentivirus and E1-deleted adenovirus have been developed to deliver N-terminal truncated Htt into cultured neurons and in vivo in rats (Senut et al., 2000; de Almeida, et al., 2002; Regulier et al., 2003; Tagawa et al., 2004; Zala et al., 2005). However, because of the large size of the huntingtin cDNA (more than 10kb) and the relatively small transgene capacity of these vectors, viral vector-mediated gene transfer of full-length htt has not been achieved so far. We took advantage of the high transgene capacity of HC-Ad vectors and generated a series of HC-Ad vectors to express truncated htt (HC-AdHtt15T and Htt128T) or full-length Htt (HC-AdHtt15 and Htt128) either with mutation (HC-AdHtt128T and Htt128) or without mutation (HC-AdHtt15T and Htt15) (Paper III, Fig. 3A). In all these vectors, the SYN1-WPRE promoter was used to achieve neuron-specific transgene expression. Transgene expression from different vectors in neuronal cells was confirmed by Western blot analysis (Paper III, Fig. 3B).

Htt inclusions are a major pathological hallmark of HD and have been used as a surrogate marker in screens for anti-HD drugs. To investigate whether HC-Ad vector mediated expression of mutant Htt modeled inclusion formation in neurons, we transduced primary cortical neuronal cells with these vectors and detected Htt expression with anti-htt antibodies at different time points (6 days and 13 days) after gene transfer. Expression of wild type truncated or full-length Htt did not lead to inclusion formation (Paper III, Fig. 4A a, c). At 6 days after transduction, inclusions had been formed in almost every primary neuronal cell expressing the truncated mutant htt (Paper III, Fig. 4A b). At this early time point, interestingly, there were no aggregations detected in neuronal cells expressing the mutant full-length htt, which was mainly diffusely distributed in the cytoplasm. However, at 13 days after
transduction, aggregates were now detected in neuronal cells expressing mutant full-length htt (Paper III, Fig. 4B b). We also observed a different localization of aggregates formed by the mutant truncated htt and mutant full-length htt: while the mutant truncated htt formed aggregates mainly in the nucleus, the mutant full-length htt formed aggregates predominantly in the cytoplasm and neurites (Paper III, Fig. 4C). We then further injected these different vectors into the mouse striatum. Two months after injection, mice were sacrificed to detect inclusion formation in the brain. Similar to the situation in vitro, injection of HC-AdHtt128T into the striatum resulted in inclusions with predominantly nuclear localization in almost every cells expressing truncated mutant htt, while injection of HC-AdHtt128 led to inclusions with mainly cytoplasmic localization only in a proportion of cells expressing mutant full-length Htt (Paper III, Fig. 7). No inclusions were detected in animals injected with HC-AdHtt15T or HC-AdHtt15, which mediated expression of normal truncated or full-length Htt (Paper III, Fig. 6). Therefore, although expression of mutant truncated or full-length Htt resulted in the formation of inclusions, the dynamics of inclusion formation, staining patterns and localization of inclusions were different between cells that received mutant truncated and full-length Htt. Mutant truncated Htt led to fast formation of inclusions mainly in the nucleus, while mutant full-length caused much slower accumulation of inclusions mainly in the cytoplasm. This observation is consistent with other in vitro and in vivo studies that the number and the localization of intracellular inclusions depend on the size of htt with the expanded polyglutamine (Hackam et al., 1998; Martindale et al., 1998). Interestingly, in post-mortem brains from HD patients, both nuclear and cytoplasmic inclusions were found (DiFiglia et al., 1997; Sapp et al., 1997; GuteKunst et al., 1999; von Roon-Mom et al., 2006). The difference between the localization of htt inclusions in HD patients and our observation are most likely due to the duration of mutant htt expression: In the post-mortem brains from HD patients results represent the chronic changes for over 30-40 years, while vector-mediated gene transfer represents the early changes with mutant htt expression in 12 days. In summary, we generated HC-Ad vectors to specifically deliver truncated and full-length Htt with or without the mutant polyQ expansion into neurons in vitro and in vivo. Gene transfection with these vectors mimicked Htt aggregate formation from mutant Htt. These vectors will be very useful tools to study Htt, particularly full-length Htt function in neuronal cell culture and in vivo including in large animals.
VI. Conclusions

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease caused by the expansion of a CAG trinucleotide repeat in exon 1 of the huntingtin (Htt) gene. Emergence and progression of HD depend on continuous expression of mutant huntingtin protein. We identified one shRNA, which efficiently inhibited huntingtin expression in cell lines and in mice *in vivo*. In addition, the formation of Htt aggregates, a hallmark of HD pathology, was attenuated in mouse models of HD, after stereotaxic injection of a high-capacity adenoviral (HC-Ad) vector expressing this shRNA into the striatum. These results indicate that an shRNA strategy could be developed for treatment of HD.

Vector-mediated delivery of N-terminal fragments of mutant Htt has been used to study the Htt function and establish HD models *in vitro* and *in vivo*. However, due to the large size of Htt cDNA vector-mediated delivery of full-length Htt has not been achieved so far. We generated a series of HC-Ad vectors, which expressed mutant and wild-type versions of N-terminal truncated and full-length Htt either *in vitro* in primary neuronal cells or in the striatum of mice. Kinetics of generation and localization of Htt aggregates differed between mutant truncated and full-length Htt. Expression of mutant truncated Htt caused very fast formation of aggregates in the nucleus but expression of mutant full-length Htt led to a much slower accumulation of aggregates in the cytoplasm. These vectors will be useful tools for studying HD and may be used to generate large animal HD models.
VII. Zusammenfassung


VIII. References


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Adenovirus-Mediated Silencing of Huntingtin Expression by shRNA

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ABSTRACT

Huntington’s disease (HD) is an inherited autosomal dominant, neurodegenerative disease that is caused by a gain of function mutation characterized by the expansion of a CAG trinucleotide repeat in exon 1 of the huntingtin (htt) gene. Since hairpin small interference RNA (shRNA) technology allows inhibition of specific gene expression in vitro and in vivo, vector-mediated expression of an shRNA directed to htt mRNA could form the basis of a new treatment modality for HD. By initial plasmid transfection of 293 cells, we identified one exon 1-targeted shRNA, which efficiently inhibited expression of an htt exon 1-GFP fusion protein and the endogenous htt gene. A replication-deficient adenovirus (Ad) vector Adie-1-1 was constructed to express this shRNA from the U6 promoter. In A549 cells expressing exon 1 of htt with an expanded CAG allele, Adie-1-1 efficiently prevented htt exon 1 expression and htt aggregate formation. In addition, in different neuronal and nonneuronal cell lines, Adie-1-1 efficiently inhibited the expression of endogenous htt. Together, this data indicates the delineation of an shRNA strategy that may become the basis for treatment of HD.

OVERVIEW SUMMARY

In Huntington’s disease, an amplified CAG trinucleotide repeat in exon 1 of the huntingtin gene codes for an expanded N-terminal polyglutamine tract that results in protein aggregation and neurodegeneration. Hairpin small interference RNAs (shRNAs) expressed from gene transfer vector might be an effective strategy to block huntingtin expression. In this study, different adenovirus vectors were constructed to either express huntingtin-GFP fusion proteins or shRNAs directed to huntingtin RNA. Cotransduction experiments in neuronal and nonneuronal cell lines indicated that, upon specific shRNA expression, huntingtin expression could be efficiently blocked and aggregation prevented.

INTRODUCTION

HUNTINGTON’S DISEASE (HD) is an inherited, autosomal dominant, neurodegenerative disease caused by the expansion of an unstable CAG trinucleotide repeat tract in the huntingtin (htt) gene, resulting in an expanded stretch of glutamines in the htt protein. Although the exact molecular mechanisms leading to neurodegeneration are not fully understood, researchers agree that the polyglutamine tract expansion in mutant htt is responsible for the observed pathology. Transgenic mice expressing full-length or truncated htt with an expanded polyglutamine develop a neurologic phenotype that is accompanied by neuropathologic alterations that, in part, reflect changes found in HD patients (Hodgson et al., 1992; Mangiarini et al., 1996; Reddy et al., 1998). Onset and severity of the disease are strongly influenced by the number of CAG repeats in the mutant allele (Ross and Hayden, 1998). In a tetracycline-inducible mouse model of HD, progression of the HD-like pathology was dependent on continuous expression of the mutant htt (Yamamoto et al., 2000). Interruption of expression of the mutant protein not only halted progression of the disease, but also reversed aggregate formation and neuronal degeneration. Thus, blocking expression of the mutant htt might be a promising therapeutic strategy.

Interference RNA has been shown to efficiently inhibit specific gene expression in different species, including Caenorhabditis elegans, Drosophila melanogaster, Trypanosoma brucei, and plants (Hutvagner and Zamore, 2002). More recently, small interference RNAs (siRNAs), which are based on double-strand RNAs of less than 30 nucleotides in length and are expressed
usually as hairpin siRNAs (shRNAs) from polymerase III promoters, have been used to inhibit gene expression in mammalian cells in vitro and in laboratory mice in vivo (Elbashir et al., 2001a,b; Hommel et al., 2003). Replication-deficient recombinant adenovirus (Ad) vectors belong to the most commonly used vectors for in vitro and in vivo gene transfer into cells of the CNS (Barkats et al., 1998; Davidson and Breakefield, 2003; Lowenstein et al., 2003). In this article, we describe the generation of E1-deleted Ad vectors for shRNA-mediated inhibition of htt expression as a tool to study htt function and with the intention to use vector-mediated expression of shRNA as a potential treatment for HD.

**MATERIALS AND METHODS**

**Recombinant plasmids**

Plasmids qp25 and qp103 were kindly provided by David Housman (Center for Cancer Research, MIT, Cambridge, MA) and distributed by the HD Foundation. These plasmids (qp25 and qp103), under transcriptional control by the hCMV promoter, express htt exon 1-EGFP fusion proteins with 25 and 103 polyglutamine repeats, respectively. Plasmid pU6 was designed to express shRNAs from the murine U6 promoter and was constructed as follows: the U6 promoter was isolated from mouse genomic DNA by PCR using primers 5'-cctcaagtttccgccgcatcta-3' and 5'-cgttggatcctggacaccaaaacgcttctcc-3'. A BbsI site (shown in bold print) was included to allow insertion of shRNA-coding sequences with the transcription beginning at the first nucleotide of the regular U6 transcript. This fragment was inserted between the BamHI and HindIII sites of plasmid pBluescript SK (Stratagene, Amsterdam, The Netherlands), resulting in plasmid pU6. Desoxyribonucleotides coding for the different shRNAs were cloned into the BbsI/XbaI sites of pU6. Four plasmids were derived to express the different shRNAs. pU6-ie-1-1 expresses a shRNA (ie-1-1) that is directed to exon 1 of the htt gene from nucleotide (nt) 313 to nt 337. pU6-ie-1-2 expresses a shRNA (ie-1-2) directed to exon 1 of the htt gene from nt 319 to nt 343. pU6-ie-2 expresses a shRNA (ie-2) that is directed to exon 2 of the htt gene from nt 601 to nt 625. pU6-ie-EGFP expresses a shRNA (ieEGFP) that is directed from nt 236 to nt 260 of the EGFP coding sequence. We designed shRNAs with 25 nts double-strand stem (bold print) and 4 nts loop (light print):

ie-1-1, 5' GCCATGGCGACCCCTGGAAAAAGCTGAAAACC-TCAAGTTTCCAGGGTGCCATGGC-3';

ie-1-2, 5' GCCGACCCCTGGAAAAAGCTGATGGAAGGAAGGC-CTTACATCAGCTTTTCCAGGGTGCC-3';

ie-2, 5' GTACCAGAAAGAGACCGTGTAATCTACC-GATTCCACAGGGTCTTTCGTTACG-3';

ie-EGFP, 5' GAAAGCGGCAAGACCTTCATGTTACCTTCAAGTCACCCGGAC-TTGAGAAGACGTGGCTGCT-3';

The plasmids pNeo/U6 and pNeo/U6-ie-1-1 contain the neomycin gene to enable selection in mammalian cells and were constructed by replacing the EGFP expression cassette of EGFP-N1 (Clontech, Palo Alto, CA) with the U6 promoter, resulting in pNeo/U6, or the U6-ie-1-1 expression cassette, resulting in pNeo/U6-ie-1-1.

**Cell lines**

HeLa cells (ATCC CCL-2) and A549 cells (ATCC CCL-185) were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (Gibco, Karlsruhe, Germany). N52E6 cells (Schienerd et al., 2000) were propagated in alpha-MEM supplemented with 10% FCS and penicillin-streptomycin. SH-SY5Y cells (ATCC CRL-2266) were grown in Dulbecco’s modified Eagle’s medium (DMEM) F12 (Gibco), supplemented with 15% FCS, 1-glutamine, and penicillin-streptomycin. For differentiation of SH-SY5Y cells, 5 × 10⁵ cells were seeded in 6 well plates. To induce differentiation, 20 μM retinoic acid (Sigma, Taufkirchen, Germany) was added one day after seeding of the cells.

**Adenovirus vectors**

To generate replication-deficient Ad vectors, the U6/ie-1-1 expression cassettes of pU6-ie-1-1 and the htt exon 1-EGFP expression cassettes of qp25 and qp103, respectively, were inserted into the adenovirus shuttle plasmid pGS66 (Schieneder et al., 2000), resulting in plasmids pGS/U6-ie-1-1, pGSqp25, and pGSqp103. pGS66 is an adenovirus shuttle plasmid for construction of E1-deleted adenovirus vectors. To rescue the viral vectors, 5 μg of pGS/U6-ie-1-1, pGSqp25, and pGSqp103 DNA were cleaved with SwaI, followed by phenol extraction and ethanol precipitation. The DNA was transfected into N52.E6 cells (Schieneder et al., 2000) by the calcium phosphate method, and the cell monolayer was overlaid with 0.5% agarose (FMC Bioproducts, Rockland, Maine) in MEM supplemented with 5% FCS, 0.05% yeast extract (Difco Laboratories, Detroit, MI), and antibiotics. After plaque purification, the vectors were produced in N52.E6 cells, purified by CsCl equilibrium density centrifugation, and desalted by PD-10 columns (Amersham, Freiburg, Germany). The particle and infectious units (iu) titers were determined by the slot blot method (Kreppel et al., 2002), and the integrity of the vector genomes was confirmed by restriction analysis. The resulting Ad vectors were called AdU6ie-1-1, Adqp25, and Adqp103. The generation of AdVB6 has been described previously (Volpers et al., 2003). AdVB6 is an E1-deleted first-generation adenovirus vector expressing lacZ from the hCMV promoter. A physical map of the recombinant adenovirus vectors used in this study is shown in Figure 2A.

**Plasmid transfection experiments**

Cells were transfected using the calcium phosphate method. For cotransfection experiments designed to screen for any inhibitory effect of shRNAs, the plasmid expressing htt exon 1-EGFP was transfected together with the shRNA expression plasmids at a ratio of 1:10. Forty-eight hours later, flow cytometry was performed using a flow cytometer (Becton Dick-
in son, Franklin Lakes, NJ) to quantify the percentage of cells showing EGFP fluorescence.

**Cotransduction experiments with different adenovirus vectors**

1 x 10^6 cells were seeded in 6 well plates one day before transduction. Cells were cotransduced with an Ad vector expressing htt exon 1-EGFP (Adqp25 or Adqp103, respectively) and with a shRNA expressing adenovirus vector (AdU6-ie-1-1) using different multiplicities of infection (MOI) ratios of the two vectors. Forty-eight hours following vector transduction, the percentage of EGFP positive cells was determined by FACS analysis.

**Western blot analysis**

The monoclonal mouse anti-huntingtin protein antibody MAB2166 (Chemicon, Temecula, CA) and the monoclonal anti-alpha-tubulin antibody N356 (Amersham, Freiburg, Germany) were used for detection of huntingtin and alpha-tubulin, respectively. Three or 5 days after transduction with the Ad vector, cells were washed twice with PBS followed by incubation with PBS, 20 mM EDTA for 10 minutes at 37°C. The cells were collected by centrifugation at 1500 rpm for 10 minutes. Thereafter, the cells were lysed in lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100) and centrifuged at 8000 rpm for 10 minutes. The protein concentration in the supernatant was determined by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Ten μg protein samples were incubated in sample buffer at 98°C for 5 minutes, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blotting onto nitrocellulose membranes (Amersham, Freiburg, Germany), the membrane was incubated overnight with blocking buffer (PBS with 5% low-fat milk powder and 0.05% Tween-20). After washing the membrane with PBST wash buffer (PBS and 0.05% Tween-20), the membrane was incubated for 1 hour at room temperature with the anti-htt antibody MAB2166 (1:5000 diluted in PBST), washed three times with PBST and then incubated for 1 hour at room temperature with a peroxidase-coupled rabbit anti-mouse Ig antibody (Sigma, Taufkirchen, Germany). Peroxidase-based detection was performed with ECL Detection Reagent (Pierce) followed by autoradiography. Signal intensities were quantified by scanning densitometry of films using ImageQuant software (Molecular Dynamics, Amersham). The ratio between the intensities of the bands corresponding to htt and to the internal control alpha-tubulin was used to estimate the cellular abundance of htt.

**Northern blot analysis**

Northern blot analysis was performed according to a standard protocol, loading 20 μg per lane of total RNA isolated with TRIzol reagent (Invitrogen, Karlsruhe, Germany) three days following vector transduction of A549 cells. Radiolabeled probes were prepared by hybridizing either to exon 1-EGFP RNA or to GAPDH RNA as an internal control. A PhosphorImager system and the software ImageQuaNT (Molecular Dynamics, Amersham) were used to quantify and compare the radioactive signals on a nylon membrane after electrophoresis. RNA transfer, and hybridization.

**RESULTS**

**Design of shRNAs directed to exon 1 of the htt RNA**

With the future objective being the validation of shRNA inhibitory strategies in the well-characterized R6 HD mouse model, in which exon 1 of the human htt with an expanded polyglutamine tract is expressed, we initially designed two shRNAs that targeted the human htt DNA sequence around the start codon in exon 1 (Fig. 1A). Expression plasmids pU6-ie-1-1 and pU6-ie-1-2 were constructed to express the shRNAs from the murine U6 promoter. 293 cells were cotransfected with the shRNA expression plasmids, together with a second set of plasmids that expressed an htt exon 1-EGFP fusion protein with a polyglutamine tract of normal (qp25) or of expanded (qp103) length. EGFP expression was evaluated by FACS analysis. As can be seen in Figure 1B, transfection of both pU6-ie-1-1 and pU6-ie-1-2 resulted in considerably lower expression of EGFP, with the more pronounced effect apparent with pU6-ie-1-1 (Fig. 1B, lane 4) with only 4% EGFP positive cells compared with 24% EGFP positive cells in the control. A similar result was obtained when plasmid qp103 with an expanded CAG repeat was cotransfected. The inhibitory effect was at least as strong as with an shRNA directed to the EGFP coding sequence (lane 2). As expected, cotransfection of qp25 with pU6-ie-2, an shRNA expression plasmid directed to exon 2 (not present in qp25), did not inhibit EGFP expression (Fig. 1B, lane 3), indicating specificity of the exon 1-directed shRNAs.

To more closely mimic the situation in the cellular environment, we investigated whether the shRNA strategy was able to also inhibit expression from the endogenous htt locus. Human 293 cells known to express htt were transfected with pNeo/U6-ie-1-1 followed by G418 selection for 7 days. Western blot analysis indicated that htt protein expression was significantly reduced (Fig. 1C, D).

**Adenovirus vector-mediated inhibition of htt expression**

In order to develop tools for studying htt function in vitro in cell lines and primary cells that frequently are difficult to transfet, we generated replication-deficient Ad vectors expressing an htt exon 1-EGFP fusion protein with either a normal (Adqp25) or an expanded allele (Adqp103). In addition, we generated an Ad vector (AdU6-ie-1-1) expressing the shRNA that, in plasmid transfection experiments, had demonstrated an inhibitory effect on htt expression. A549 cells were cotransduced with AdU6-ie-1-1 together with either Adqp25 or Adqp103 at different ratios (Fig. 2). At a ratio of 1:1, htt exon 1-EGFP expression was inhibited by more than 60%, as evaluated by FACS analysis (Fig 2B). At a ratio of 5:1 of AdU6-ie-1-1 and Adqp25 or Adqp103, only about 20% EGFP positive cells were detected compared with the control. This result was confirmed by microscopy to detect EGFP fluorescence (Fig. 2C). With 5-fold excess of AdU6-ie-1-1 htt, exon 1-EGFP expression was suppressed almost to background levels. Interestingly, while the cytoplasm of Adqp25 transduced cells was diffusely EGFP-positive (Fig. 2C, arrows) and visible aggregates were completely absent, Adqp103 transduced cells contained a large number of aggregates (Fig. 2C, arrowheads).
Increasing the AdU6ie-1-1 vector dose further did not result in additional reduction of htt exon1-EGFP expression. Control experiments, in which AdU6ie-1-1 was replaced with AdVB6 (an unrelated vector expressing lacZ), indicated that with high vector doses there was an unspecific activation of expression from Adqp25 and Adqp103 (Fig. 2B). The activation of htt exon 1-EGFP expression by high vector doses described above and detected by FACS analysis was confirmed by detecting EGFP fluorescence by microscopy (data not shown) and is probably due to activation of the hCMV promoter by adenoviral functions at high vector doses, as it has been similarly observed in other systems.

Northern blot analysis of cellular RNA following cotransduction experiments demonstrated that in A549 cells there was a decrease in htt exon 1-EGFP RNA levels (Fig. 2D and E), indicating RNA cleavage as the mechanism for the decrease in htt exon 1-EGFP expression. Similarly, endogenous htt RNA levels were significantly decreased following transduction of A549 cells with AdU6-1-1 (data not shown).

Inhibition of aggregate formation

Cellular aggregation of htt is a pathological hallmark of HD in humans and also in the murine model of HD (Davies et al., 1997; Difiglia et al., 1997). The degree of aggregate formation depends on expression levels of mutant htt and the size of the polyglutamine expansion. Since transduction of cells with Adqp103 resulted in a large number of aggregates containing EGFP-positive material (Fig. 3A, lane 1), we analyzed the effect of adenovirus-mediated shRNA expression on aggregate formation. Upon cotransduction of A549 cells at the same time with Adqp103 and AdU6ie-1-1, aggregate formation 72 hours after transduction was almost completely prevented (Fig. 3A, lane 2). In separate experiments (and to analyze whether shRNA technology was suitable to decrease the number of aggregates after they had formed), A549 cells were first transduced with Adqp103. Forty-eight hours later, after aggregates had become visible (Fig. 3A, lanes 3 and 4, and Fig. 3B, top panels), the cells were transduced with AdU6ie-1-1, followed by incubation for an additional 72 hours (Fig. 3A, lane 6, and Fig. 3B, lower right panel). Compared with a control (cotransduction of Adqp103 with AdVB6 [Fig. 3A, lane 5, and Fig. 3B, lower left panel]), there was a significant reduction in the number of aggregates in the presence of shRNA expressed from AdU6ie-1-1.

Adenovirus-mediated downregulation of endogenous htt expression in different cell lines

The potential to inhibit endogenous htt expression was analyzed by transduction of different cell lines with AdU6ie-1-1.
FIG. 2. Adenovirus-mediated inhibition of htt exon 1-EGFP expression. (A) Map of E1-deleted Ad5-based adenovirus vectors with the different expression cassettes. (B) To determine inhibitory effects of Adie-1-1 on htt expression, A549 cells were cotransduced with AdVB6 and Adqp25, AdU6ie-1-1 and Adqp25, or AdU6ie-1-1 and Adqp103 at MOI ratios of 0:1, 1:1, 5:1, 10:1, and 25:1, as indicated. After 48 hours, the percentage of EGFP-positive cells was determined by fluorometry. The results are presented as relative percentage of EGFP-positive cells compared with the control, which had not been transduced with AdU6ie-1-1. Each value represents the mean from duplicate experiments. (C) A549 cells were transduced with 25 MOI of Adqp25 or Adqp103, together with either 125 MOI of AdVB6 (left panel) or 125 MOI of AdU6ie-1-1 (right panel). Seventy-two hours following transduction, photographs were taken using a digital camera and a fluorescence microscope. EGFP-positive cells with diffuse staining of the cytoplasm are indicated by arrows, and htt aggregates are indicated by arrowheads. (D) To investigate the mechanism of inhibition, A549 cells were cotransduced with 25 MOI of Adqp25, together with either 125 MOI of AdVB6 (lane 1) or with 125 MOI of AdU6ie-1-1 (lane 2). RNA levels were evaluated by Northern blot analysis, using an exon 1-EGFP probe or a GAPDH probe as internal control for detection. (E) Quantification of above data, represented as relative intensities between htt exon 1-EGFP and GAPDH RNA expression.
As evaluated by Western blot analysis (Fig. 4A and B), it is apparent that following gene transfer with AdU6-ie-1-1, both in A549 and in HeLa cells, there was very significant reduction of htt protein levels. The strongest effects were observed 5 days after transduction, at a time when there was a more than 20-fold reduction of htt protein levels.

Finally the experiment was repeated with the neuronal cell line SH-SY5Y, a human catecholaminergic neuroblastoma cell line. SH-SY5Y cells were differentiated by retinoic acid. Following transduction with AdU6-ie-1-1, there was strong reduction of htt protein levels in SH-SY5Y cells, similar to the results obtained with the nonneuronal cell lines described above (Fig. 4C and D).

**DISCUSSION**

HD is a chronic neurodegenerative disorder caused by a CAG repeat expansion in exon 1 of the htt gene. The dominant phe-
notype has been directly linked to the polyglutamine tract of increased length in the N-terminal of htt. Insoluble htt aggregates have been observed in mammalian cells in vitro, in transgenic animals, and in brain tissues from patients with HD (Davies et al., 1997; DiFiglia et al., 1997; Scherzinger et al., 1997). Although whether there is a causal relationship between aggregate formation and disease is still debated, most evidence indicates that clearing aggregates can result in reduced cell death (Bates, 2003). Thus, therapeutic intervention to decrease htt aggregate formation might contribute to the treatment of HD.

Previous results from transgenic animal models indicate that prevention of mutant htt expression (Yamamoto et al., 2000) or mutant htt protein aggregation (Tanaka et al., 2004) leads to an improved phenotype, suggesting that there might be different therapeutic strategies to inhibit the formation of aggregates. Antisense approaches have been used to reduce htt expression in cell culture (Boado et al., 2000; Nellenmann et al., 2000; Hague and Isacson, 1997), but with limited success. Since more recently the use of siRNA technology allowed strong and consistent inhibition of specific gene expression, we tested whether this strategy could be adopted to inhibit expression of htt. Since HD is a chronic disorder, it is unlikely that in vivo transfection of siRNAs will be a realistic option. Rather, vector-mediated expression of inhibitory RNAs in the form of shRNA might be suitable to achieve a long-term effect. We designed shRNAs that were directed to exon 1 of htt. The decision not to target the CAG repeat was based on the rationale that it was unlikely to develop a siRNA strategy selectively targeting the expanded CAG repeat in htt without negatively interfering with the expression of other CAG repeat-containing genes.

In initial transfection experiments, we identified one shRNA that strongly inhibited htt expression. Since adenovirus vectors are easy to manipulate, can be produced to high titers, allow transduction of noncycling cells, and have been shown to result in long-term gene expression in the CNS (Thomas et al., 2003), we tested whether shRNA expression from this vector was functional. By different criteria we showed that adenovirus-mediated shRNA strongly inhibited htt expression when expressed from a second vector or from the endogenous allele. As demonstrated by Northern blot analysis, the inhibition of htt protein expression was due to decreased RNA levels. Importantly, the formation of aggregates was strongly inhibited by shRNA, even if their formation had already been initiated. Together, these data indicate that in principle vector-mediated inhibition of htt expression could be developed into a realistic therapeutic option, as has also been recently demonstrated with an AAV-based vector in an animal model of polyglutamine-induced neurodegeneration (SCA1) (Xia et al., 2004). This therapeutic strategy, however, will likely not be based on an E1-deleted Ad vector, but rather on vectors with a higher safety profile, including “gutless” Ad vectors or vectors based on AAV or lentivirus (see, for example, Thomas et al., 2000; Davidson and Breakefield, 2003; Deglon and Hantraye, 2005).

A cautionary note: The presented strategy would, without discrimination, inhibit expression of both the mutant and the normal htt allele. It is possible, and has previously been discussed (Martindale et al., 1998; O’Kusky et al., 1999), that in addition to gain of function effects by the mutant htt, subnormal levels of htt expressed from the normal allele might contribute to the pathology observed in HD. Thus, at the present...
time negative consequences of a strong inhibition of normal hit expression by shRNA technology cannot be excluded. Future experiments will address this question. One of the potential strategies to overcome this hurdle capitalizes on allele-specific differences at the nucleotide level, such as single-nucleotide polymorphisms (Miller et al., 2003).

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High-Capacity Adenoviral Vector-Mediated Reduction of Huntingtin Aggregate Load In Vitro and In Vivo

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ABSTRACT

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease caused by the expansion of a CAG trinucleotide repeat in exon 1 of the huntingtin (htt) gene. Emergence and progression of HD depend on continuous expression of mutant Huntingtin protein (Htt). Therefore, blocking expression of mutant Htt might be a promising therapeutic strategy. We generated a high-capacity adenoviral (HC-Ad) vector expressing a short hairpin RNA (shRNA) targeted to exon 1 of the htt gene. In vitro, this vector efficiently inhibited Htt expression in neuronal and nonneuronal cell lines. In addition, the number of Htt-immunoreactive (IR) aggregates, a hallmark of HD pathology, was significantly reduced after gene transfer with this vector. Importantly, the attenuation of aggregate formation by shRNA was observed in vivo after stereotaxic injection into the striatum of mouse models of HD. The vector was tested in two models: the R6/2 transgenic mouse model and a mouse model based on the local injection of an adenoviral vector expressing a truncated version of mutant Htt. In both models an efficient reduction in mutant Htt aggregate load measured by decreased Htt-IR aggregate formation was observed. Our results support the further development of shRNA for HD therapy.

OVERVIEW SUMMARY

An expanded glutamine tract at the N terminus of huntingtin protein (Htt) results in a chronic neurodegenerative disease characterized by neuronal dysfunction, cell death, and deposition of aggregated Htt fragments. Here, a high-capacity “gutless” adenoviral vector was constructed to express a short hairpin RNA (shRNA) directed to the htt mRNA. Gene transfer experiments performed in vitro and in vivo, including the transgenic R6/2 mouse model, demonstrated efficient blockade of mutant Htt expression and a reduced mutant Htt load.

INTRODUCTION

Huntington’s disease (HD), an autosomal dominant neurodegenerative disorder, is caused by the expansion of a CAG trinucleotide repeat tract in exon 1 of the huntingtin gene (htt), resulting in an extended polyglutamine [poly(Q)] tract in the N-terminal part of the huntingtin protein (Htt). The exact molecular mechanisms leading to neurodegeneration are not yet fully understood. However, there is consensus that the expanded poly(Q) tract in mutant Htt directly or indirectly causes the observed pathology. Transgenic mice or rats expressing full-length or truncated versions of mutant Htt develop a neurological phenotype and neuropathological alterations that resemble alterations observed in HD patients (Mangiarini et al., 1996; Reddy et al., 1998; Hodgson et al., 1999; von Horsten et al., 2003). Onset and severity of the disease correlate with the number of CAG repeats in the mutant allele (Ross and Hayden, 1998). Interruption of expression of the mutant protein in a tetracycline-inducible mouse model of HD not only halted progression of the disease but also reversed aggregate formation and neuronal degeneration (Yamamoto et al., 2000). Therefore, current research includes efforts to block expression of mutant Htt.

Short interfering RNA (siRNA) technology is increasingly used to inhibit gene expression in mammalian cells in vitro and in vivo. From gene transfer vectors they are expressed as short
hairpin RNA (shRNA) (Elbashir et al., 2001a,b; Hommel et al., 2003). Adeno-associated virus (AAV)-mediated shRNA expression and also direct injection of siRNA were shown to inhibit Htt expression both in cell culture and in vivo. In HD mouse models neuropathological abnormalities were attenuated and motor impairments were improved (Harper et al., 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005; Machida et al., 2006).

Various vectors have been used for gene transfer into the CNS (Davidson and Breakfield, 2003; Lowenstein et al., 2003). Recombinant adenoviral vectors are easy to construct, can be produced to high titers, and have a large cloning capacity. They have been used frequently for gene transfer into the brain in different species. Some limitations of E1-deleted adenoviral vectors, namely, their inflammatory potential and immunogenicity, have been addressed with the development of high-capacity (“gutless”) adenoviral (HC-Ad) vectors, in which all viral coding sequences have been deleted (Imperiale and Kochanek, unpublished results), resulting in the HC-Ad shuttle vectors. The cells were subsequently infected with the helper virus AdLC8cluc (Parks et al., 1996) at a multiplicity of infection (MOI) of 5. As described previously (Schiedner et al., 1998), aliquots of crude vector lysate were serially amplified in 293Cre66 cells, purified by CsCl equilibrium density centrifugation, and desalted by passage through PD-10 columns (Amersham/GE Healthcare Life Sciences, Freiburg, Germany). Infectious titers, particle titers, and helper virus contamination were determined by slot-blot analysis as described (Kreppel et al., 2002). The inverse bioactivity of all vector preparations was 10 or better and the helper virus contamination was less than 3% in all cases. The integrity of the vector genomes was confirmed by restriction analysis.

Materials and Methods

Recombinant plasmids and cell lines

pU6-ie-1-1 expresses an shRNA (ie-1-1) that is directed to exon 1 of htt and pU6-iEGFP expresses an shRNA (iEGFP) that is directed to enhanced green fluorescent protein (EGFP) as described (Huang and Kochanek, 2005).

HeLa cells (CCL-2; American Type Culture Collection [ATCC], Manassas, VA) and A549 cells (CCL-185; ATCC) were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin (GIBCO, Karlsruhe, Germany). SH-SY5Y cells (CRL-2266; ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM)-F12 (GIBCO) supplemented with 15% FCS, 1-glutamine, and penicillin–streptomycin.

Adenoviral vectors

HC-Adβgal is an HC-Ad vector with a CMV-lacZ expression cassette (Biermann et al., 2001). HC-AdHB01 is an HC-Ad vector expressing EGFP from the neuron-specific SYN1-WPRE promoter and HC-AdHB07 is an HC-Ad vector expressing, from the same promoter, the N-terminal 171 amino acids of Htt with an expanded poly(Q) tract of 128 glutamines (Huang et al., 2005, unpublished data).

A 1.2-kb DNA fragment (LB) from the human lamin B2 locus (Abdurashidova et al., 2000; Kreppel and Kochanek, 2004) was cloned into the Sviar site of HC-Ad plasmid pSTK129 (S. Kochanek, unpublished results), resulting in the HC-Ad shuttle plasmid pHBO3, which, by increasing the size of the stuffer DNA to 29.5 kb, was designed to generate HC-Ad vectors with small expressing cassettes. The U6-ie-1-1 expression cassette of pU6-ie-1-1 was inserted into pHBO3, resulting in pHBO4. The U6-iEGFP expression cassette of pU6-iEGFP was inserted into pHBO3, resulting in pHBO5.

To generate HC-AdHB04 expressing the shRNA ie-1-1 targeted to htt exon 1 and HC-AdHB05 expressing the shRNA iEGFP targeted to EGFP, pHBO4 and pHBO5 DNAs were linearized by Pme1 digestion and transfected into 293Cre66 cells expressing Cre recombinase. The cells were subsequently infected with the helper virus AdLC8cluc (Parks et al., 1996) at a multiplicity of infection (MOI) of 5. As described previously (Schiedner et al., 1998), aliquots of crude vector lysate were serially amplified in 293Cre66 cells, purified by CsCl equilibrium density centrifugation, and desalted by passage through PD-10 columns (Amersham/GE Healthcare Life Sciences, Freiburg, Germany). Infectious titers, particle titers, and helper virus contamination were determined by slot-blot analysis as described (Kreppel et al., 2002). The inverse bioactivity of all vector preparations was 10 or better and the helper virus contamination was less than 3% in all cases. The integrity of the vector genomes was confirmed by restriction analysis.

Adq25 and Adq103 are E1-deleted Ad vectors expressing htt exon 1–EGFP fusion proteins with 25 and 103 glutamines, respectively, under the control of the cytomegalovirus (CMV) promoter (Huang and Kochanek, 2005). Maps of viral vectors used in this study are depicted in Fig. 1.

Western blot analysis

The monoclonal mouse anti-Htt antibody MAB2166 (Chemicon International, Temecula, CA) was used for detection of Htt. Western blot analyses were performed as described (Huang and Kochanek, 2005). In brief, 3 days after transduction with HC-Ad vectors, cells were lysed and 10-μg protein samples were denatured in sample buffer at 98°C for 5 min, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After blotting to nitrocellulose membranes, membranes were incubated overnight with blocking buffer (phosphate-buffered saline [PBS] with 5% low-fat milk powder and 0.05% Tween 20). Membranes were washed with PBST wash buffer (PBS and 0.05% Tween 20). Membranes were incubated for 1 hr with the anti-Htt antibody MAB2166 (diluted 1:5000 in PBST), followed by 1 hr of incubation with a peroxidase-coupled rabbit anti-mouse IgG antibody (Sigma, Taufkirchen, Germany). Peroxidase-based detection was performed with enhanced chemiluminescence (ECL) detection reagent (Pierce Biotechnology, Rockford, IL) followed by autoradiography.

Sterotoxic injection into mouse striatum

The R6/2 line of transgenic mice [strain name: B6CBA-TgN(HDexon1)62Gpb/J] was introduced by G. Bates (Mangiari et al., 1996) and breeding pairs (ovarian transplant hemizygote × B6CBAF1/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). The line was maintained by backcrossing to CBA × C57BL/6 F1 in the animal facilities of the Medical Hospital of RWTH Aachen (Aachen, Germany). Animal Protection Regulations were applied and adhered to closely. Five-week-old hemizygous transgenic or littermate wild-type mice were used in all experiments as was confirmed by polymerase chain reaction. Mice were housed together in groups of transgenic and nontransgenic littermates.
Viral vector HC-AdHB04 or HC-AdHB05 (1 × 10^7 infectious units [IU] in 1 μl of PBS–10% glycerol), each mixed with HC-AdHB07 (2 × 10^7 IU) and HC-AdHB01 (2 × 10^7 IU), was injected into the left or right striatum of wild-type mice, respectively. Vector HC-AdHB07 is an Ad vector expressing the Htt N terminus (171 amino acids) with a mutant poly(Q) tract of 128 glutamines from the neuron-specific SYN1-WPRE promoter (Huang et al., unpublished data). HC-Adβgal is an Ad vector expressing LacZ from the hCMV promoter. All Ad vectors contain 20 kb of stuffer DNA derived from the human hypoxanthine phosphoribosyltransferase (HPRT) locus (HUMHPRTB [GenBank accession no. M26434], gene map positions 1777 to 21729), a 6.5-kb stuffer DNA fragment from locus HUMDXS455A [GenBank accession no. L31948], map positions 10205 to 16750, the left and right termini of Ad5 (inverted terminal repeats [ITRs]), and the packaging signal from Ad5 (φ). HC-AdHB04 and HC-AdHB05 contain an extra 1.2 kb of stuffer DNA from the human lamin B2 locus (LB) to increase the vector genome size. Adqp25 and Adqp103 are E1-deleted replication-deficient adenoviral vectors expressing, from the hCMV promoter, htt exon 1–EGFP fusion proteins with either a normal (25Q) or a mutant (103Q) polyglutamine tract.

To perform stereotaxic injections, mice were placed into a stereotaxic frame and anesthetized continuously with isoflurane, using a Univentor 400 anesthesia unit (Univentor, Zejtum, Malta). Viral vector was injected into the striatum through a small hole with a 2-μl Hamilton microliter syringe over a period of 5 min; the needle was left in place for a further 2 min and then was slowly withdrawn. Coordinates used were as follows: 1.1 mm forward from the bregma, 2.4 mm lateral from the bregma, and 2.5 mm vertical from the dura. Four weeks after injection the animals were killed, followed by perfusion with 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed, postfixed in 4% PFA for 4 hr, and subsequently cryoprotected in 30% sucrose. Sections (thickness, 20 μm) were cut with a cryostat (Leica Microsystems, Wetzlar, Germany) and slices were stored in PBS in 24-well plates.

**Immunohistochemistry**

Free-floating PFA-fixed sections were washed three times for 15 min before blocking for 2 hr in blocking buffer (3% normal goat serum, 1% Triton, and 1% bovine serum albumin [BSA] in 0.1 M PBS). Sections were then incubated with the primary antibody (monoclonal anti-Htt antibody 2B4 [MAB5492; Chemicon International, Temecula, CA], diluted 1:500 in ABD buffer [1% BSA in 0.1 M PBS containing 1% Triton]) with gentle agitation. After overnight incubation, sections were washed three times for 15 min in PBS, incubated for 2 hr with the secondary antibody (Alexa 594-conjugated goat anti-mouse IgG antibody diluted 1:500 in ABD buffer containing 1% Triton), washed again three times in PBS, and mounted onto slides with fluorescence mounting medium. Images were captured with a Zeiss Axioskope 2 microscope (Carl Zeiss, Oberkochen, Germany).

**RESULTS**

**Generation of HC-Ad vector expressing shRNA directed to huntingtin mRNA**

In a previous study we demonstrated that the shRNA ie-1-1, when expressed from an E1-deleted adenoviral vector, effi-
ciently inhibited Htt expression (Huang and Kochanek, 2005). To generate an HC-Ad “gutless” vector inhibiting Htt expression that, because of decreased toxicity, would allow long-term expression in vivo, we wished to insert the U6-ie-1-1 expression cassette into the adenovirus shuttle pSTK129, which in several studies has formed the vector basis. However, probably because of the small size of the U6-shRNA expression cassette, we were unable to propagate the viral vector without instability of the vector genome. Therefore, a 1.2-kb fragment from the human lamin B2 locus was inserted into pSTK129, resulting in pHB03 with an increased size of the stuffer DNA of 29.5 kb. After insertion of the U6-ie-1-1 expression cassette, the resulting HC-AdHB04 vector was successfully amplified to a high titer without rearrangement. Similarly, the U6-shRNA cassette directed to EGFP was inserted into pHB03 and the resulting vector HC-AdHB05 was produced.

To analyze whether HC-AdHB04 inhibited Htt expression, A549 cells were cotransduced with HC-AdHB04 together with Adqp25 (an E1-deleted adenoviral vector expressing an htt exon 1–EGFP fusion protein without mutation) at a ratio of 5:1. htt exon 1–EGFP expression was suppressed almost to background levels compared with a control 3 days after transduction (Fig. 2A). We also transduced several cell lines to determine whether HC-AdHB04 inhibited endogenous Htt expression. As evaluated by Western blot analysis (Fig. 2B), endogenous Htt expression in A549 cells, HeLa cells, and the neuronal cell line SH-SY5Y was significantly reduced after gene transfer with HC-AdHB04 compared with controls.

**Inhibition of Htt aggregate formation in vitro**

Cellular aggregation of mutant Htt is a major pathological hallmark of HD in humans and also in murine models of HD (Davies et al., 1997; DiFiglia et al., 1997). Because transduction of cells with Adqp103 resulted in a large number of aggregates containing EGFP-positive material (Fig. 3A and Fig. 3C, column 1), thereby confirming previous results (Huang and Kochanek, 2005), we analyzed the effect of HC-Ad shRNA-mediated shRNA expression on the formation of EGFP-tagged Htt aggregates. On cotransduction of A549 cells with Adqp103 and HC-AdHB04, hardly any EGFP-positive aggregates were detectable 72 hr after transduction (Fig. 3A and Fig. 3C, column 2), suggesting that the formation of EGFP-tagged Htt aggregates can be prevented by HC-AdHB04. To explore whether HC-AdHB04 can decrease the number of already formed EGFP-tagged aggregates, A549 cells were first transduced with Adqp103 to induce aggregate formation. Forty-eight hours later, when EGFP-positive aggregates had started to form (Fig. 3B

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**FIG. 2.** HC-Ad-mediated inhibition of Htt expression by an shRNA directed to exon 1 of the htt RNA. (A) To determine any inhibitory effects of HC-AdHB04 on Htt expression, A549 cells were transduced at an MOI of 25 of Adqp25 (a and b), together with either an MOI of 125 of HC-AdβGal (a) or an MOI of 125 of HC-AdHB04 (b). Seventy-two hours after transduction, the cells were analyzed by fluorescence microscopy. EGFP-positive cells with diffuse staining of the cytoplasm are indicated by arrows. (B) HC-AdHB04-mediated inhibition of endogenous Htt expression in various cell lines. A549 cells, HeLa cells, and SH-SY5Y cells were transduced with an MOI of 50 of HC-AdHB04 or HC-AdHB05, the latter being a control vector expressing an shRNA directed to the EGFP RNA. Five days after transduction the cells were harvested and extracts were subjected to Western blot analysis, using antibody MAB2166 for detection of Htt and the anti-α-tubulin antibody N356 as an internal control. Lanes 1, untransduced cell controls; lanes 2, cells transduced with HC-AdHB04; lanes 3, cells transduced with HC-AdHB05 as a control.
[parts c and d] and Fig. 3C, columns 3 and 4), the cells were transduced with HC-AdHB04, followed by incubation for an additional 72 hr after gene transfer. Cells with diffuse staining of the cytoplasm are indicated by arrows and Htt aggregates are indicated by arrowheads. (B) A549 cells were transduced with Adqp103. Forty-eight hours later, when aggregates had formed (e and d), the cells were either transduced with HC-Adβgal (e) or transduced with HC-AdHB04 (f). Another 72 hr later photographs were taken. (C) Quantification of aggregates from A (columns 1 and 2) and from B (columns 3–6). The number of EGFP-positive aggregates was determined by fluorescence microscopy by taking the mean of aggregates from 10 randomly selected fields per dish. Each value represents the mean from duplicate experiments.

**FIG. 3.** shRNA-mediated inhibition of Htt–EGFP aggregate formation in A549 cells. (A) A549 cells were transduced with an MOI of 25 of Adqp103 together with either an MOI of 125 of HC-Adβgal as a control (a) or with an MOI of 125 of HC-AdHB04 (b). Photographs were taken 72 hr after gene transfer. Cells with diffuse staining of the cytoplasm are indicated by arrows and Htt aggregates are indicated by arrowheads. (B) A549 cells were transduced with Adqp103. Forty-eight hours later, when aggregates had formed (c and d), the cells were either transduced with HC-Adβgal (e) or transduced with HC-AdHB04 (f). Another 72 hr later photographs were taken. (C) Quantification of aggregates from A (columns 1 and 2) and from B (columns 3–6). The number of EGFP-positive aggregates was determined by fluorescence microscopy by taking the mean of aggregates from 10 randomly selected fields per dish. Each value represents the mean from duplicate experiments.

**Reduction of Htt aggregates in vivo**

In HD patients Htt-IR aggregates are formed mainly in neuronal cells of the brain (DiFiglia et al., 1997). We have injected HC-AdHB07 expressing from the SYN1-WPRE promoter the N-terminal 171 amino acids of Htt, including an expanded poly(Q) tract of 128 glutamines, into mouse striatum and observed the emergence of Htt aggregates in neuronal cells in the vicinity of the injection site (Huang et al., unpublished data). To explore whether HC-AdHB04 could interfere with the formation of EGFP-tagged Htt aggregates in vivo, we coinjected viral vectors HC-AdHB07 and HC-AdHB01, the latter expressing EGFP under the control of the SYN1-WPRE promoter, as internal control (Huang et al., unpublished data), either together with HC-AdHB04 (expressing anti-htt shRNA) into one side of the striatum or with HC-AdHB05 (expressing anti-EGFP shRNA) into the other side of the striatum. Note that adenoviral vectors transduce both neuronal and glial cells and that use of the RNA polymerase III (polIII) U6 promoter ensures shRNA expression in both cell types. Four weeks after injection of HC-AdHB04 into the striatum no Htt aggregates were observed (Fig. 4b), whereas control animals injected with HC-AdHB05 displayed numerous Htt aggregates (Fig. 4a) but no EGFP expression (Fig. 4c), indicating the specificity of the shRNAs used. The results suggested that the HC-AdHB04 vector efficiently reduced mutant Htt load and therefore attenuated the neuronal formation of Htt aggregates in vivo.
In further experiments the potential of the HC-AdHB04 vector was tested in the R6/2 HD mouse model. The R6/2 model is a well-characterized transgenic mouse model of HD that is used extensively to evaluate therapeutic strategies for HD (Li et al., 2005). R6/2 transgenic mice express exon 1 of the human HD gene with a large expanded CAG repeat, which in humans invariably produces the juvenile-onset HD phenotype. Aggregates begin to be detectable in neuronal cells of about 4-week-old animals and are the most striking neuropathological alterations observed in this model (Morton et al., 2000; Meade et al., 2002). We injected HC-AdHB04 vector into the striatum of 5-week-old R6/2 mice to investigate whether HC-AdHB04 could interfere with Htt aggregate formation in vivo. Injected HC-AdHB04 vector was mixed with HC-AdHB01 expressing EGFP under neuron-specific promoter control at a ratio of 5:1 so that the area of HC-AdHB04 transduction was marked by EGFP expression. Four weeks after injection, neurons at the injection sites (delineated by EGFP fluorescence) displayed significantly less Htt-IR aggregates compared with the control (cells transduced with HC-Adβgal) (Fig. 5A). EGFP-positive neuronal cells in the striata of R6/2 mice were analyzed with respect to the presence or absence of Htt-IR aggregates. Less than 10% of the EGFP-positive cells contained aggregates in the part of brain injected with HC-AdHB04 compared with more than 80% EGFP-positive cells in control areas that received HC-Adβgal instead of HC-AdHB04 (Fig. 5B). Together these data demonstrated that HC-AdHB04 efficiently reduced the number of Htt-IR aggregates forming in striatal neurons of the R6/2 mouse model.

**DISCUSSION**

HD is caused by a CAG repeat expansion mutation located in exon 1 of the *htt* gene encoding an expanded poly(Q) tract close to the N terminus of Htt. During the course of the disease, preferentially neuronal Htt-IR aggregates form (Davies et al., 1997; DiFiglia et al., 1997; Scherzinger et al., 1997). The precise role of aggregates within the cascade of pathophysiological events in HD continues to be a matter of debate. It is still unclear whether Htt aggregates represent a protective cellular mechanism by sequestering toxic soluble Htt species (Arrasate et al., 2004) or whether Htt aggregates themselves cause detrimental effects (Bates, 2003). However, there is consensus that the formation of aggregates detectable by light microscopy reflects an increased burden of mutant Htt fragments in excess of the degrading capacity of the respective cell in which they gradually develop (Yamamoto et al., 2000). Encouraged by observations in our earlier study, in which an shRNA expressed from a first-generation E1-deleted adenoviral vector and directed to exon 1 of the *htt* gene prevented aggregate formation in vitro (Huang and Kochanek, 2005), we explored the efficacy

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**FIG. 4.** shRNA-mediated inhibition of HC-Ad vector induced Htt aggregate formation in neurons of mouse striatum. HC-AdHB04 or HC-AdHB05 (1 × 10⁴ IU) were each mixed with 2 × 10⁶ IU of HC-AdHB07 and 2 × 10⁶ IU of HC-AdHB01, followed by injection into the left or right striatum of 5-week-old mice. Four weeks after injection, brain sections were analyzed with Htt antibody 2B4 to analyze aggregate formation. (a) Left striatum injected with HC-AdHB05 (control) and detection of Htt with the 2B4 antibody. (b) Right striatum injected with HC-AdHB04 and detection of Htt with the 2B4 antibody. (c and d) Merged pictures of aggregates (red) and EGFP (green) from slides (a) and (b).
of the shRNA construct further by engineering the construct into a safer vector type that would allow testing in vivo. Previously, HC-Ad vector-mediated LacZ expression in the brain of immunocompetent rats has resulted in stable gene expression without inflammation (Thomas et al., 2000). The results of this study indicated that an HC-Ad vector expressing an anti-htt shRNA from the polIII U6 promoter not only effectively prevented aggregate formation in vitro but also in vivo, suggesting a reduction in mutant Htt load. In cellular model systems we could demonstrate that cells subjected to gene transfer with HC-AdHB04 displayed no or a markedly reduced number of Htt aggregates even after aggregates had started to form at the time of gene transfer. Comparable results were obtained in vivo, using two distinct HD mouse models, one generated by vector-mediated local expression of a mutant Htt fragment (Huang et al., unpublished data), the other representing a widely used transgenic model of HD with panneuronal expression of an N-terminal fragment of mutant Htt under the control of the human htt promoter, that is, R6/2 mice (Mangiarini et al., 1996). In the latter model, the formation of aggregates was strongly decreased locally at the injection site.

Several groups have successfully used AAV-mediated expression of shRNA or direct siRNA injection for inhibition of Htt expression in vitro and in vivo (Harper et al., 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005; Machida et al., 2006). Harper et al. (2005) reported that AAV-mediated shRNA
improved motor and neuropathological abnormalities in the N171-82Q HD mouse model. Promising data with an shRNA strategy have also been observed in a model for SCA1, another model of a dominant polyglutamine disorder (Xia et al., 2004). Together, our data and the above-mentioned published experiences with AAV vector systems support the notion that shRNA-based strategies have significant potential for HD gene therapy. Although not addressed in this paper, potential side effects of shRNAs in both target (neuronal) cells and nontarget (glial cells) due to off-target effects of shRNAs or to oversaturation of cellular micro-RNA pathways (Grimm et al., 2006) must be carefully evaluated. Another significant hurdle for the transfer of this strategy from preclinical studies to the clinical situation is the relatively poor distribution of vector particles on direct injection into the brain. Although our data indicated excellent inhibition of htt aggregate formation in neuronal cells at the site of injection, vector distribution did not go beyond a few millimeters surrounding the needle track, so that behavioral testing was not thought to be reasonable. Our current efforts therefore include attempts to improve distribution and targeting of Ad vectors to neuronal cells after intracranial injection.

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Inducing huntingtin inclusion formation in primary neuronal cell culture and in vivo by high-capacity adenoviral vectors expressing truncated and full-length huntingtin with polyglutamine expansion

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Keywords Huntington's disease; huntingtin; polyglutamine; adenovirus; gene transfer

Abstract

Background Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disease caused by the expansion of a CAG trinucleotide repeat in exon 1 of the huntingtin (htt) gene. Vector-mediated delivery of N-terminal fragments of mutant htt has been used to study htt function in vitro and to establish HD models in rats. Due to the large size of the htt cDNA vector-mediated delivery of full-length htt has not been achieved so far.

Methods High-capacity adenoviral (HC-Ad) vectors were generated expressing mutant and wild-type versions of N-terminal truncated and full-length htt either in vitro in primary neuronal cells or in the striatum of mice.

Results In vitro these vectors were used for transduction of primary neuronal cells isolated from E17 mouse embryos. Expression of mutant htt resulted in the formation of htt inclusions, a surrogate marker of the HD pathology. Kinetics of generation and localization of htt inclusions differed between truncated and full-length htt carrying identical mutations. Following injection into the striatum vector-mediated delivery of mutant truncated htt led to prominent accumulation of htt inclusions in cell nuclei, while inclusions formed upon expression of mutant full-length htt localized to the cytoplasm.

Conclusions These results indicate that HC-Ad vector-mediated in vitro and in vivo delivery of truncated and full-length mutant htt results in prominent inclusion formation in neuronal cells but in different cell compartments. These vectors will be useful tools for studying HD and may be used to generate large animal HD models. Copyright © 2007 John Wiley & Sons, Ltd.

Introduction

Huntington's disease (HD) is an autosomal dominant disease that is caused by the expansion of a CAG trinucleotide repeat in exon 1 of
the huntingtin (htt) gene [1]. The classic clinical features of HD include cognitive impairment, psychiatric disturbances and motor disability irreversibly progressing to death 10–20 years after the onset of the symptoms [2]. Although htt is quite ubiquitously expressed in neuronal and non-neuronal tissues the most prominent abnormalities are found in the brain, in particular in the striatum and the cortex. These areas are characterized by neurodegeneration and atrophy, and, as a morphological hallmark of HD, by the presence of nuclear and cytoplasmic inclusions in neuronal cells [3–6]. Huntingtin is a very large protein of 350 kDa [7], and is believed to be a multifunctional protein, which is not surprising considering its large size. Many studies have focused on the N-terminus of htt harboring a glutamine expansion in the case of mutation, since toxic N-terminal fragments of mutant htt have been shown to play a crucial role in the pathogenesis of HD [8–10]. However, it is also apparent that toxicity is significantly influenced by other parts of the protein [11–14].

To gain insight into the molecular function of htt, understand the pathogenesis, and test potential therapeutic strategies, cDNAs coding for full-length htt or fragments thereof have been expressed under the control of endogenous or heterologous promoters and have formed the basis for the establishment of in vitro models including transgenic mice, flies and C. elegans [15]. In addition, viral vectors based on AAV, lentivirus and E1-deleted adenovirus have been used to deliver N-terminal fragments of mutant htt into cultured cells and also in vivo in rats [16–20]. Vector-mediated expression complements research performed in transgenic animal models since localized expression of htt is possible and high levels of regulated htt expression can be achieved. In addition, the parallel generation and testing of a large number of different htt expression constructs is possible in a relatively short period of time. A further advantage may lie in the possibility to use viral vectors for htt delivery in large animal models such as non-human primates. Due to the large size of the htt cDNA, however, viral vector-mediated gene transfer of full-length htt has not been achieved so far.

Here we describe the generation of a set of high-capacity adenoviral (HC-Ad) vectors that were designed to deliver and express truncated and full-length versions of the huntingtin (htt) gene with 15 or 128 glutamines, excised from pCI10366-15 or pCI10366-128 (a kind gift from Dr. M. Hayden, Centre for Molecular Medicine and Therapeutics, University of British Columbia, CA, USA) and were inserted into the Xhol site of pBSK/s-2 to generate pBSK/S-httN171-15Q and pBSK/S-httN171-Q128; similarly, cDNAs coding for full-length huntingtin with 15 or 128 glutamines, excised from pCI10366-15 or pCI10366-128, were inserted into the Xhol/EcoRV sites of pBSK/S-2, resulting in pBSK/S-httQ15 or pBSK/S-httQ128.

Finally, HC-Ad plasmids were generated as follows: the expression cassettes from pBSK/S-EGFP, pBSK/S-httN171-15Q and pBSK/S-httN171-Q128 were cloned as NotI fragments into the NotI site of the HC-Ad shuttle plasmid pSTK129 (S. Kochanek, unpublished observation), resulting in pHB01, pHBHtt15T and pHBHtt128T (Note: names of plasmids or adenoviral vectors expressing truncated versions of huntingtin end with a ‘T’); the expression cassettes from pBSK/S-httQ15 and pBSK/S-httQ128 were cloned into the NotI site of the HC-Ad shuttle plasmid pSTK119 (S. Kochanek, unpublished observation), resulting in pHBHtt15 or pHBHtt128.

pFK7 was kindly supplied by Dr. Kreppel [21]. It is an HC-Ad plasmid carrying an hCMV-EGFP expression cassette.

For production of the HC-Ad vectors HC-AdHB01, HC-AdFK7, HC-AdHtt15T, HC-AdHtt128T, HC-AdHtt15 and HC-AdHtt128, the different plasmids pHB01, pHBFK7, pHBHtt15T, pHBHtt128T, pHBHtt15 and pHBHtt128 were cleaved with Pmel followed by transfection into 293Cre66 cells. The cells were subsequently infected with the helper virus AdLC8luc [22]. Production and purification followed published protocols [23]. Infectious and particle titers and contamination with helper virus were determined by the slot blot method [24]. The inverse bioactivity of all vector preparations was 10 or better and the helper virus contamination was below 1.5% in all cases. The integrity of vector genomes was confirmed by

Materials and methods

Construction and production of HC-Ad vectors

HC-Ad vectors were designed to express different transgenes from the SYN1-WPRE promoter. Their construction was started with generation of plasmid pBSK/s-1 by inserting a NotI-MluI-EcoRI-NotI multiple cloning site into the Sacl and Kpnl sites of pBluescript SK (pBSK, Stratagene, Amsterdam, The Netherlands). Into the resulting plasmid three additional elements were inserted: the bovine growth hormone polyadenylation signal derived from PIRESnueo2 (Clontech, Palo Alto, CA, USA) was inserted into the EcoRV site; a MluI/EcoRI fragment of pXCSynl-WPRE (kindly provided by Dr. James B. Uney, University of Bristol, Bristol, UK) containing the SYN1 promoter was cloned into the MluI and EcoRI sites; and an EcoRI fragment of pXCSynl-WPRE containing the WPRE element was inserted into the EcoRI site. The resulting plasmid with the SYN1-WPRE promoter, multiple-cloning site and polyA signal was called pBSK/s-2.

Into pBSK/s-2 different transgenes were inserted: the EGFP cDNA from pEGFP-N1 (Clontech) was inserted into the Kpnl/EcoRV site to create pBSK/s-EGFP; cDNAs coding for the N-terminal 171 amino acids of huntingtin with 15 or 128 glutamines were excised from pCI10366-15 or pCI10366-128 (a kind gift from Dr. M. Hayden, Centre for Molecular Medicine and Therapeutics, University of British Columbia, CA, USA) and were inserted into the Xhol site of pBSK/s-2 to generate pBSK/S-httN171-15Q and pBSK/S-httN171-Q128; similarly, cDNAs coding for full-length huntingtin with 15 or 128 glutamines, excised from pCI10366-15 or pCI10366-128, were inserted into the Xhol/EcoRV sites of pBSK/S-2, resulting in pBSK/S-httQ15 or pBSK/S-httQ128.

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Figure 1. HC-Ad vector-mediated gene transfer in murine primary cortex cells isolated from E17 embryos. (A) Maps of the HC-Ad vectors used in this experiment: both vectors express EGFP, HC-AdHB01 from the SYN-1-WPRE promoter and FK7 from the hCMV promoter. Both vectors have identical stuffer DNA backbones derived from the human human HPRT and the C346 locus. The inverted terminal repeats (ITRs) and the packaging signal (ψ) are indicated. (B) Seven days after plating primary cortical neuronal cells were transduced with a MOI of 50 of HC-AdHB01 (a–c) or FK7 (d–f). Five days after gene transfer, cells were reacted with an antibody detecting the neuronal cell marker beta III tubulin. (a, d) EGFP fluorescence; (b, e) immunofluorescence using an antibody detecting beta III tubulin; (c, f) merged fluorescence from a/b and d/e.

Primary neuronal cell culture

Cerebral cortices or striata isolated from BALB/C mice at embryonic (E) day 17 were dissociated by 0.1% trypsin (Gibco, Karlsruhe, Germany) digestion and trituration with a fire-polished Pasteur pipette. Cells were seeded at a concentration of 2.5 × 10^5 cells/well into polyornithine (Sigma, Taufkirchen, Germany) coated 24-well plates or polyornithine-coated glass coverslips. The cells were grown in Neurobasal medium (Gibco) supplemented with B27 (Gibco), 300 µM glutamine, 25 µM β-mercaptoethanol and penicillin-streptomycin. Every 3 days 50% of the medium was exchanged.

Antibodies

Monoclonal anti-beta III tubulin and anti-NeuN antibodies (Chemicon, Temecula, CA, USA) were used to detect β-tubulin and NeuN, respectively. A monoclonal anti-GFAP antibody (Sigma, St. Louis, MO, USA) was used to detect glial fibrillary acid protein (GFAP). Polyclonal anti-microtubule-associated protein 2 (MAP2, Chemicon) and anti-ubiquitin (Dako, Hamburger, Germany) antibodies were used to detect MAP2 and ubiquitin, respectively. The monoclonal MAB2166 (Chemicon) recognizes an epitope between amino acids 181 and 810 of huntingtin and was used for the detection of full-length htt. The monoclonal antibody 2B4 (MAB5492, Chemicon) recognizes the N-terminal 82 amino acids of htt and was used to detect the N-terminally truncated htt.

Western blot analysis

Western blot analysis was performed as described [25]. In brief, 2 days after transduction with HC-Ad vectors, cells were lysed and 10 µg protein samples were denatured in sample buffer at 98°C for 5 min, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After blotting onto nitrocellulose membranes, the membranes were incubated overnight with blocking buffer (phosphate-buffered saline (PBS) with 5% low-fat milk powder and 0.05% Tween-20). Membranes were washed with PBST wash buffer (PBS and 0.05% Tween-20). Thereafter, the membranes were incubated for 1 h with the anti-htt antibodies MAB2166 (1 : 5000 diluted in PBST) or MAB5492 (1 : 2000 diluted in PBST), followed by an 1-h incubation with a peroxidase-coupled rabbit anti-mouse Ig antibody (Sigma, Taufkirchen, Germany).
Peroxidase-based detection was performed with ECL detection reagent (Pierce, Rockford, IL, USA) followed by autoradiography.

Immunocytochemistry

EGFP fluorescence and immunofluorescence were detected with a Zeiss Axioskope 2 microscope (Zeiss, Göttingen, Germany). Seven days after plating primary cortical or striatal neuronal cells were transduced with HC-Ad vectors at a multiplicity of infection (MOI) of 50. Five days after gene transfer, the primary cultures were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and blocked with blocking buffer (PBS, 2% bovine serum albumin (BSA), 5% fetal bovine serum (FBS)). The cells were incubated with the primary antibody for 1 h, washed with PBS, incubated with the secondary antibody (goat anti-mouse IgG Alexa546 conjugated antibody, 1:500 or goat anti-rabbit IgG Alexa488 conjugated antibody, 1:500) for 1 h, washed with PBS and mounted with fluorescence mounting medium. Images were taken with the CCD camera of the IF microscope and pictures were merged with Image J software.

Stereotaxic injection into the mouse striatum

C57BL/6J (B6) mice (5 weeks old) were used for in vivo experiments. The animals were anaesthetized with isoflurane by a Univentor 400 anaesthia unit (Univentor, Malta) and were placed in a stereotaxic frame. Vectors were injected into the left and right striatum through a small hole with a 2 µl Hamilton microliter syringe. A volume of 2 µl containing a total of 2 × 10⁷ infectious units of vector was injected over a period of 5 min; the needle was left in place for another 2 min before it was slowly withdrawn. Coordinates used were 1.1 mm forward from bregma, 2.4 mm lateral from bregma and 2.5 mm vertical from dura. Animals were sacrificed 7 days or 2 months after injection, followed by perfusion with 4% PFA in PBS. The brains were removed, post-fixed in 4% PFA for 4 h and subsequently cryoprotected in 30% sucrose. Sections of 20 µm were cut with a cryostat (Leica, Wetzlar, Germany) and slices were stored in PBS in 24-well plates. All animal experiments were performed following animal protection regulations.

Immunohistochemistry

Free-floating, PFA-fixed sections were washed three times for 15 min before blocking for 2 h in blocking buffer (3% goat serum, 1% Triton and 1% BSA in PBS). Sections were transferred directly into the solution containing the primary antibody diluted in ABD buffer (1% BSA in PBS) with 1% Triton. After overnight incubation with gentle agitation, sections were washed three times for 15 min in PBS, incubated for 2 h with the secondary antibody (goat anti-mouse IgG Alexa594 conjugated antibody 1:500 diluted in ABD buffer with 1% triton), washed again three times in PBS and mounted onto slides with fluorescence mounting medium. Images were captured with a Zeiss Axioskope 2 microscope.

Results

HC-Ad vector-mediated neuronal cell-specific transgene expression in vitro and in vivo in the striatum of mice

Huntingtin has been shown to be ubiquitously expressed. However, the clinical phenotype is believed to be mainly caused by specific degeneration of neuronal cell types in the striatum and, likely, also in other areas of the central nervous system (CNS). As a first step in the generation of HC-Ad vectors expressing full-length htt in neuronal cells we initially compared expression of an EGFP reporter in primary neuronal cells and in cell lines using either the hCMV promoter (FK7) or the SYN1-WPRE promoter (HC-AdHB01) to control transgene expression (Figure 1A). In the context of E1-deleted Ad vectors the SYN1-WPRE promoter has been shown to be a neuron-specific promoter [26,27]. In the non-neuronal cell lines HeLa and A549 HC-AdHB01-mediated gene transfer did not result in detectable gene expression, while transduction with FK7 resulted in very strong EGFP expression (data not shown). Next, primary cortical cells isolated from E17 mouse embryo consisting mainly of neuronal cells with a smaller mixture of glial cells (below 5%) were transduced either with HC-AdHB01 or FK7. As shown in Figure 1B, gene transfer with HC-AdHB01 resulted in exclusive expression in neuronal cells, as defined by morphological criteria and labeling with the neuron-specific markers beta III tubulin (Figure 1B a–c) and NeuN (data not shown). In contrast, gene transfer with FK7 resulted in gene expression only in cells that both morphologically and by recognition with an anti-GFAP antibody (data not shown) corresponded to glial cells (Figure 1B d–f).

Cell-type-specific gene expression was confirmed in vivo following vector injection into the striatum and analysis 7 days later. Injection of HC-AdHB01 led to EGFP expression almost exclusively in cells that were NeuN-positive (Figure 2A a–c), while injection of FK7 resulted in EGFP expression almost exclusively in non-neuronal cells (Figure 2A d–f). Morphological criteria confirmed that EGFP was expressed in neuronal cells following injection of HC-AdHB01 while gene transfer with FK7 resulted in EGFP expression in glial cells, as was confirmed with an anti-GFAP antibody (Figure 2B). Cell-type specificity and duration of transgene expression were maintained for at least 2 months, as shown by additional in vivo injection experiments (Figure 2C). Together, this
Figure 2. Cell-specific EGFP expression in the striatum following stereotaxic injection of HC-AdHB01. Mice were analyzed 7 days (A, B) or 2 months (C) after vector injection for EGFP fluorescence and using a neuronal cell-specific NeuN antibody and an astrocyte-specific GFAP antibody. (A) Injection of HC-AdHB01 or FK7. (a, d) EGFP fluorescence; (b, e) NeuN antibody; (c, f) merged data from a and b or d and e. (B) Injection of FK7. (a) EGFP fluorescence; (b) GFAP antibody; (c) merged data from a and b. (C) EGFP fluorescence in the striatum 2 months after injection with HC-AdHB01 (a) and FK7 (b).

Data demonstrates neuron-specific transgene expression from an HC-Ad vector in vitro and in vivo through the use of the SYN1-WPRE promoter for transcriptional control.

**Generation of HC-Ad vectors expressing full-length htt or truncated huntingtin with or without mutation**

Different HC-Ad vectors were constructed to express truncated htt (HC-AdHtt15T and Htt128T) or full-length htt (HC-AdHtt15 and Htt128) either with mutation (HC-AdHtt128T and Htt128) or without mutation (HC-AdHtt15T and Htt15) (Figure 3A). In all vectors the SYN1-WPRE promoter was used to assure neuron-specific transgene expression. Western blot analysis was performed to analyze transgene expression from the different vectors in primary neuronal cells (Figure 3B). Htt-specific signals were detected, as expected, as bands of small (HC-AdHtt15T and Htt128T) or very large (HC-AdHtt15 and Htt128) molecular weight.

**Inclusion formation in primary neuronal cells following HC-Ad vector-mediated expression of mutant but not wild-type alleles of truncated and full-length huntingtin**

Cellular inclusions of htt are a pathological hallmark of HD and are observed not only in patients, but also in murine models of HD. Htt inclusions are also used as a surrogate marker in screens for the identification of drugs to treat HD. We investigated whether expression of mutant htt resulted in inclusion formation in neuronal cells. Primary striatal neuronal cells were transduced with the different vectors and...
Figure 3. HC-Ad vector-mediated neuronal cell-specific expression of truncated and full-length huntingtin with or without an expanded polyglutamine track. (A) Maps of adenoviral vectors. HC-AdHtt15T and HC-AdHtt128T express the N-terminal 171 amino acids of htt with 15 or 128 glutamines, respectively. HC-AdHtt15 and HC-AdHtt128 express full-length htt with 15 or 128 glutamines, respectively. (B) Primary cortical neuronal cells isolated from embryos were transduced with a MOI of 50 of the different vectors. Two days after gene transfer, the cells were harvested and cell extracts were subjected to Western blot analysis using the anti-htt antibody 2B4 (lanes 1, 2 and 3) or MAB2166 (lanes 4, 5 and 6). Lanes 1 and 4: untransduced cells as controls.

Figure 4. Inclusion formation in primary neuronal cells following HC-Ad vector-mediated expression of truncated and full-length htt with mutation but not without mutation. (A) Primary neuronal cells were transduced with HC-AdHtt15T (a), HC-AdHtt128T (b), HC-AdHtt15 (c), or HC-AdHtt128 (d). Six days after transduction, htt expression was detected with an anti-htt antibody. At this time point inclusions indicated by arrowheads were only detected in cells expressing truncated htt with an expanded polyglutamine (b). (B) Htt expression 13 days following gene transfer with HC-AdHtt15 (a) and HC-AdHtt128 (b). Inclusions indicated by arrowheads were detected only in cells expressing mutant htt (b). (C) Differences in localization of htt inclusions following expression of mutant truncated htt (a) and mutant full-length htt (b). Co-staining cells with the anti-MAP2 antibody (green fluorescence to stain neuronal cells), the anti-htt antibody (red fluorescence) and DAPI (blue fluorescence to stain the nucleus). Inclusions formed by the mutant truncated htt (a) are localized mainly in the nucleus. Inclusions formed by the mutant full-length htt (b) are mainly localized in the cytoplasm including in neurites. Inclusions are indicated by arrowheads.
were not detected in neuronal cells expressing the wild-type alleles of truncated or full-length htt (Figure 4A, a, c). At this early time point, interestingly, expression of mutant full-length htt with HC-AdHtt128 (Figure 4A d) did not lead to inclusions although strong staining of htt with a diffuse cytoplasmic distribution was detected. However, at 13 days after transduction, inclusions were now also detected in neuronal cells expressing mutant full-length htt (Figure 4B b) but not in cells expressing full-length htt without mutation (Figure 4B a). In cells with inclusions, immunostaining revealed a decrease in diffusely distributed cytoplasmic htt. Another observation relates to differences in inclusion morphology of mutant versions of truncated or full-length htt: while expression of mutant truncated htt resulted in inclusions in the nucleus, expression of mutant full-length htt led to the formation of inclusions localized predominantly in the cytoplasm of the cell body and in neurites (Figure 4C). Staining of the primary neuronal cells expressing normal or mutant full-length htt with the 2B4 antibody resulted in the same staining pattern (data not shown) as with the MAB2166 antibody.

We performed a time-course study to quantify the formation and localization of htt inclusions in primary striatal cells at different time points (6, 13 and 20 days) after transduction with HC-AdHtt15T, HC-AdHtt128T, HC-AdHtt15 or HC-AdHtt128 (Table 1). Inclusions were not detected in cells expressing normal truncated or full-length htt at all time points. In cells expressing the mutant truncated htt, inclusions were detected in most of cells (97%) already at 6 days after transduction and further in all cells (100%) at 20 days after transduction. The formation of htt inclusions was considerably delayed in cells expressing mutant full-length htt. For the first time and also only in 15.2% of cells inclusions were detected at 13 days. There was a further moderate increase to 25.3% inclusion-containing cells at day 20. Nuclear inclusions (NIs) were detected in all inclusion-positive cells expressing mutant truncated htt. Although 9.6% of NI-positive cells with expression of mutant truncated htt also contained cytoplasmic inclusions (CIs) 6 days after transduction, the frequency of CIs in NI-positive cells decreased to 3.2% at day 13 and 0% at day 20. In contrast, only CIs and no NIs were detected in cells expressing the mutant full-length htt. Inclusions formed after vector-mediated expression of mutant truncated and full-length htt also reacted with the anti-ubiquitin antibody (Figure 5).

### Table 1. Time-course study of the formation and localization of htt inclusions in primary neuronal cells transduced with HC-AdHtt15T, HC-AdHtt128T, HC-AdHtt15, or HC-AdHtt128

<table>
<thead>
<tr>
<th>Days</th>
<th>Vector</th>
<th>Inclusion-positive cells with NIs (%)</th>
<th>Inclusion-positive cells with CIs (%)</th>
<th>Inclusion-positive cells with NIs and CIs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>HC-AdHtt15T</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>HC-AdHtt128T</td>
<td>97.0</td>
<td>100.0</td>
<td>9.6</td>
</tr>
<tr>
<td>6</td>
<td>HC-AdHtt15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>HC-AdHtt128</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>13</td>
<td>HC-AdHtt15T</td>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>13</td>
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<td>100.0</td>
<td>3.2</td>
</tr>
<tr>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
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</tr>
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<td>HC-AdHtt128T</td>
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<tr>
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<td>HC-AdHtt128</td>
<td>25.3</td>
<td>0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Immunofluorescence analysis was performed using the anti htt 2B4 antibody and DAPI for nuclear staining at different time points (6, 13 and 20 days) after transduction. Htt-expressing cells and cells with inclusions were counted and their localization determined in five randomly selected fields per well under the IF microscope. Each value represents the mean from duplicate experiments. NI, nuclear inclusion; CI, cytoplasmic inclusion.
Discussion

A major quest in current HD research is to decipher the function(s) of htt at molecular and cellular levels and to understand the pathophysiological events that lead to neurodegeneration when a mutant form of htt is expressed [28,29]. Although it has been known for many years that neuronal cells are predominantly affected in HD, the reasons for this cell-type specificity are only partially delineated. Both cellular and transgenic animal models have been developed and form the basis for a better understanding of the molecular events leading to neurodegeneration [15,30,31]. More recently, viral vector-mediated gene transfer has been used to express truncated htt both as wild-type and mutant alleles in vitro [19,20] and in vivo in rats [16–18]. Viral vector-mediated htt expression recapitulated major pathogenic steps of HD in vitro and in vivo. So far, viral vector-mediated expression of full-length huntingtin has not been achieved because of the larger size of the huntingtin cDNA (more than 10 kb) and the relatively small transgene capacity of most viral vectors. Also, in vivo gene transfer with non-viral vectors has not been possible because of the low transduction efficiency inherent with current non-viral vector systems. Here, we used HC-Ad vectors to express truncated and full-length huntingtin, each either as a 15Q or 128Q allele.

Polyglutamine containing inclusions are a key pathology hallmark of HD [3,32]. Initial findings supported the concept that inclusions themselves might trigger neurodegeneration [33–35]. Other studies have dissociated inclusion formation and cell death [9,36] or even suggested that inclusion formation may serve as a protective mechanism for affected cells [8,10]. Whether there is a causal relationship between inclusion formation and disease is still debated. However, most evidence indicates that clearing inclusions may result in reduced cell death [37,38] and inclusions have been widely used as a surrogate marker in drug screens [39].
Figure 6. Inclusion formation in vivo following stereotaxic injection of HC-Ad vectors expressing truncated and full-length htt with expanded polyglutamine tracks. Five-week-old mice were injected into the striatum with $1 \times 10^7$ infectious units (i.u.) of HC-AdHtt15T (a, b, c), HC-AdHtt128T (d, e, f), HC-AdHtt15 (g, h, i), or HC-AdHtt128 (j, k, l). To mark vector-transduced neuronal cells $1 \times 10^6$ i.u. (1/10) of the EGFP-expressing HC-AdHB01 were added to each htt-expressing vector. Mice were analyzed 2 months after injection using anti htt antibodies as described in the Materials and methods section to determine extent and localization of htt expression. a, d, g, j: htt expression. b, e, h, k: EGFP fluorescence from the same section as a, d, g or j. c, f, i and l: merged data from a and b, d and e, g and h or j and k. An arrowhead points to one neuron with inclusion staining formed from mutant full-length htt.

In the present study expression of truncated and full-length htt with the Q128 allele – but not of normal htt – resulted in the formation of inclusions in neuronal cells in vitro and in vivo. The dynamics of inclusion formation, staining patterns and localization of inclusions differed between cells that either received the truncated or the full-length versions of mutant htt. Mutant truncated htt caused very fast formation of inclusions mainly in the nucleus, a staining pattern that similarly has been observed in other instances of expression of a mutant N-terminal htt fragment including in the R6/2 transgenic mouse model [40]. Expression of mutant full-length htt, however, led to a much slower accumulation of inclusions that were localized in the cytoplasm rather than in the nucleus. This was true not only in vitro but also in vivo following striatal injection of the vector expressing mutant full-length htt (see Figure 7B). Our observation is consistent with results from earlier studies in vitro [41] and in vivo [11] that the localization of intracellular inclusions depends on the size of htt expressed with the expanded polyglutamine. In theses studies expression of mutant full-length htt led to inclusions in the perinuclear part of the cytoplasm. Interestingly, in brains from HD patients both nuclear and cytoplasmic inclusions have been detected [3–6]. The exclusive cytoplasmic localization of inclusions following production of mutant full-length htt is different from what has been observed in patients and transgenic mice. The difference between the localization of inclusions in patients and transgenic animals (both in the nucleus and in the cytoplasm) and our findings (in the cytoplasm) most likely can be attributed to the duration of expression: while results in patients and transgenic animals by nature represent chronic changes, vector-mediated gene transfer allows the study of the early changes that go along with mutant htt expression. This interpretation is supported by earlier in vitro findings in an inducible cellular model of htt expression [42], in which at early time
points following induction of htt expression inclusions were mainly detected in the cytoplasm, while later the number of cytoplasmic inclusions were reduced in favor of nuclear accumulation of inclusions. Also in human postmortem brain htt-positive inclusions have been described in the nucleus and also extranuclear preferentially in dystrophic neurites [3]. Interestingly, extranuclear inclusions were the predominant form in presymptomatic HD mutation carriers (whereas in late disease stages nuclear inclusions were found more frequently) suggesting earlier development of the extranuclear inclusion form.

In an attempt to determine whether expression of mutant forms of htt resulted in detectable cytotoxicity in vitro, we assayed the viability of primary neuronal cells either by the WST-1 reagent (measuring metabolic activity) or by directly counting neuronal cells at different time points (6, 13, 20 days) after transduction with different vectors. However, cytotoxic effects due to expression of mutant truncated or full-length htt were not observed (data not shown) presumably for two reasons: First, different to the study by Zala et al. [43], in which cytotoxicity was observed at 2 months after gene transfer with a lentiviral vector, the latest time point analyzed in our study was 3 weeks. Second, the SYN1-WPRE promoter used in our study is a relatively weak promoter, potentially explaining less pronounced effects at early times.

In summary, we describe the generation and initial evaluation of HC-Ad vectors expressing truncated and full-length htt with or without mutant polyQ expansion. In vitro and in vivo experiments point to differences in the localization of htt inclusions, when expressed as N-terminal and full-length mutant htt. The in vitro and in vivo results also support the concept that the cellular localization of htt inclusions depends on the size of htt. We suggest that the materials described in this study will be valuable for functional studies in vitro and in vivo including in large animals.

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References


Erklärung über die in Anspruch genommenen Hilfen aus Zusammenarbeiten

Die *in vivo* Experimente in Labormäusen, beschrieben in den Publikationen II und III, wurden gemeinsam mit der Arbeitsgruppe von Prof. Dr. C. Kosinski, RWTH Aachen durchgeführt.
ERKLÄRUNG

Ich versichere hiermit, dass ich die vorliegende Arbeit selbstständig verfasst, und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie wörtlich oder inhaltlich übernommene Textpassagen als solche gekennzeichnet habe.

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