

HUNTINGTON'S DISEASE MODELING AND TREATMENT: FROM PRIMARY NEURONAL CULTURES TO RODENTS

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PAR

Diana ZALA

ingénieure chimiste diplômée EPF
de nationalité suisse et originaire de Brusio (GR)

acceptée sur proposition du jury:

Prof. P. Aebischer, Dr N. Déglon, directeurs de thèse
Dr J. Caboche, rapporteur
Prof. E. Cattaneo, rapporteur
Prof. R. Luthi-Carter, rapporteur

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ABSTRACT

Huntington's disease (HD) is a mid-life-onset neurodegenerative disorder characterized by involuntary movements, personality changes and dementia. It progresses to death within 10-20 years after onset. There is currently no cure to treat this fatal disease. In HD patients, the protein huntingtin contains an abnormal expansion of a polyglutamine tract, which leads to the selective death of striatal neurons. The functions of huntingtin, as well as the dysfunctions induced by the mutation are still poorly understood

The first chapter of this thesis describes the state of the art in the study of Huntington's disease: huntingtin (htt), the protein which induces the disease; the hypothesis of toxic pathways induced by the mutant htt and the proposed therapeutic strategies to interfere with them; the available cellular and animal models and finally; the promise of gene therapy for neurodegenerative diseases such as Huntington's disease.

The second chapter presents a new cellular model of the developed by infecting primary cultures with lentiviral vectors expressing a mutant htt fragment. This model is characterized by generalized neuronal transgene chronic pathology, expression, neuronal dysfunction and finally cell death. The slow progression in this model allows the study of the cascade of events leading to cell death. These unique characteristics allow the investigation of the pathological events induced by htt expression with analytical techniques on the entire neuronal population. The chapter ends with the description of an experiment showing the neuroprotective effects of ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) on these neurons.

The third chapter describes the delivery of CNTF in transgenic HD mice which express the human full-length htt (YAC72). CNTF was delivered directly to the affected neurons by striatal lentiviral injection. The sustained one-year delivery was not associated with side effects; it was correlated to reduced hyperactivity and a reduction in degenerating neurons. However, a neuroprotective effect was difficult to establish, due to the very mild and subtle pathology occurring in these animals.

The fourth chapter considers delivery of BDNF in two genetic rodent HD models: in the mild phenotypic HD mice (YAC128) and a more severe rat model based on the lentiviral delivery of mutant htt in the striatum. No neuroprotective effect of BDNF could be detected in either animal model.

The conclusions of this thesis discuss the potential of lentiviral vectors in modeling neurodegenerative diseases *in vitro* and *in vivo*, as well as their potential role in the treatment

of such diseases. The perspective gained by the developed cellular model toward the understanding of the molecular events induced by mutant huntingtin is discussed, as well as the difficulties in testing neuroprotective approaches on animal models of HD.

RIASSUNTO

La corea di Huntington (HD) è una malattia genetica neurodegenerativa caratterizzata da movimenti involontari, cambiamenti di personalità e da demenza. La malattia progredisce fino alla morte del paziente, da 10 a 20 anni dopo la manifestazione dei primi sintomi. Non esiste nessuna cura in grado di arrestare o rallentare il corso della malattia. Nei pazienti affetti da HD, la proteina huntintina contiene un'espansione anormale di una ripetizione di poliglutamine che induce la morte neuronale dei neuroni dello striato. Le funzioni dell'huntintina e le disfunzioni indotte dalla mutazione sono ancora sconosciute.

Il *primo capitolo* descrive lo stato attuale della ricerca sulla corea di Huntington, la proteina coinvolta nella malattia: la huntintina mutata; le ipotesi sui meccanismi della sua tossicità e le possibilità terapeutiche, seguono i modelli cellulari e animali disponibili ed infine le possibilità di terapia genica per delle malattie neurodegenerative.

Il *secondo capitolo* descrive un nuovo modello cellulare ottenuto infettando delle culture primarie striatali con dei vettori lentivirali che esprimono un frammento della huntintina mutata. Questo modello è caratterizzato da una patologia cronica, un'espressione generalizzata a tutti i neuroni e da una disfunzione seguita da morte neuronale. Queste caratteristiche proprie al nuovo modello lo rendono idoneo allo studio della patologia con tecniche analitiche su tutta la popolazione neuronale, non più limitate a singole osservazioni cellulari. In oltre, la sua progressione rende possibile discernere la sequenza dei differenti avvenimenti. Il capitolo termina mostrando come questi neuroni destinati alla morte possono essere salvati da due fattori neurotrofici, il CNTF ed il BDNF.

Il *terzo capitolo* descrive la somministrazione di CNTF in topi transgenici YAC72, i quali esprimono l'huntintina umana mutata in tutta la sua lunghezza. Abbiamo dunque iniettato direttamente nello striato di topi transgenici il vettore lentivirale CNTF. L'espressione di CNTF costante per almeno un anno, non è stata correlata da effetti collaterali, ma ha cambiato l'attività locomotrice dei topi rendendoli meno iperattivi. Lo studio istologico ha dimostrato che il numero di neuroni in degenerazione è ridotto alla metà. Purtroppo l'effetto protettivo non si è potuto provare dato l'esiguo numero di casi di morte neuronale.

Il *quarto capitolo* descrive un esperimento analogo: il BDNF è stato amministrato in topi transgenici YAC128. Questi topi avrebbero dovuto manifestare una patologia più acuta dei topi utilizzati in precedenza. Le stesse difficoltà legate alla mancanza di patologia

neuronal ci hanno impedito di determinare l'efficienza del BDNF. Abbiamo dunque utilizzato un modello molto più severo, l'espressione di un frammento della huntingtina mutata tramite vettori lentivirali nello striato di ratti, infatti il BDNF non è neuroprotettore in questo paradigma sperimentale.

La conclusione di questa tesi di ricerca discute la capacità dei vettori lentivirali di modellare *in vitro* ed *in vivo* le malattie neurodegenerative e la loro potenzialità per curare le stesse malattie. I risultati insoddisfacenti ottenuti sui modelli animali inducono a sviluppare nuovi paradigmi sperimentali per verificare le potenzialità di fattori terapeutici non soltanto su modelli cellulari ma anche animali.

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1. INTRODUCTION

The central nervous system (CNS) is the most complex, amazing and still mysterious structure of our body, controlling functions such as voluntary movements and perception, but also learning, creativity, abstract thinking and emotions. Multiple pathways are orchestrating these functions in networks of highly specialized neurons. Evolution has provided a robust skull and backbone to protect the CNS from outside mechanical injuries, and a molecular obstacle, the blood-brain barrier, to protect it from endogenous chemical agents. However, the brain is still vulnerable, as revealed by the emergence of neurodegenerative diseases associated with dramatic increases in human life expectancy. Dysfunctions in this complex system can lead to a wide range of different severe pathologies such as Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis or Huntington's disease. These disorders are characterized by a specific degeneration of neurons in well-defined areas of the CNS. The molecular bases of these specific neuronal losses are not yet fully understood, but major medical research efforts are concentrated on finding therapies to slow down or stop the course of these disorders.

Cellular and animal models have been developed to mimic these complex pathologies. Models faithfully reproducing a human disease are indispensable tools to analyze, understand, and finally, test interventions against the disorder. This helps explain the great research interest in Huntington's disease (HD). Although it is rare compared to Parkinson's and Alzheimer's, it is the paradigm of a dominant monogenetic disease. The genetic factor, underlying HD was identified in 1993 as a mutation in a unknown protein: a polyglutamine expansion in huntingtin. This is the primary cause of neuronal dysfunction, accumulation of insoluble proteins and neuronal death, which are events common to most of chronic neurodegenerative diseases. With the technological advances in molecular biology and gene transfer techniques, mutant huntingtin can be delivered to living cells to model HD in cell cultures but also in animals. These genetic models are important tools to understand the toxic mechanisms leading to neuronal death, as well as to test therapeutic approaches.

The first goal of this thesis was to develop a cellular genetic model for Huntington's disease, closely mimicking the typical features of the pathology which can be used for fundamental research as well as a platform to develop potential therapies. This goal was strongly inspired

by the seminal paper of Saudou *et. al.* in 1998, which described a new cellular model by transfecting a mutant huntingtin fragment in primary striatal neurons. In that model, neurons experience similar chronic molecular events and death as is observed in the human pathology. The fast kinetics and the scarce transfection yield limit the analysis of pathological events to single cell analysis. To overcome this disadvantage, we used a lentiviral vector, which can efficiently infect non-dividing cells such as neurons and therefore increase the neuronal population expressing mutant htt. The elegant, slow kinetics of the pathological events leading to neuronal death, combined with a generalized neuronal transgene expression are unique features of this cellular paradigm which allow the analysis of population dysfunctions at different time points and open new avenues to understanding the impact of mutant htt.

The second goal of this thesis was the evaluation of therapeutic strategies, first in the new cellular model, then in genetic animal models. Neurotrophic factors have been proposed to slow down, or arrest disease progression or even promote regeneration in neurodegenerative diseases. CNTF and BDNF have shown a specially strong protection spectrum in different paradigms of striatal neuron injuries, both *in vitro* and *in vivo*. We have therefore tested if the lentiviral mediated delivery of these factors could represent an efficient treatment for HD. Complete rescue from neuronal death *in vitro* and long-term delivery of trophic factors *in vivo* without any side effects are encouraging with regard to both gene therapy and the possible beneficial effects of these trophic factors.

1.1.HUNTINGTON'S DISEASE

1.1.1. Eponym Huntington: introduction to the disease

Huntington's disease is a fatal neurodegenerative disease named after George Summer Huntington (Figure 1) who first described the disorder in 1872 (Huntington, 2003). His original paper published under the title "On Chorea" is brief, complete and still up-to-date in major aspects. Going through Huntington's publication represents an excellent introduction to the disorder.



Figure 1: Photograph of George Summer Huntington

Born 1850, East Hampton, New York, USA –1916, died in Cairo, New York, USA (Okun, 2003).

Inheritance

'When either or both the parents have shown manifestations of the disease, and more especially when these manifestations have been of a serious nature, one or more of the offspring almost invariably suffer from the disease if they live to adult age. But if by any chance these children go through life without it, the thread is broken and the grandchildren and the great-grandchildren of the original shakers may rest assured that they are free from the disease.'

This description of the autosomal dominant trait of the mutation is remarkable, because the knowledge of genetics was poorly integrated by the scientific community at that time and Mendel's Laws describing the principle of hereditary transmission were still largely ignored,

although they had been published seven years before in 1865 in a book under the title *Versuche über Pflanzen-hybride* (Treaties on Plant Hybrids). These theories were rediscovered in the early 20th century, when the cell and chromosome structures were better understood, and significant interest for HD, especially its hereditary aspect, was stimulated by Huntington's paper.

Dementia

'The tendency to insanity, and sometimes that form of insanity which leads to suicide, is marked. I know of several instances of suicide of people suffering from this form of chorea, or who belonged to families in which the disease existed. As the disease progresses the mind becomes more or less impaired, in many amounting to insanity, while in others both mind and body gradually fail until death relieves them of their sufferings. At present I know of two married men, whose wives are living, and who are constantly making love to some young lady, not seeming to be aware that there is any impropriety in it. They are suffering from chorea to such an extent that they can hardly walk, and would be thought, by a stranger, to be intoxicated. They are men of about fifty years of age, but never let an opportunity to flirt with a girl go past unimproved. The effect is ridiculous in the extreme.'

A second aspect of the hereditary chorea he observed is dementia and depression. Mood and behavioral disturbance, memory impairment and personality changes are typical clinical features of the disorder, occurring in general before the onset of chorea. Indeed, suicide attempts are more frequent in persons carrying the HD mutation. These secondary treats of the disorder are often ignored as they are less impressive than the abrupt motor dysfunctions and can only be perceived by relatives living close to HD patients. Antidepressant and antipsychotic drugs are often being prescribed to HD patients.

Adult onset

'Its third peculiarity is its coming on, at least as a grave disease, only in adult life. I do not know of a single case that has shown any marked signs of chorea before the age of thirty or forty years, while those who pass the fortieth year without symptoms of the disease are seldom attacked.'

Huntington’s disease is predominantly an adult disorder, with the average onset between 35 and 42 years (Quinn and Schrag, 1998). However, the disease can start at any age and 6% are juvenile forms, with an onset before the age of 20 (Figure 2). These early onsets have quite a different clinical description with the absence of abrupt movements and chorea. As it will be discussed in chapter 1.2.2 the age of onset is closely correlated with the extent of the mutation in the *huntingtin* gene.



Figure 2: Proportion of HD subjects living and deceased

Data are from a sample of 2492 patients, collected as part of the National research Roster for Huntington (Foroud et al., 1999)

Movement disorders

It begins as an ordinary chorea might begin, by the irregular and spasmodic action of certain muscles, as of the face, arms, etc. These movements gradually increase, when muscles hitherto unaffected take on the spasmodic action, until every muscle in the body becomes affected (excepting the involuntary ones), and the poor patient presents a spectacle, which is anything but pleasing to witness. I have never known a recovery or even an amelioration of symptoms in this form of chorea; when once it begins it clings to the bitter end. No treatment seems to be of any avail, and indeed nowadays its end is so well known to the sufferer and his friends, that medical advice is seldom sought. It seems at least to be one of the incurables’.

These uncontrolled and involuntary movements are the most peculiar trait of the disorder. They were probably the cause for the famous witch trials in Salem, Massachusetts in 1692, where several girls were judged and executed as witches possessed by the devil (Trask, 1997). George Huntington concluded his essay with the sad statement that no cure is available and that the death of the affected persons is inevitable. More than a century after Huntington's paper, and more than ten years after the discovery of the HD gene, still no effective treatment is available and the disease progresses uninterrupted to the death of the patient.

1.1.2. Epidemiology of HD and the discovery of huntingtin

Huntington's disease is the most prevalent disorder in a family of nine neurodegenerative diseases that are caused by a polyglutamine expansion in the associated proteins. HD affects both sexes with the same frequency. The highest prevalence is in Europe and North America, with 4-8 cases per 100'000 (Squitieri et al., 1994). In Switzerland about 400 persons are affected by the disorder (Laccone et al., 1999). HD is notably rare in Finland and in Japan (Harper, 1992). An extremely high occurrence was found within the 15'000 members of a large group of inter-related families living in fishing villages along the borders of Lake Maracaibo in Venezuela (Negrette, 1955). The common ancestor of these families was a woman with *el mal de San Vito*, as the locals named Huntington's disease, who had 10 children about 200 years ago. This sad peculiarity led to the foundation of the Venezuela Huntington's disease project with Nancy Wexler as principal investigator. The aim of that project was to identify the chromosomal region, and ultimately the gene, responsible for HD. These big families were therefore genetically screened, and in 1983 a transcript called IT15 (interesting transcript 15) located on the 4th chromosome was shown to be associated with the disease (Gusella et al., 1983). This was the first gene linked to a disease to be mapped. Ten years later, in 1993, the gene was finally isolated and called the *HD gene* or *huntingtin* (*htt*) (The Huntington's Disease Collaborative Research Group, 1993). The mutation leading to HD was identified as a polyglutamine expansion in the N-terminal region of the protein. The discovery of *htt* was a major breakthrough for HD research allowing the development of genetic models both *in vitro* and *in vivo*, and also opened the possibility to perform genetic screening for the mutation on individuals at risk, including human fetuses or even for *in vitro* fertilized eggs prior to implantation.

1.1.3. Clinical aspects of HD

Huntington's disease is a progressive neurodegenerative disorder characterized by chorea (which means dance in Greek) rigidity and dementia; moreover its juvenile form is frequently associated with seizures. This chapter describes the typical decline of an HD patient from the onset until death.

Psychiatric symptoms are believed to occur before the development of neurological symptoms, but a study performed by Shiwach and Norbury concluded that asymptomatic HD gene carriers do not have a greater incidence of psychiatric disorders than non-gene carriers born from a HD parent (Shiwach and Norbury, 1994). However, what the study revealed is that at-risk persons have often psychiatric episodes, probably associated with the uncertainty of having inherited the mutation. The authors conclude that neither depression nor psychiatric disorders are significant pre-neurological indicators of the disease. In contrast to this, attention, learning and planning are affected even before the onset of clinical symptoms (Rosenberg et al., 1995).

The definition of onset is often unclear and imprecise. A common definition is the time of the first appearance of persistent motor abnormalities, which can be of different nature. Ocular motor abnormalities, especially the saccadic movement, are observed in early HD and are often tested to assess the onset of the disease (Berardelli et al., 1999).

The initial symptoms vary from person to person. In general, involuntary movements of face, fingers, feet or thorax manifest the onset. Symptoms develop gradually and are initially very subtle and hard to detect, as they only weakly influence the normal life of the affected person, who keeps his independence. In concomitance, abrupt mood changes, unusual apathy, irritability, anger and depression are commonly observed. Minor motor abnormalities usually precede the obvious signs of dysfunction, which follow at least 3 years later (Folstein et al., 1986; Penney et al., 1990).

As HD progresses, the physical, intellectual and emotional symptoms become more marked. In many cases, the affected person develops overt choreiform movements of the head, neck, arms and legs. These characteristic movements are present in 90 percent of patients. In juvenile forms, the patient will often experience muscle rigidity and bradykinesia without chorea. This form of the disease is also called 'Westphal variant' of HD and affects 6% of HD patients (Hayden et al., 1981; Gervais et al., 2002; Zackowski et al., 2002). Weight loss is one of the features of advanced HD and is not related to a poor dietary intake (Morales et al., 1989).

Bradykinesia, rigidity and dystonia gradually appear and often dominate the final stage of the disease, in which the patient will become severely rigid, grossly akinetic and dysphagic. About 20 percent are incontinent. A global decline in cognitive capabilities is present at the final stage. Due to severe dementia and progressive motor dysfunction, patients with advanced HD may become unable to walk, have poor dietary intake, eventually cease to talk, and become unable to care for themselves, potentially requiring long-term institutional care. Fatal complications may result from injuries related to serious falls, poor nutrition, infection, choking, inflammation of the lungs and heart failure (<http://www.wemove.org>).

1.1.4. The neuropathology of HD

The neurodegenerative process starts in the striatum, which is so called because of its striped structure (*Figure 3*). More than 95% of striatal neurons are medium spiny projecting neurons (MSN), which use gamma-aminobutyric acid (GABA) as their primary inhibitory neurotransmitter (Chesselet and Delfs, 1996). These neurons are most severely affected, resulting in atrophy of the striatum, first in the caudate nucleus, then in the putamen. Post-mortem brains of advanced state HD patients are recognized by the dilatation of the lateral ventricles due to striatal shrinkage. Glial proliferation is observed in concomitance with neuronal cell loss (Robitaille et al., 1997).

The first degenerating subpopulation of striatal neurons belongs to the so-called ‘indirect’ pathway (*see chapter 1.1.5*). These neurons express enkephalin and are enriched in dopamine receptor D2 (Reiner et al., 1988; Albin et al., 1991; Richfield et al., 1995; Sapp et al., 1995). In contrast, striatal interneurons, including large spiny cholinergic cells, medium aspiny neurons as well as somatostatin-, neuropeptide Y-, nitric oxide synthase (NOD)- and parvalbumin positive neurons are spared (Ferrante et al., 1985; Graveland et al., 1985; Ferrante et al., 1987a; Ferrante et al., 1987b; Sieradzan and Mann, 2001). The neuronal loss in the cortex is less severe. Large neurons in layer V and VI, which project to the striatum, are mostly affected (Hedreen et al., 1991).

Vonsattel *et al.* have proposed a system for grading the severity of neuropathology by macroscopic and microscopic criteria (Vonsattel et al., 1985). The grades correlate closely with the extent of clinical disability and are ranking from grade 0 (no discernible neuropathology) to grade 4 (95% of neuronal loss in caudate nuclei).

Characteristic insoluble protein aggregates are found in HD but also in other polyglutamine diseases as well as other neurological disorders, such as the Lewy bodies in Parkinson's disease, the plaques and tangles in Alzheimer's disease or the Bunina bodies in ALS. These aggregates, which are ubiquitinated, are called neuronal nuclear inclusions (NIIs) or dystrophic neuritic inclusions (DNIs), depending on their sub-cellular localization (DiFiglia et al., 1997). Inclusions are mainly found in the striatum and cortex and partially overlap with the neuronal pathology. Their direct impact on the disease is still under debate, as both protective as well as toxic functions have been described (Klement et al., 1998; Saudou et al., 1998; Sisodia, 1998; Kim et al., 1999; Rubinsztein et al., 1999; Yang et al., 2002).

1.1.5. Link between chorea and striatal degeneration

The primary motor dysfunctions in HD are due to the degeneration of a subset of striatal neurons. The striatum belongs to a system called basal ganglia (*Figure 3*), which are a collection of subcortical nuclei that are involved in the control of movement. The basal ganglia do not receive direct sensory input and send little output to the spinal cord. The major flow of information arrives from the cortex into the striatum and returns to the cortex through the thalamus, which is the major output structure of the basal ganglia.

A classical model developed by Albin *et. al.* in 1989 described two major neuronal motor pathways, which act in opposition, and correctly predicts the motor impairment in Huntington's disease. The 'direct pathway' promotes movements by a relay of two inhibitory synapses, first in the globus pallidum internal segment and the substantia nigra pars reticulata (Gpi/SNr) and second in the thalamus; the 'indirect pathway' inhibits movement by three inhibitory connections in the globus pallidum external segment (Gpe), the substantia nigra pars compacta (SNc) and the thalamus with a relay of GABAergic inhibition between the SNc and SNr. A more recent view of the basal ganglia functions indicates that in addition from the striatum, the subthalamic nucleus also receives input from the cortex (Mink and Thach, 1993; Nambu et al., 2000) and sends output to the Gpi/SNr. This new cortico-subthalamo-pallidal loop is called 'hyperdirect pathway' (Nambu et al., 1996) and acts by inhibiting the thalamus and cortex. As this loop is not modified in HD, the model proposed by Albin correctly predicts the motor defects present in Huntington's disease (*Figure 3*) as well as in Parkinson's disease and ballism.

According to this model, the prevalent loss of striatal GABAergic neurons projecting to the Gpe, in the early stage of HD reduces the 'inhibitory pathway', thus causing an imbalance in

favor of the direct pathway. Movements are therefore initiated, but can neither be controlled nor stopped (Albin et al., 1989). At later stages, the general loss of the GABAergic medium size spiny neurons (MSN), including those projecting through the ‘direct pathway’, induces a drastic motor dysfunction leading to bradykinesia.

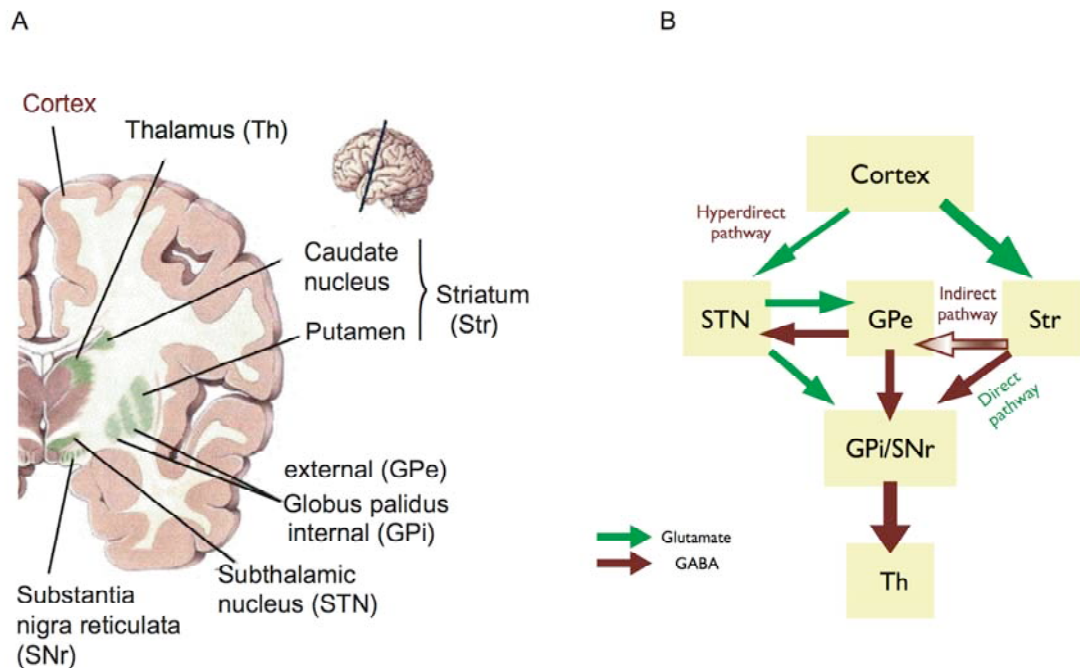


Figure 3: Basal ganglia, their associated structures and the motor loops

(A) Schema of a coronal section of the human brain, showing the position of the basal ganglia and their different sub-nuclei in green. Adapted from (Bear et al., 1996)

(B) Schematic diagram of the cortico-STN-Gpi/SNr ‘hyperdirect’ pathway, cortico-striato-Gpi/SNr ‘direct’ pathway, and cortico-striato-Gpe-STN-Gpi/SNr ‘indirect’ pathway. Green filled arrows represent excitatory glutamatergic, brown arrows represent the inhibitory GABAergic (GABA) projections and the light-brown arrow represents the loss of GABAergic neurons projecting to the Gpe, which are impaired in the early stage of HD. Gpe, external segment of the globus pallidus; Gpi, internal segment of the globus pallidus; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; Str, striatum; Th, thalamus. Adapted from (Nambu et al., 2002).

1.2.HUNTINGTIN

1.2.1. Huntingtin: searching for functions

The human huntingtin gene contains 67 exons spanning over 200 kb and is located in the beginning of the short arm of chromosome 4 at position 4p16.3 (Figure 4). The translated protein contains 3144 amino acids with a molecular weight of 347855 Da (reference with 23 polyQ). Despite the fact that more than 10 years have past since the discovery of htt, this protein is still keeping its secret. Htt has very little homology to other proteins and its functions are not yet known. There are no distinguishable features which could help predict its biological role: no membrane spanning domains, and no apparent enzymatic activity.



Figure 4: Position of huntingtin

Human chromosome 4 with indicated the location of the HD gene at the top of the short arm (Entrez accession number P42858)

Htt is widely expressed within the body with the highest levels in brain and testis. In the brain, it can be found in highest levels in the cerebellar cortex, the neocortex, the striatum and the hippocampus (Schmitt et al., 1995). Htt is expressed predominantly in neurons (Sapp et al., 1997) and is required for normal embryogenesis, as knockout mice die at an early developmental stage (E 7.5) (Nasir et al., 1995). Conditional knockouts have demonstrated that htt also has an essential role at postnatal stages, as the inactivation of the gene in brain and testis leads to degeneration of these two tissues (Dragatsis et al., 2000). These studies indicate that htt is required for cell survival and suggest that a loss of function of htt could induce neurodegeneration, although with a pathology different from HD. Humans with a partial deletion of the distal part of the chromosome 4p16.3, which includes the HD gene, are affected by the Wolf Hirschhorn Syndrome, which is characterized by growth and mental retardation and a premature death at 2 years, but lacks a HD-like phenotype. It is, however, worth noting that knockout neurons *in vitro* differentiate and develop functional synapses (Metzler et al., 1999).

<i>Interacting protein</i>	<i>PolyQ-length dependence</i>	<i>Region of htt involved</i>	<i>Function</i>	<i>References</i>
α -adaptin/C/HYP-J	Yes ↓	NT (aa 1-550)	Endocytosis (CT)	(Faber et al., 1998)
Akt/PKB	No	S421	Kinase (S)	(Humbert et al., 2002)
β -tubulin	No	Unknown	structure, vesicle transport (CT)	(Hoffner et al., 2002)
CA150	No	Unknown	Transcriptional activator (T)	(Holbert et al., 2001)
Calmodulin	Yes ↑	Unknown	Calcium-binding regulatory protein	(Bao et al., 1996)
CBP	Yes ↑	NT (aa 1-588)	Transcriptional co-activator with acetyltransferase activity (T)	(Steffan et al., 2000; Chai et al., 2001)
CIP4	Yes ↑	NT (aa 1-152)	cdc42-dependent signal transduction	(Holbert et al., 2003)
CtBP	Yes ↓	PLDLS motif (aa 182-186)	Transcription factor (T)	(Kegel et al., 2002)
Cystabthionine β -synthase	No	NT (aa 1-171)	Generation of cystein (M)	(Boutell et al., 1998)
FIP2/HYP-L	Unknown	NT (aa 1-150)	Cell morphogenesis (S)	(Faber et al., 1998; Hattula and Peranen, 2000)
GAPDH	Yes ↑	Polyproline	Glycolitic enzyme (M)	(Burke et al., 1996)
GRb2	Unknown	Polyproline	Growth factor receptor-binding protein (S)	(Liu et al., 1997)
HAP40	Unknown	CT	Unknown	(Peters et al., 2002)
HIP1	Yes ↓	NT (aa 1-540)	Endocytosis, proapoptotic (CT)	(Kalchman et al., 1997; Wanker et al., 1997; Karpuj et al., 1999)
HIP2	No	NT (aa 1-540)	Ubiquitin-conjugated enzyme (M)	(Kalchman et al., 1996)
HIP14/HYP-H	Yes ↓	NT (aa 1-550)	Trafficking, endocytosis (CT)	(Singaraja et al., 2002) (Faber et al., 1998)
HYP-A,B	Yes ↑	Polyproline	RNA splicing factors (T)	(Faber et al., 1998)
HYP-C	Yes ↑	Polyproline	Transcription factor (T)	(Faber et al., 1998)
IP ₃ 1	Yes ↑	NT (aa 1-171)	Calcium release channel (S)	(Tang et al., 2003)
MLK2	Yes ↑	First three exons	JNK activator (S)	(Liu et al., 2000)
N-CoR	Yes ↑	NT (aa 1-171)	Nuclear receptor co-repressor (T)	(Boutell et al., 1999)
NF κ B	Unknown	HEAT repeats	Transcription factor (T)	(Takano and Gusella, 2002)
p53	No	PRD	Transcription factor (T)	(Steffan et al., 2000)
PACSIN1	Yes ↑	PRD	Endocytosis, actin cytoskeleton (CT)	(Andrade and Bork, 1995)
PSD-95	Yes ↓	PRD	Synaptic scaffolding protein (CT)	(Sun et al., 2001)
RasGAP	Unknown	PRD	Ras GTPase activating protein (S)	(Liu et al., 1997)
REST/NRSF	Yes ↓	Unknown	repressor element 1 transcription factor (T)	(Zuccato et al., 2003)
SC35			Spliceosome (T)	(Kegel et al., 2002)
SGK		S421	Kinase (S)	(Rangone et al., 2004)
SH3GL3	Yes ↑	PRD	Endocytosis (CT)	(Sittler et al., 1998)
Sin3a	Yes ↑	NT (aa 1-171)	Transcription repressor (T)	(Steffan et al., 2000)
SP1	Yes ↑	NT (aa 1-171), NT (aa 1-480)	Transcription factor (T)	(Dunah et al., 2002; Li et al., 2002)
TAFII-130	No	NT (aa 1-480)	Transcription factor (T)	(Dunah et al., 2002)
TBP	Unknown	Unknown	Basal transcription factor (T)	(Huang et al., 1998)

Table 1: Proteins interacting with huntingtin

The proteins found so far to interact with huntingtin are listed in alphabetical order. The table shows if these interactions are increased (\uparrow) or decreased (\downarrow) by the polyglutamine expansion, the domain where htt interact and the principal function of these proteins. (CT: cellular trafficking, M: metabolism, PRD: proline rich domain, S: signaling and T: transcription. The table was adapted and completed from (Petérsen et al., 1999; Cattaneo et al., 2001; Harjes and Wanker, 2003; Li and Li, 2004).

Htt functions are not well known, but the list of proteins interacting with huntingtin is growing. To date, 35 proteins have been described in the literature (**Table 1**). By regrouping these proteins, four functional families can be formed: the ‘transcription’ family (T) with 15 proteins is the largest, the ‘cellular trafficking’ family (CT) has 9 members, the ‘signaling’ family (S) has 8 proteins and the ‘metabolic’ family (M) has 3 members. The high number of transcription factors interacting with htt suggests an important role of htt in gene control. Except for REST, all the interacting factors are nuclear and hence required the presence of htt in the nucleus. It is therefore probable that these interactions are relevant in HD pathology as the mutation induces a nuclear translocation of htt. The second largest family of proteins interacting with huntingtin are involved in cellular trafficking. This is in agreement with htt colocalization in the endoplasmic reticulum, microtubules, mitochondria, clathrin-coated vesicles and synaptosomal fraction (DiFiglia et al., 1995; Sharp et al., 1995; Gutekunst et al., 1998; Velier et al., 1998). Indeed, wild type huntingtin is essentially a cytoplasmic protein (DiFiglia et al., 1995; Trottier et al., 1995), however it is also found in minor quantities in the nucleus (Wheeler et al., 2000; Tao and Tartakoff, 2001; Kegel et al., 2002).

The protein contains 36 HEAT-like repeats (Huntingtin, Elongation factor 3, regulatory A subunits of protein phosphatase 2A and TOR1), which are distributed across the entire protein (Takano and Gusella, 2002). HEAT motifs are sequences of about 40 amino acids, which form hydrophobic α helices and assemble into an elongated superhelix (Andrade et al., 2001) (Figure 5). The function of HEAT repeats is still unclear, although they are found predominantly in proteins involved in intracellular trafficking and chromosomal segregation. This suggests that huntingtin may act as a scaffold molecule facilitating the formation of variety of complexes, analogous to other HEAT repeat proteins (Takano and Gusella, 2002).



Figure 5: Structure of HEAT repeats

Tertiary structure of HEAT repeats of the protein phosphatase 2A PR65/A from *H. sapiens*, which is an open solenoid-like structure. The structure of huntingtin is probably analogous to this protein. Individual motifs are composed of a pair of α -helices that assemble in a mainly linear, repetitive fashion to form an elongated molecule characterized by a double layer of α -helices. Left-handed rotations generate a novel left-hand superhelical conformation. The protein interaction interface is formed from the intrarepeats that are aligned to form a continuous hydrophobic ridge (Groves et al., 1999).

One of these functional complexes is composed of HAP1, dynactin p150 and is involved in microtubule-dependent transport of vesicles and interacts with beta-tubulin (Engelender et al., 1997; Li et al., 1998; Gauthier et al., 2004). A second complex is formed with HIP1, actin and clathrin and participates in vesicle endocytosis (Waelter et al., 2001). A IP_3 1-HAP1A-htt ternary complex might be involved in calcium signaling in the endoplasmic reticulum (Tang et al., 2003). PACSIN 1 and SH3GL3, the binding partners for dynamin 1, are both involved in synaptic vesicle recycling and both interact with htt (Sittler et al., 1998). In addition, htt associates with postsynaptic density 95 (PSD-95), a scaffold protein that causes clustering and activation of receptors in the postsynaptic membrane (Sun et al., 2001). Htt could also be implicated in gene regulation by acting in the cytoplasm to inhibit gene transcription by associating with the repressor element-1 transcriptional factor (REST), which would prevent its nuclear translocation. In the nucleus, REST inhibits gene transcription by binding to the NRSE-consensus sequence, which is found in the promoters of several neuronal genes. Interestingly, the expression of one of these regulated genes is the neurotrophic factor BDNF (Zuccato et al., 2003), which is decreased in HD brains which and has been described as playing an important role in the survival of striatal neurons (see 1.2.3).

1.2.2. Mutant huntingtin: when length does matter

Downstream amino acid 17 of htt is a polymorphic glutamine/proline (Q/P) rich domain. The expansion of a glutamine stretch encoded by the nucleic acids $(CAG)_n$ will cause

Huntington's disease. There is a strong inverse correlation between the number of CAG repeats found in the *htt* gene and the age of disease onset (Figure 6). However, other genetic and environmental factors influence the age of appearance of the first HD symptoms (Rubinsztein et al., 1997; Holbert et al., 2001; Wexler et al., 2004). To date, 11 disorders with CAG expansion in the coding region of proteins have been found. Interestingly all are neurodegenerative diseases, almost all are characterized by the presence of insoluble ubiquitinated aggregates and a degeneration of specific brain regions, despite the ubiquitous expression of the corresponding proteins. These other members of the triplet repeat disease family are: spinobulbar muscular atrophy (SBMA), spinocerebellar ataxias (SCA1,2,3,6,7,8,12 and 17) and the dentatorubralpallidoluysian atrophy (DRPLA).

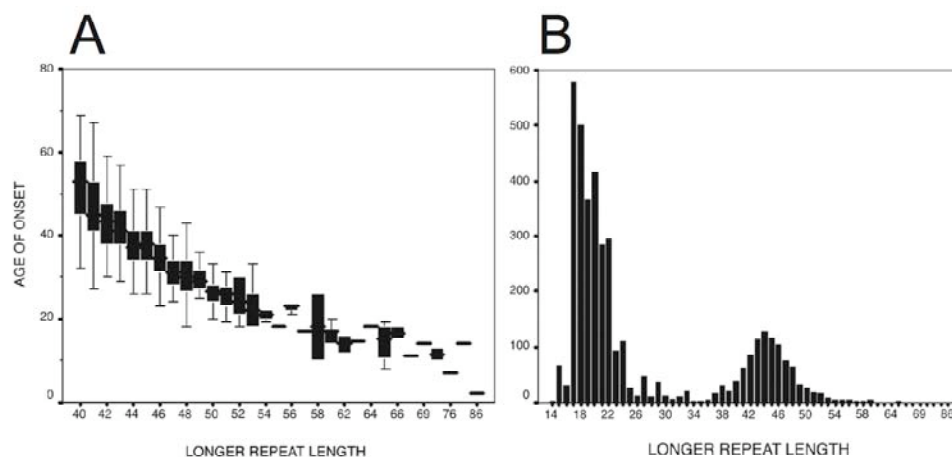


Figure 6: Frequency of CAG repeat size on normal and HD chromosomes

(A) Box plot of age of onset and repeat length of the longer allele. The curvilinear relationship between the two variables can be observed. It also is important to note the large variability of age of onset values, even within each repeat length. (B) Histogram of the longer allele repeat length in the Venezuelan HD kindred. Repeat length ranges are defined as normal (14–34 CAGs), incompletely penetrant (35–39 CAGs), and fully penetrant (40 CAGs) (Wexler et al., 2004).

In HD, CAG repeat numbers between 8-28 are associated with the normal allele, 29-35 are pre-mutations, 36-40 have incomplete penetrance and the pathological threshold of the disease is defined at 40 CAGs. More than 60 repeats are associated with a *juvenile* onset (before 20 years). The frequency of the repeat sizes on normal and HD chromosomes is illustrated in Figure 6B. There is no correlation between CAG repeat length and survival after onset. Homozygous mutations do not lower the age at onset of symptoms, but it more severely affects the phenotype and the rate of disease progression (Squitieri et al., 2003). The CAG repeats may also undergo a somatic expansion with a frequency related to the length of the

repeat. Indeed different expansion lengths have been found in cells from the same transgenic mouse (Ishiguro et al., 2001). During meiosis, the risk of expansion is more frequent in spermatogenesis as compared to oogenesis, which leads to the so-called paternal anticipation. It is, therefore, possible that a 'pre-mutation', which is not associated with the disorder, will be amplified and will reach the pathological threshold in the offspring (Trottier et al., 1994).

1.2.3. Possible toxic mechanisms of mutant huntingtin

HD is an autosomal dominant disorder. This suggests that the mutation leads to a toxic gain of function. Accordingly, several cellular and animal models were developed by over-expressing mutant huntingtin. But there is evidence that a loss of protective function of huntingtin could act synergistically with the gain of toxic functions. The more puzzling aspect of HD is to understand why mutant htt, which is not particularly highly expressed in the striatum as compared to other brain regions, leads to a specific neuronal degeneration. In this chapter I will discuss several non-exclusive hypotheses that have been proposed to describe the toxic mechanisms induced by mutant htt.

a) The toxic fragment hypothesis

The toxic fragment hypothesis is based on the observation that mutant huntingtin is cleaved both in cellular models, in transgenic animals and in HD patients (Sieradzan et al., 1999). The cleavage produces N-terminal fragments bearing the polyglutamine (polyQ) expansions which are considered to be the toxic compounds. Several experiments have shown that the expression of different N-terminal fragments with expanded polyQ is sufficient to induce a HD-like pathology, whereas longer fragments are less toxic in cellular and animal models (Lunkes and Mandel, 1998; Saudou et al., 1998; Karpuj et al., 1999; de Almeida et al., 2002). Short fragments may induce toxicity in the cytoplasm by promoting aberrant interactions with proteins and by inducing formation of aggregates. In addition, once cleaved, htt fragments can translocate from the cytoplasm into the nucleus, where they aberrantly interact with several transcription factors and form inclusions (Ross, 2002). This is supported by the fact that cleavage of mutant htt promotes aggregate formation (Cooper et al., 1998; Li and Li, 1998; Martindale et al., 1998). The toxic fragment hypothesis is however non-exclusive and does not contradict other postulated mechanisms, such as the aggregation or the transcriptional dysregulation hypothesis.

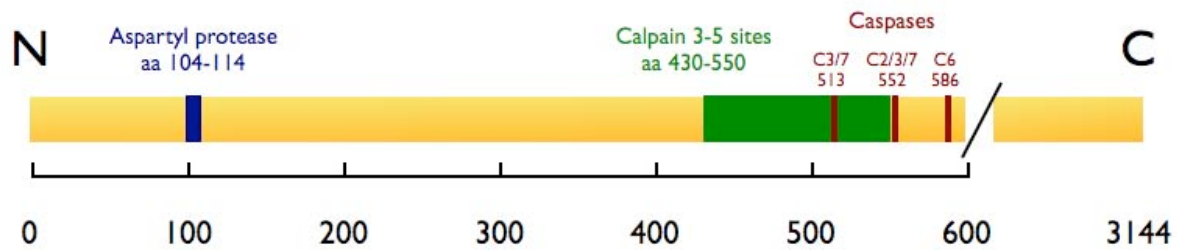


Figure 7: huntingtin consensus cleavage sites

The schema shows the huntingtin protease domains and the position of the known cleavage sites of caspases, calpains and aspartyl proteases. Adapted from (Wellington et al., 2003).

Several consensus cleavage sites have been identified in htt (Figure 7). Experiments have shown that preventing proteolysis by inhibition of caspase or calpaine activation or by modifying the consensus cleavage site in htt reduces mutant huntingtin toxicity *in vitro* and *in vivo* HD models (Ona et al., 1999; Sanchez et al., 1999; Wellington et al., 2000; Gafni and Ellerby, 2002). Based on this hypothesis, the inhibition of huntingtin proteolysis could be an effective therapy against HD. Interestingly, the phosphorylation of huntingtin prevents mutant htt cleavage, aggregation and finally toxicity (Saudou et al., 1998; Humbert et al., 2002; Rangone et al., 2004). Therefore factors such as IGF-1, which induces the phosphorylation of htt, could be effective against HD.

b) The aggregation and protein turnover hypothesis

Using electron microscopy and X-ray diffraction Perutz and collaborators demonstrated that polyQ forms cylindrical β -sheets strongly interacting via hydrogen bonds, which could act as 'polar zippers' leading to protein aggregation and precipitation (Perutz, 1994; Perutz et al., 1994; Perutz et al., 2002). The threshold for huntingtin aggregation *in vitro* appears to be 35-48 glutamine residues, which closely approximates the critical range for the development of HD (Scherzinger et al., 1997). Progressive accumulation of abnormal protein aggregates associated with neuronal loss is a common molecular event observed in all polyQ diseases, but also in other neurodegenerative diseases such as PD, AD or ALS. A slow increase in protein accumulation could occur if the protein turnover is affected or the processing of the mutant form is less efficient. Huntingtin turnover could be tightly regulated by cleavage, and the proteolysis might be involved in the pathology in several ways. For example, the toxic fragment might be resistant to further degradation. The fact that the ubiquitin-proteasome pathway (UPP) is impaired by polyQ expansions strongly suggests that the protein turnover is

indeed affected in polyglutamine diseases (Bence et al., 2001). A generalized proteasomal inhibition was recently described to occur, not only in brain regions affected by HD, but in other CNS territories and also in fibroblasts from HD patients (Seo et al., 2004). UPP impairments are also associated with other neurodegenerative diseases. A mutation in a protein of the ubiquitin pathway, the ubiquitin carboxyl terminal hydrolase L1 (UCHL-1) and mutations in parkin, which is a ubiquitin ligase (E3), cause familial forms of Parkinson's disease (von Bohlen und Halbach et al., 2004). Several studies in cellular and animal models, and also data from human brains, have shown that large aggregates colocalized with ubiquitin and chaperones, suggesting that the mutant protein is recognized as misfolded and is targeted for protein degradation. Overexpression of the chaperone HSP70 in a fly model of SCA3 suppresses polyQ-induced pathology without reducing aggregation, but by decreasing the soluble mutant protein content (Warrick et al., 1999; Pollitt et al., 2003). This suggests that aggregation has a protective function by sequestering the toxic compound. The suppression of aggregates by the expression of a dominant negative ubiquitin-conjugating enzyme in a cellular model of HD increased mutant htt toxicity (Saudou et al., 1998). On the other hand, aggregates could sequester important transcription factors, leading to transcriptional abnormalities, as discussed below. Finally, they could block axonal and dendritic trafficking, leading to neuronal dysfunction (Gunawardena et al., 2003; Lee et al., 2004).

A current view of α -synuclein toxicity imputes small aggregates, rather than mature fibrils, to be the pathogenic factor (Lashuel et al., 2002). Similarly, mutant htt oligomers, but not htt aggregates, may inactivate transcriptional factors. In support of this view, a recent study with purified proteins expressed in *Echerichia coli* showed that soluble mutant htt interacts with TBP and CBP prior to aggregation, and htt aggregates lose the ability to bind these transcription factors (Schaffar et al., 2004), thus adding new evidence that monomers and/or oligomers, but not aggregates are the toxic compounds. All this data seems to show that at a first step, aggregate formation is protective as it inactivates the toxic species, but at a later stage, the accumulation of large aggregates leads to a physical impairment.

Several compounds reducing aggregation have been tested *in vitro* and *in vivo*, such as the dye congo red or more recently sugar trehalose, which decreased aggregation and neuronal pathology in the transgenic mouse model R6/2 (Smith et al., 2001; Sanchez et al., 2003; Tanaka et al., 2004).

c) Transcriptional dysregulation

The hypothesis that mutant htt, but also other polyQ proteins affect nuclear functions, emerged when it was noted that nuclear translocalization increases toxicity in cell culture models and transgenic mice (Klement et al., 1998; Saudou et al., 1998; Peters et al., 1999). In addition, the disease progression in humans and animal models is correlated with the accumulation of nuclear aggregates that may sequester transcription factors and other cellular proteins (Boutell et al., 1999; Steffan et al., 2000; Zoghbi and Orr, 2000; Nucifora et al., 2001; Ross, 2002).

The majority of transcription factors (T) interacting with htt associate with the domain rich in glutamine residues and the interaction is polyQ-length-dependent (Table 1). Moreover, the ectopic presence of mutant htt fragment in the nucleus strongly modifies the physiology of htt activity. The expanded htt protein interacts with the acetyl transferase domain of different transcription factors such as CBP (cAMP-response element binding protein (CREB) binding protein) and p300/CBP Associated Factor (P/CAF), which affects histone deacetylase, chromatin structure and finally gene transcription (Steffan et al., 2001). Other transcription factors which interact with htt are the TATA box binding protein (TBP) and Sin3A (Dunah et al., 2002). Recently, it was shown that monomers and small soluble oligomers of mutant htt exon 1 inactivate transcription factors, including CBP and TBP, by a polyQ-mediated interaction (Schaffar et al., 2004). Dysregulations of gene transcription are, therefore, considered to play an important role in HD. Indeed, DNA-microarray experiments on transgenic mice and cell cultures have revealed several transcriptional changes, most often a relative reduction of transcripts, induced by the polyQ expansions (Luthi-Carter et al., 2000; Nucifora et al., 2001; Wyttenbach et al., 2001; Sipione et al., 2002). Besides the downregulation of important mediator of cell survival via CRAB, transcriptional dysfunctions may also induce the overexpress of proapoptotic genes via activation of the JNK/c-Jun module (Garcia et al., 2002). To restore normal transcriptional activity, the over-expression of transcription factors such as CBP and CA150 has been considered to be neuroprotective against mutant htt toxicity (Steffan et al., 2000; Holbert et al., 2001; Nucifora et al., 2001). In addition, chemical compounds that such as inhibit histone deacetylase, such as sodium butyrate and suberoylanilide hydroxamic acid, reduced polyglutamine toxicity in cell lines, transgenic flies and mouse models of HD (McCampbell et al., 2001; Steffan et al., 2001; Ferrante et al., 2003; Hockly et al., 2003).

d) The loss of function hypothesis

The hypothesis that a loss of function could play a role in HD pathology was first reported when it was shown that wild type huntingtin has anti-apoptotic function (Rigamonti et al., 2000; Rigamonti et al., 2001). In addition, its over-expression rescues mutant-htt-induced toxicity in cellular models, suggesting that the mutation could interfere with an endogenous pro cell-survival function of htt (Cattaneo et al., 2001; Sipione and Cattaneo, 2001; Rubinsztein, 2002). This protection could occur through the interaction with REST (see 1.2.1), which is decreased by the polyglutamine expansion. Interestingly, REST controls cortical BDNF expression. BDNF is anterogradely transported to the striatum and is decreased in HD, transgenic mice and cellular models (Ferrer et al., 2000; Zuccato et al., 2001; Zuccato et al., 2003). This chronic depletion could contribute to HD pathology and partially explain the preferential striatal vulnerability. Conditional knockout mice of forebrain BDNF confirmed the importance of this trophic factor for striatal dendrite morphology and for a long-term survival of MSNs (Baquet et al., 2004). In addition, these knockout mice show noticeable analogies to transgenic HD mice, with a reduction of striatum volume, a late neuronal loss and a clasping phenotype. These data suggest that restoring striatal BDNF may represent a therapeutic approach for HD.

The anti-apoptotic effect of wild-type htt may also occur via the sequestration of pro-apoptotic molecules such as Hip1, which once dissociated from huntingtin forms a complex with Hipp1 and activates caspase 8 (Gervais et al., 2002). In accordance with the loss of function hypothesis, wild type htt has been proposed as therapeutic factor (Leavitt et al., 2001; Cattaneo, 2003).

e) Mitochondrial impairment

Energy metabolism impairments have been described in affected brain regions of HD, including an increase in lactate levels, a decrease in N-acetylaspartate and creatine and a reduction of complex-II and complex-III activities. In addition, HD patients progressively lose weight despite high caloric intake. All these findings strongly suggest a mitochondrial dysfunction in Huntington's disease (for review see (Beal, 2000)). Mitochondrial impairment could contribute to excitotoxic processes, oxidative damage, and altered gene regulation.

Striatal neurons are highly innervated by cortical excitatory input and require important energy supplies to maintain a physiological depolarized state. Energy failure would over-activate striatal NMDA receptors inducing so-called ‘secondary excitotoxicity’. Striatal vulnerability to mitochondrial impairment was accidentally discovered after the intoxication of Chinese children with 3-nitropropionic acid (Ming, 1995). This toxin irreversibly inhibits succinate dehydrogenase (mitochondrial complex-II) and leads to a severe neurological disease resembling HD, with a degeneration of basal ganglia and movement dysfunctions characterized by dystonia, chorea and hypokinesia (Ludolph et al., 1991; Alexi et al., 1998). A generalized mitochondrial impairment seems to occur in HD; lymphoblast mitochondria of HD patients and transgenic mice have a lower membrane potential (Panov et al., 2002; Panov et al., 2003). It was shown that htt associates with the mitochondrial outer membrane and that mutant htt N-terminus decreases the calcium threshold required to induce mitochondrial permeability transition (MPT) pore opening and release of cytochrome c (Choo et al., 2004), which can activate apoptotic death pathways. Interestingly, neurons with high calcium buffer (e.g. calbindin positive) are spared in the striatum (Ferrer et al., 1994). Potential neuroprotective strategies are the administration of creatine, which may exert neuroprotective effects by increasing phosphocreatine levels or by stabilizing the mitochondrial permeability transition, the administration of co-enzyme Q10, a carrier for electron-transfer in the mitochondrial membrane or remacemide an NMDA antagonist. These compounds are neuroprotective in transgenic mouse model R6/2 and are currently being tested in clinical trials (Ferrante et al., 2000; Ferrante et al., 2002).

e) Excitotoxicity hypothesis

Excitotoxicity could play an important role in HD, as cortical glutamatergic processes massively innervate the striatum. The overactivation of N-methyl-D-aspartate (NMDA)-type glutamate receptors allows high levels of calcium entry can results in the death of MSN neurons (Ferrante et al., 1985; Lipton and Rosenberg, 1994). Calcium triggers different downstream events, such as activation of calpain, protein kinase C, DNAase, phospholipase and can induce cell death either by necrosis or apoptosis (Lynch and Guttman, 2002). Notably, significant increases in the NMDAR-mediated current density associated with an increase of intracellular free calcium levels were found in MSNs from several mouse models for HD (Levine et al., 1999; Cepeda et al., 2001; Zeron et al., 2002; Zeron et al., 2004). At a

molecular level, huntingtin interacts with PSD-95, a post-synaptic protein that can modulate excitatory signaling through the interaction with NMDA receptors (Aarts et al., 2002). Mutant htt promotes sensitization of NMDA and the over-expression of the wild type reduce NMDA or kainate-mediated toxicity in a neuronal cell line (Gratacos et al., 2001a; Sun et al., 2001).

1.2.4. Dysfunction and death of striatal neurons

As discussed in the previous chapter, several mechanisms of mutant huntingtin toxicity have been proposed, which partially fit with clinical data gathered from HD patient as well as from molecular, cellular and animal experiments. These different mechanisms could participate synergistically in the pathology or be subordinate to one of them. The fact that there are generalized biochemical abnormalities in cells from HD patients, but only a specific cell death (Seo et al., 2004), suggests that several accomplices are needed to finally induce striatal neuronal death.

Whether the cell death in the striatum is cell-autonomous or involves other brain regions is still an open question. Dysregulation of cortical glutamatergic input could lead to excitotoxicity, and the decrease of cortical BDNF delivery either by transcriptional dysregulation or by impairment in vesicle transport could lead to a trophic starvation of striatal neurons. In addition, the massive release of dopamine in the striatum could activate oxidative stress pathways and autophagy (Petérsen et al., 2001a). On the other hand, cell autonomous dysfunctions have been observed, such as mitochondrial impairment, aggregation, gene transcriptional changes and htt cleavage, which would speak more for a cell-autonomous death. Again, the combination of these two factors could lead together to this specific cell death.

During the last 30 years, cell death was usually classified either as apoptosis or as necrosis (Kerr et al., 1972). Classical necrosis is unlikely to be responsible for the cell loss in HD, because it involves cell swelling and rupture, electing an inflammatory response, a violent and quick form of degeneration affecting extensive cell populations. Programmed cell death is therefore more likely to be involved in HD neuronal death. Caspase activation is one of the indicators of apoptotic mediated cell death and as previously discussed is involved in the cleavage of htt and is activated in HD (Ona et al., 1999; Sanchez et al., 1999). In addition, fragmented chromatin was described in HD striatal tissues. Despite this, evidence of apoptotic neuronal death in HD is scarce. The simplest dichotomy, necrosis or apoptosis, was shown to

be insufficient and autophagy was added as an alternative type of cell death. Autophagy is a primary mechanism of cell physiology that digests long-lived stable proteins and organelles, but it is also a potential mechanism of cell death. Mutant huntingtin can be taken up and degraded by autophagic vacuoles (Michalik and Van Broeckhoven, 2003; Qin et al., 2003). Accumulation of proteasomal-resistant htt aggregates could therefore lead to a chronic activation of this pathway, which could induce cell death by autophagy. Indeed, a number of studies have demonstrated the presence of autophagy in HD brain and in animal models of HD (Larsen and Sulzer, 2002). Cellular models of HD have highlighted the presence of autophagic bodies (Kegel et al., 2000; Petérsen et al., 2001a). In conclusion, several features of apoptotic and autophagic cell death are present in HD, it is possible that a cross-talk of these two alternative cell death pathways are involved in the mutant htt mediated cell death.

1.3. MODELING HD: FROM CHEMICAL TO GENETIC INSULTS

Before the emergence of genetic models, different toxins were delivered to rodents and primates to reproduce a HD-like phenotype (Brouillet et al., 1999). The over-stimulation of glutamate receptors with excitatory amino acids such as ibotenic acid, kainic acid, N-methyl-D-aspartate (NMDA) or quinolinic acid, induces neuronal death by excitotoxicity (Bruyn and Stoof, 1990). The activation of this pathway, as discussed in chapter 1.2.3, could be relevant to HD pathology.

The second strategy to induce striatal degeneration consists in the systemic administration of mitochondrial blocker 3-nitropropionic acid, which inhibits succinate dehydrogenase, leads to ATP depletion, and reproduces a specific striatal degeneration of GABAergic neurons. This model is based on the hypothesis that chronic impairment of the mitochondrial metabolism may induce neuronal death (see chapter 1.2.3).

These chemically induced models were, and still are, useful tools to test therapeutic strategies which can spare striatal neurons and could therefore be interesting for HD. On the other hand, these models do not reproduce the progressive chronic neuronal degeneration and lack the genetic component of HD. Therefore their interest has decreased in favor of huntingtin-based models.

1.3.1. Molecular models

Almost 40 years ago, the aggregation properties of polyglutamine stretches in a test tube were discovered (Krull et al., 1965) and, without being aware of it, the first polyQ model was concomitantly developed. These cell-free systems are suitable to investigate the biochemical parameters leading to conformational change, protein sequestration and aggregation, as well as to being screen for chemical compounds which could interfere with this processes. (Perutz, 1994; Perutz et al., 1994; Scherzinger et al., 1997; Perutz et al., 2002; Schaffar et al., 2004).

1.3.2. In vitro models

These models are obtained either from knock-in mice or by the expression of mutant huntingtin, a huntingtin fragment or a polyQ expansion in different cell types, including yeast, cell lines, or primary cultures. Yeast, *Saccharomyces cerevisiae*, provide a useful tool for the screening of genes involved in aggregate formation, to study potential polyQ-induced toxicity and for the screening of chemical compounds (Lindquist et al., 2001; Muchowski et al., 2002). For example, the importance of chaperone-mediated folding of polyglutamine expansions was discovered in yeast. The molecular chaperone Hsp104 was reported to be essential for the aggregation of polyQ peptides containing proteins, and over-expression of Hsp70 and Hdj1 (yeast homologue of Hsp40) suppressed the formation of fibrous deposits, but promoted amorphous, detergent-soluble aggregates (Krobitsch and Lindquist, 2000; Muchowski et al., 2000).

Several mammalian cell lines that are stably over-expressing mutant or wildtype huntingtin were developed (Lunkes and Mandel, 1998; Jana et al., 2000; Rigamonti et al., 2000; Wytenbach et al., 2001). Neuronal cell lines or primary neuronal cultures obtained from htt knock-in mice (see 1.3.3) are particularly interesting because of the physiological expression of mutant htt, but the absence of a severe phenotype is a major limitation to study HD pathology (Trettel et al., 2000). Other models obtained by culturing dissected striata from transgenic mice were characterized by mild pathology and no cell death under stable culture condition (Petérsen et al., 2001b; Zeron et al., 2004). Saudou et al. (1998) were the first to develop a model in primary striatal neurons transfected with mutant huntingtin, and to reproduce the typical features such as protein aggregation and neuronal death. However the low transfection rate is a major limitation of that model. In summary, all these different models show that mutant huntingtin is toxic when over-expressed in different cell types, but

endogenous or low expression induces only subtle changes. A balance between survival of the culture *in vitro* and the level of htt expression should be found to obtain a progressive, but severe pathology.

1.3.3. *In vivo* genetic animal models

A breakthrough in HD research was the development of transgenic models. These animals are valuable tools for determining the first molecular changes associated with the disease, highlighting which toxic pathways are activated and finally testing effective therapies.

With only 302 neurons, the nematode *Caenorhabditis elegans* is the simplest genetic animal model of HD (Faber et al., 1999; Holbert et al., 2001; Parker et al., 2001; Faber et al., 2002). Biochemical and morphological studies, as well as behavioral tests can be performed in this classical genetic animal model. In addition, its transparency allows longitudinal live imaging studies *in vivo* with standard microscopy equipment.

The fruit fly, *Drosophila melanogaster*, is a second invertebrate model for HD, where degeneration of photoreceptors and motor function are often measured (Jackson et al., 1998; Steffan et al., 2001; Gunawardena et al., 2003). PolyQ-expressing flies form nuclear inclusions and undergo a progressive neurodegeneration, leading to early cell death. The ease in developing different transgenic flies or worms makes them an ideal platform to screen for genetic or chemical modifiers, which would interfere with the polyQ-induced pathology *in vivo*.

Mammals and especially the mouse, *mus musculus*, are the favorite animals chosen to model a human pathology. Transgenic mice expressing the first exon of *htt* with 115 CAG (strain line R6/1) or 155 CAG (strain line R6/2) repeats, develop progressive behavioral symptoms and neuropathology, but little evidence for cell loss could be found (Mangiarini et al., 1996; Murphy et al., 2000; Turmaine et al., 2000; van Dellen et al., 2000). Various other mouse lines have been developed thereafter, each characterized by different promoter, expression levels, htt length and polyQ expansion (see **Table 2**). Knock-in mice with the murine htt bearing polyQ expansion are close models of the HD genetic state, as they express mutant huntingtin at endogenous levels. Similarities between all these mouse strains have been reported with respect to inclusion formation, brain atrophy, tremors, hyperactivity and clasping phenotype. A few studies reported cell loss but only at an old age, for example in the YAC72 and YAC128, which express the full-length htt with 72 and 128 glutamines, respectively.

In addition, a transgenic rat model of Huntington's disease was developed, showing phenotype similar to transgenic mice, and allowing extensive longitudinal study *in vivo* with high resolution imaging (von Horsten et al., 2003), but again this model displayed little neuronal loss.

Finally, a new strategy for generating genetic animal models has been developed by the striatal injection of viral vectors encoding portions of the mutated protein. The first model was obtained by the delivery of a polyQ-GFP fusion protein mediated with an AAV viral vector (Senut et al., 2000). Moreover, lentiviral-mediated striatal expression of mutant htt fragments in rats and primates represents a good tool to analyze the impact of the mutation in a specific brain region. These models have finally shown, for the first time, a severe neuronal degeneration followed by cell death (de Almeida et al., 2002; Régulier et al., 2003). Transgenic mice may therefore be more suitable to analyze early pathological dysfunctions and lentiviral- based models may be more appropriate to study neuroprotective strategies.

<i>Group</i>	<i>Promoter gene size polyQ expression</i>	<i>Onset behavior</i>	<i>Inclusions</i>	<i>Cell loss and brain atrophy</i>	<i>References</i>
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Transgenic HD mice

Bates (R6/1-2)	HD exon1 115Q-155Q < endogenous	5 weeks Clasping, tremors, abnormal gait, learning deficit, hypokinesia, diabetes	NII and DNI	Fewer dendritic spines, few cells lost, overall brain atrophy	(Mangiarini et al., 1996; Turmaine et al., 2000)
Hayden (YAC)	HD full-length 72Q 2x endogenous	3 months Clasping, hyperactivity and circling	Inclusions in the striatum	Cell loss in the striatum	(Hodgson et al., 1999)
	HD full-length 128Q 6x endogenous	Motor abnormalities, hyperactivity, end stage hypoactivity	Inclusions	Striatal neuronal loss Striatal and cortical atrophy	(Slow et al., 2003)
Ross/ Borchelt	PrP N171 18,44, 82Q 5x endogenous	4 months Clasping, tremors, abnormal gait, hypokinesia, weight loss, early death	Inclusions, diffuse nuclear accumulation of htt	dark cells Overall brain atrophy	(Schilling et al., 1999)
Tagle	CMV full-length 6, 48, 89Q 1-2x endogenous	4 months Circling, hyperactivity, end stage hypoactivity, urinary incontinence	Few inclusions	20% cell loss in striatum in some animals	(Reddy et al., 1998; Reddy et al., 1999)
	CMV exons 1-3 89Q 1/5x endogenous	4 months Prolonged hyperactivity	Few inclusions	not described	Unpublished?
Aronin/DiFiglia	rat NSE 3 kb fragment 18,48,100Q > endogenous	3-4 months Clasping, hyperactivity and endstage hypoactivity	Inclusions	20% cell loss and brain atrophy in some animals	(DiFiglia et al., 1997)
Hen/Yamamoto	tet-off(camKII α -tTA) HD exon1 97Q < endogenous	2.5 months Clasping, late onset tremor and gait abnormality	Inclusions and reactive astrocytes	Brain and progressive striatal atrophy	(Yamamoto et al., 2000)

Knock-in HD mice

MacDonald	Hdh 50,92,111Q endogenous	No abnormal behavior	htt nuclear relocalization and inclusions	not described	(White et al., 1997)
	neo and HDh 29, 111Q < 1/2 endogenous	2 months tremor, abnorm gait	htt nuclear relocalization and inclusions	Brain atrophy	(Wheeler et al., 1999; Wheeler et al., 2000)
Myers	Hdh prom, 71,82Q 2x endogenous	early onset aggressive behaviour	Late inclusions, LTP impaired, repeat instability in striatum	not described	(Shelbourne et al., 1999)
Zeitlin	Hdh 71, 94Q, 150Q ~ 2 endogenous	no phenotype	NMDA sensitivity	No inclusions smaller striatal cell	(Levine et al., 1999)

Transgenic HD rat

Riess	rat HD 1962 bp rat htt 51Q Tet/off	2 months reduction in anxiety, decline in spatial learning test, motor dysfunctions	htt inclusions	Focal lesion in striatum, ventricle enlargement	(von Horsten et al., 2003)
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Lentiviral mediated HD rat

Dégion/ Aebischer	CMV, PGK 171, 853, 1520AA 19,44,66,82Q not determined	No behaviour	htt inclusions	Striatal atrophy and severe neuronal loss	(de Almeida et al., 2002)
	tTA1-PGK 853 AA 19, 82Q not determined	No behaviour	htt inclusions	Striatal atrophy and severe neuronal loss	(Régulier et al., 2003)

Table 2: Genetic rodent models of Huntington's disease

The table recapitulates the major characteristics of the available transgenic and knock-in mice as well as the transgenic and lentiviral-mediated models of Huntington's disease. (Adapted and completed from <http://www.hdfoundation.org/PDF/hdmicetable.pdf>)

1.4. THE HUNT FOR A CURE

Several hypotheses describe the mechanism of how huntingtin may act to induce neuronal death (see 1.2.3 Possible toxic mechanisms of mutant huntingtin). Thus, several different ‘deterministic’ approaches, which could directly interfere with mutant huntingtin toxicity have been proposed (Peschanski et al., 2004). They are summarized below:

- a) Blockade of htt proteolysis with caspases and/or calpain inhibitor
- b) Reduction of aggregates and/or mutant htt toxicity with chaperones and chemical compounds
- c) Up-regulation of transcription with histone deacetylase inhibitors
- d) Restoring the loss of with wild-type htt functions
- e) Improving the energetic state with metabolic enhancers
- f) Blocking excitotoxicity with NMDA antagonist
- g) Blocking the expression of mutant htt by RNA interference

On the other hand, restorative or ‘pragmatic’ strategies are also proposed, which in contrary to the deterministic approaches try to interfere directly with some effect of the disease, typically with the striatal neuronal loss (Peschanski et al., 2004). For example, neuroprotective strategies such as the delivery of neurotrophic factors could represent a valid option; as such proteins have shown a strong protection of neurons in various animal models of striatal injuries. Alternatively, the reconstruction of neuronal circuits in the brains by intrastriatal transplantation of striatal neuroblasts from human fetuses could represent a valid option for HD (Peschanski et al., 2004).

1.4.1. Neurotrophic factors

Neurotrophic factors are proteins which promote the survival of specific neuronal populations. They act by inducing physiological effects such as morphological differentiation, enhancing nerve regeneration, neurotransmitter expression, gene expression and altering the physiological characteristics of neurons. It has been proposed that neurotrophic factors could slow down or arrest the course of neurodegenerative diseases, or even promote regeneration. Neurotrophins, glial cell line-derived neurotrophic factor family members and ciliary

neurotrophic factor have shown potent neuroprotective effects on different neuronal populations of the striatum. Thus, these neurotrophic factors may be suitable for the development of a neuroprotective therapy for neurodegenerative disorders such as Huntington's disease (Alberch et al., 2004). In particular, two neurotrophic factors have caught our interest. One is the ciliary neurotrophic factor (CNTF), which in several different animal models has proven a high rescue potential and has been tested in a clinical trials (Bloch et al., 2004); the other is the brain-derived neurotrophic factor (BDNF), which is a potent neurotrophic factor for GABAergic neurons and is decreased in HD brains.

1.4.2. CNTF

Ciliary neurotrophic factor purified from chick eye was originally described to rescue neurons from embryonic chick ciliary ganglia *in vitro* (Barbin et al., 1984). CNTF is not critical for the development and for the postnatal and adult maintenance of neurons, since knockout mice develop normally, with only subtle changes in motor survival (Masu et al., 1993). In fact 2,5% of the Japanese population have a homozygous null mutation of this factor without any associated neurological abnormalities (Takahashi et al., 1994). On the other hand, the knockouts of the genes coding for the CNTF receptors (CNTFR α , gp130 or LIFR β) led to prenatal death of mice or severe motor neuron deficits (DeChiara et al., 1995). Later, a new heterodimeric ligand for CNTF receptor alpha (CNTFR α) the stable secreted complex of cardiotrophin-like cytokine (CLC) and the soluble receptor cytokine-like factor-1 (CLF) was discovered, thus resolving the apparent paradox between the results of knocking out the neurotrophic factor or its receptor (Elson et al., 2000).

CNTF is a member of an α -helical cytokine superfamily that includes interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and leptin. CNTF lacks a signal peptide and is found in adults in glia cells within the CNS and PNS. Its expression is highly regulated following neuronal traumas or ischemia and is restricted to astrocytes adjacent to the lesion (Ip et al., 1993; Asada et al., 1995). CNTF is considered an 'injury factor' and numerous studies have shown neuroprotection in different models of neurodegenerative diseases (Alberch et al., 2004).

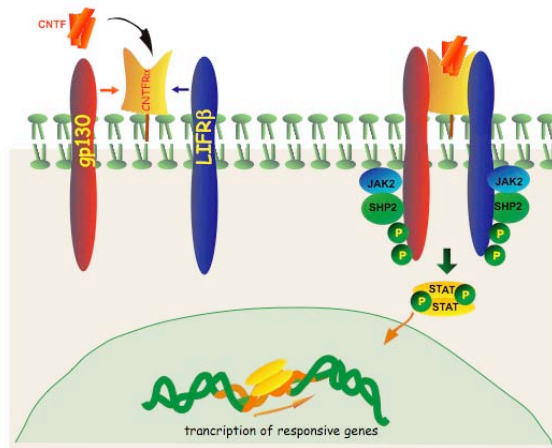


Figure 8: CNTF and its receptors: mode of action

The schema shows how CNTF acts in the cell. The binding of CNTF with the receptor CNTFR α induces the formation of the heterodimeric complex with gp130 and LIFR β . This allowing tyrosine phosphorylation of JAKs, activation of STAT (STAT-3) and translocation of STAT homodimers to the nucleus, where they bind to specific DNA sequences promoting gene transcription.

CNTF acts through a heteromeric receptor complex formed by the subunits CNTFR α , gp130 and LIFR β (Figure 8). The CNTFR α lacks transmembrane and cytoplasmic domains and is attached to the cell membrane by a glycosyl-phosphatidylinositol anchor. The receptor subunit exists in two functional forms, either bound to the membrane or soluble after cleavage by phospholipidase c. CNTFR α has no enzymatic activity, but forms a functional complex with CNTF that promotes its binding with gp130 and LIFR β , ultimately leading to tyrosine phosphorylation, STAT (signal transducers and activators of transcription) activation, dimerisation and translocation to the nucleus, where they bind to specific DNA sequences, leading to enhanced transcription of responsive genes, e.g. VIP, somatostatin, Substance P and ChAT (Stahl and Yancopoulos, 1994; Stahl et al., 1995). Several lines of evidence indicate that CNTF may be a powerful mitigator against to injury of the nervous system. In experimental animals, CNTF has been delivered by direct infusion using osmotic pumps, by daily injections, via implanted cells engineered to express CNTF or encapsulated in a polymer membrane, and by viral vectors. In all cases, CNTF was shown to proserve striatal neurons in a chemically-induced rat model of HD (Anderson et al., 1996; Emerich et al., 1996; Emerich et al., 1997a; de Almeida et al., 2001). The neuroprotective effect of CNTF on GABAergic striatal neurons was also tested in the primate lesion models QA and 3-NP (Emerich et al., 1997b; Mittoux et al., 2000). In a genetic *in vitro* model, CNTF was also shown to protect striatal neurons from death (Saudou et al., 1998).

This led to the initiation of a clinical trial in order to determine the effect of CNTF in HD patients (Bachoud-Levi et al., 2000). In this trial, CNTF was delivered *ex vivo* by

encapsulated BHK cells implanted into the lateral ventricle of HD patients. The trial has demonstrated the feasibility of long-term delivery of CNTF in human brain with the encapsulation technique. However, the diffusion of the factor from the ventricle to the parenchyma is limiting as was shown in primates (Mittoux et al., 2000), therefore providing only little benefit to the patients (Bloch et al., 2004) .

1.4.3. BDNF

Brain-derived neurotrophic factor (BDNF) was the second protein with neurotrophic activities that isolated after the nerve growth factor (NGF) (Barde et al., 1982). Together with NGF, NT-3, NT-4/5, it belongs to the family of neurotrophins, which act through the interaction with two distinct receptors. All four bind to the p75 neurotrophin receptor ($p75^{NTR}$) and to a specific member of the tyrosine kinase (Trk) receptor family, the TrkB. Two alternative splice variants of the TrkB receptor exist: a full-length and a truncated variant. The latter is thought to act as dominant inhibitor and therefore to modulate the trophic effect of neurotrophin. Binding of neurotrophin homodimers causes receptor homodimerization, followed by its phosphorylation at several tyrosine residues (Figure 9). Various signaling cascades are then activated: the PI3K/Akt kinase pathway, the mitogen-activated protein kinase MAPK/ERK pathway and the PLC- γ 1 pathway (Barbacid, 1994).

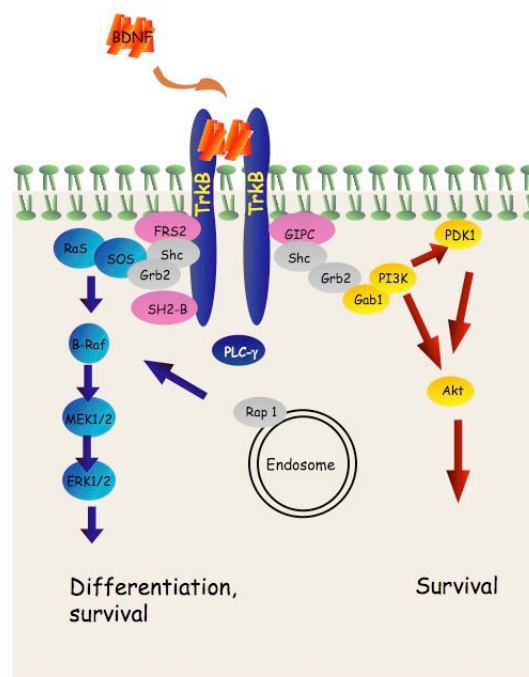


Figure 9: BDNF and its receptors: mode of action

The schema shows how BDNF acts in the cell. The cellular actions of BDNF are mediated through two types of receptors: a high-affinity TRKB and a low-affinity pan-neuretrophin p75 receptor. Binding of BDNF initiates TRKB dimerization and transphosphorylation of tyrosine residues in its cytoplasmic domain. The phosphotyrosine residues of TRKB receptors function as binding sites for recruiting specific cytoplasmic signaling and scaffolding proteins. Binding of Shc (Src Homology domain-containing adaptor protein) results in recruitment of a complex of adaptors like Grb2 and SOS, which activate Ras. Ras in turn activates the PI3K, MEK1/2 pathway.

BDNF is produced by cortical neurons in layers V and VI, anterogradely transported, released and finally taken up by GABAergic striatal neurons (Marty et al., 1997). It is found in the striatum at a concentration of 7 ng/g of tissue, which is decreased by half in aged rats (Kato-Semba et al., 1998; Yurek and Fletcher-Turner, 2001). The receptor TrkB is expressed and is present in both forms in the striatum. BDNF is a potent survival factor for striatal neurons (Ventimiglia et al., 1995) and induces the differentiation of striatal GABA-, calbindin- and DARPP-32 neurons (Nakao et al., 1995). BDNF and TrkB are upregulated after QA, 3-NP or colchicine lesions in the striatum and might serve, therefore, as an endogenous neuroprotective system (Alberch et al., 2004). BDNF knock-out mice die prematurely (Jones et al., 1994; Ivkovic et al., 1997) and the cortical postnatal ablation of BDNF induces early striatal dendrite deficits reminiscent of HD transgenic mice (Baquet et al., 2004). Intra-striatal administration of BDNF protects striatal neurons from excitotoxicity *in vivo* (Martinez-Serrano and Bjorklund, 1996; Bemelmans et al., 1999; Perez-Navarro et al., 2000b; Gratacos et al., 2001b). BDNF may play a direct role in HD, since it is decreased in affected patients, transgenic mice models and cell cultures (Zuccato et al., 2001). In addition, BDNF induces neurogenesis in the adult brain. Therefore it could have the dual effect of being neuroprotective and also promoting neurogenesis (Zigova et al., 1998; Pencea et al., 2001). BDNF is therefore one of the interesting candidates for gene therapy strategies in HD because of its implication in the disorder and its protective effects on striatal projection neurons. However, its neuroprotective properties still need to be verified in genetic animal models of HD.

1.5. DELIVERY OF NEUROTROPHIC FACTORS IN THE CNS

The systemic delivery of neurotrophic factors has major disadvantages. It is often associated with side effects as their receptors are widely distributed in the organism and the proteins do not cross the blood-brain barrier. Therefore direct CNS delivery is proposed to avoid the limitations of systemic delivery. Recombinant protein delivery, especially of neurotrophic

factors, has shown promising success in animal models of neurodegenerative diseases and is currently being tested in clinical trials with GDNF for PD (Gil et al., 2002), CNTF for HD (Bachoud-Levi et al., 2000) and NGF for AD (<http://www.clinicaltrials.gov/ct/gui/show/NCT00017940>).

Several different strategies have been developed to directly deliver proteins in the brain. The choice of method depends on the application, the delivery area, the treatment duration and the balance of risks versus benefits. The delivery of GDNF with mechanical pumps has brought new prospects to PD patients, although long-term problems could be associated with the infusion device and the high amount of delivered GDNF. Alternatively, *ex-vivo* gene therapy approaches could be designed to locally deliver neurotrophic factors from encapsulated cells or a graft of engineered cells. Finally, gene therapy with viral vectors could bring an efficient alternative to the issues of long-term delivery of protein in the CNS. In our studies we have chosen the viral vector approach, because it was shown to efficiently infect striatal neurons and constantly deliver proteins, which is particularly interesting for long-term experiments with transgenic mice. Moreover, due to the small size of mice, the implantation of mechanical devices in the brains would be very difficult to realize technically. In addition, viral vectors have the valuable advantages that after production they can be stored for later use and that the surgery is simple compared to other devices.

1.5.1. Viral vectors for gene therapy

Gene therapy is a novel approach to treat diseases by directly modifying gene expression. Thus, genes become the *pharmaceutical agent* and gene therapy the tool for drug delivery (Kay et al., 2001). Replacement of defective genes, expression of novel genes or even silencing of toxic genes can be considered. Although this may be seen as a simple concept, this new technique implies technical, ethical and bio-safety concerns. The challenge is to develop approaches for the delivery of genetic material to the target cells that operate in an efficient, specific and safe way.

The feasibility and efficacy of gene therapy was demonstrated for the first time with the clinical trial of a therapy for severe combined immunodeficiency (SCID) (Bordignon et al., 1995; Bordignon, 1998; Cavazzana-Calvo et al., 2001; Aiuti et al., 2002). Bone-marrow stem cells taken from the patient were genetically engineered to express the defective gene (ADA or SCID) with viral vectors. The successful treatment of the first patients was much welcomed in the scientific and medical community. The sudden and sad news of two treated children who developed a leukemia-like disease turned this first trial into a general alarm against gene therapy. Genetic analysis of the malignant cells showed that the gene was inserted in a locus which activates oncogene LMO2 (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b; Cavazzana-Calvo et al., 2004) and the clinical protocol was inducing a clonal selection. Thus the balance of risks and benefits has to be evaluated individually, because gene therapy represents a life-saving treatment for most of the SCID children.

In vivo gene therapy for neurodegenerative diseases presents additional difficulties. The brain is probably the organ that is the least accessible to therapeutic intervention. In addition, the terminally differentiated state of neurons precludes the use of conventional retroviral vectors, which require cell replication for stable integration into the genome. Several viral vectors have been developed to overcome this barrier, such as viruses derived from the Herpes Simplex Virus, the Adeno-associated Virus (AAV) and the Lentivirus (Lv). These last two vectors have interesting properties for a long-term and sustained gene delivery in the brain. The choice of adequate vector strictly depends on the therapeutic or experimental goal. High transduction efficiency in the striatum combined with a large cloning capacity makes Lv the ideal vector to model HD and to evaluate therapeutic strategies. On the other hand, AAVs (serotype 2) have shown a high affinity for tyrosine hydroxylase (TH)-positive substantia nigra neurons, which could be an advantage for PD therapy (Kirik and Bjorklund, 2003;

Nakai et al., 2003). Moreover, the property of being retrogradely transported from the muscle nerve terminal to the spinal cord makes this vector attractive for ALS (Kaspar et al., 2003). Recently, a rabies-G pseudotyped equine infectious anaemia virus (EIAV) expressing VEGF, which was injected in muscles and retrogradely transported to motoneurons in the spinal cord, showed efficient gene delivery associated with behavioral improvement and survival of SOD-1 transgenic mice (Azzouz et al., 2004).

1.5.2. Lentiviral vectors

Replication-defective and multiply attenuated lentiviral vectors have several features suitable for gene delivery into neurons. They efficiently infect quiescent cells and long-term sustained transgene expression is observed. Moreover, the absence of sequences encoding toxic viral proteins and the large cloning capacity of about 9 kb (Naldini, 1998) makes them ideal for gene therapy. Lentiviral vectors are therefore interesting for neurodegenerative disease research, since they can be used not only to model genetic diseases (such as Parkinson's disease and Huntington's disease (de Almeida et al., 2002; Lo Bianco et al., 2002; Kirik and Bjorklund, 2003; Régulier et al., 2003)), but also to administer therapeutic molecules in various animal models of these diseases (Kordower et al., 1999; Bensadoun et al., 2000; Déglon et al., 2000; de Almeida et al., 2001; Lo Bianco et al., 2002; Palfi et al., 2002).

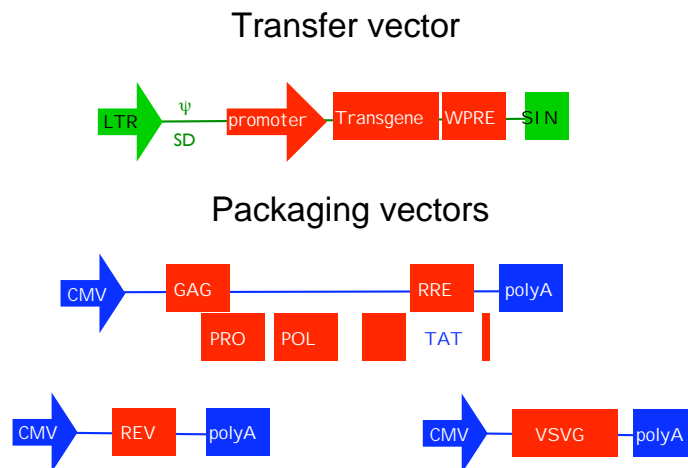


Figure 10: The four plasmids used to generate lentiviral vectors

The third generation of HIV-1-derived lentivirus vector is produced by cotransfection of four plasmids, consisting of the split tat-less packaging vectors, the REV plasmid, the heterologous envelope vesicular stomatitis virus G-glycoprotein (VSV-G), and the improved SIN transfer vector.

As human lentiviral vectors are derived from the HIV-1 virus, bio-safety is one of the major concerns for potential gene therapy applications. Several strategies in vector design have been applied to minimize the remaining risk and a first clinical trial is current underway with this vector (Dropulic, 2004)(NIH 0107-488). Alternatively, equine (EIAV), simian (SIV) and feline (FIV) viruses are developed as vectors to minimize the risk of viral genome recombination with wild type HIV. The standard strategy is to delete viral genes that are not essential for gene delivery. The essential genes are then divided in several plasmids (rev, gag-pol, env) plus one plasmid containing the gene of interest, which is called transfer vector (Figure 10). Deletion of the packaging sequence Ψ in the packaging plasmids will produce vectors without viral genomes. Only the transfer vector will be packed in the viral particle. A partial deletion of the 3' LTR leads to the production of self-inactivating vectors (SIN) where the promoter is inactivated.

This first generation of viral vectors was improved using several strategies. The wild-type envelope gene (env) was deleted and replaced with a gene encoding the heterologous envelope protein from the vesicular stomatitis virus (VSV-G), expressed in a separate plasmid. This makes the viral vector more robust, allowing purification with ultracentrifuges and increasing the host range. A significant increase in transduction efficacy is achieved by including the central polypurine tract (cPPT), which increases nuclear translocation of the viral vector genome (Follenzi et al., 2000; Sirven et al., 2000; Zennou et al., 2000). An enhanced transgene expression is achieved upon introducing the Woodchuck post-transcriptional regulatory element (WPRE), which stabilizes the RNA-protein complex enhancing protein expression (Zufferey et al., 1999; Déglon et al., 2000). Finally, major efforts to design regulated transgene expression single vectors are currently underway.

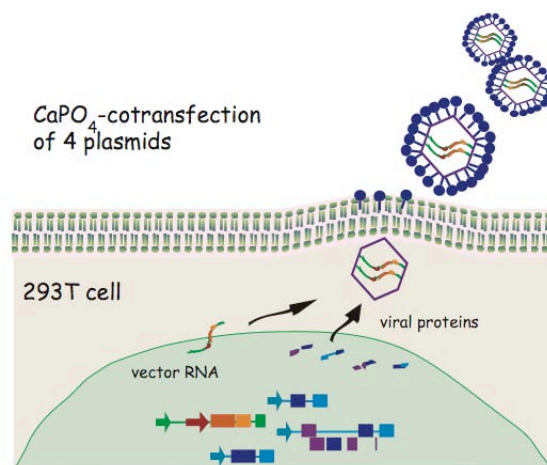


Figure 11: Lentiviral vector production

Upon transfection of all four plasmids into human 293T cells, high titers of replication-defective, self-inactivating vectors are produced.

The recombinant viruses are produced by transient transfection of human 293T cells (**Figure 11**). The viral vectors are produced by the cells and secreted in the medium. The viral vector is concentrated by ultracentrifugation or purified by ultrafiltration. The preparation can then be used for *in vitro* applications by just adding the viral preparation to the medium or it can be delivered to animals. In both cases, the infection is an abortive process as the viral genome is deleted in the recombinant Lv. Infected cells are therefore unable to produce new viral vectors (**Figure 12**).

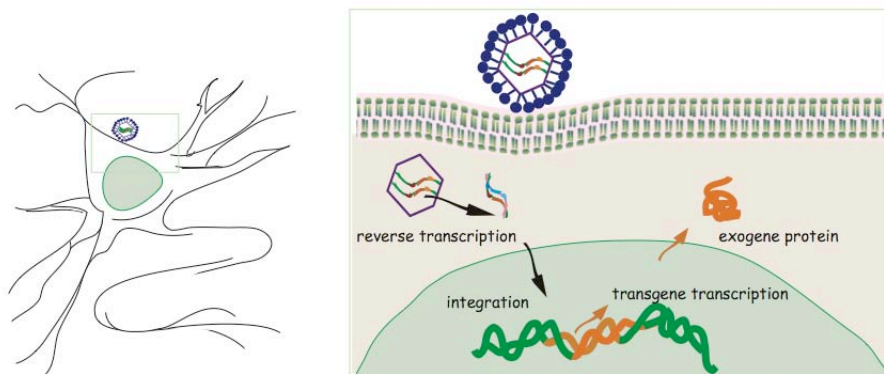


Figure 12: Lentiviral-mediated production of exogenous protein in a target cell

VSV-G pseudotype interacts with the membrane of the target cell (here a neuron) and allows entry to the cytoplasm by the endocytic pathway (Aiken, 1997). The virion core is uncoated to expose the viral nucleoprotein complex. The modified virion is reverse transcribed. This virion is also called a pre-integration complex in which viral cDNA intermediates are synthesized and integrated into the host chromosome. The integration site within the host cell genome appears to be random, although a preference for kinked or distorted DNA has been observed (Frankel and Young, 1998). The transgene can now be transcribed in the host cells. As the packaged virion does not contain any encode for viral proteins, the virus itself will not be produced in the infected cell.

1.6. AIMS AND STRATEGIES OF THIS THESIS

Despite major research efforts on Huntington's disease (more than 3'000 Pubmed references in the last 10 years), no clear pathological mechanisms and no validated treatment have been proposed. To gain insight into the pathology, the main aim was to develop a new cellular model of the disease, characterized by a general chronic and severe pathology in striatal neurons, thus allowing an extensive dissection of the pathology with a large range of technologies, spanning from immunocytochemistry, biological, enzymatic activity tests and gene expression analysis. Once this goal was achieved, therapeutic strategies were evaluated first in this cellular model, and then in genetic animal models.

To develop the new cellular model, primary rat striatal cultures were infected with lentiviral vectors to express a mutant huntingtin fragment. The two-month survival of the culture combined with the transgene expression in most of neuronal cells are both indispensable elements, which allow the reproduction of major features of Huntington's disease neuropathology. Once the model was characterized, neuroprotective strategies were tested. The effects of two neurotrophic factors CNTF and BDNF, were assessed. Both were previously described to protect striatal neurons in other experimental paradigms. The success of neuronal protection was then tested on genetic animal models, both transgenic mice and lentiviral-mediated rat models showing neuronal death. Lentiviral vector injections in parenchyma assured the long-term delivery of the trophic factors in the rodent striatum. Neuronal protection was evaluated with behavioral and immunohistochemical analysis.

2. PRIMARY STRIATAL CULTURES EXPRESSING MUTANT

HUNTINGTIN: CHRONICLE OF A FORETOLD DEATH

Submitted as:

Neuronal dysfunction and cell death in primary striatal cultures expressing a mutant huntingtin fragment via a lentiviral vector

Diana Zala, Alexandra Benchoua, Emmanuel Brouillet, Anne D. Zurn, Patrick Aebischer and Nicole Déglon

2.1. ABSTRACT

Several cellular models have been developed following the cloning of *huntingtin* (*htt*), the gene that is mutated in individuals with Huntington's disease (HD). However, none of these models allows biochemical and molecular dissection of mutant *htt*-induced cell death in striatal neurons, the most relevant cell type for HD pathology. In this study, we have used a lentiviral vector expressing the first 171 amino acids of the mutant *htt* protein (*htt171-82Q*) to generate a chronic model of HD in rat primary striatal neurons and reproduce the pathological events leading to cell death. The transduction of primary cultures with lentiviral vectors allowed the analysis of long-term cultures with more than 95% of neurons infected. The first sign of pathology in cells expressing the *htt* mutant fragment was the appearance of nuclear and neuritic inclusions at 4 weeks post-infection. These inclusions progressively accumulated thereafter, with a concurrent loss of neurofilaments. By 8 weeks, the number of NeuN- and MAP-2-positive cells was significantly lower in *htt171-82Q*-infected cultures than in controls. The decrease in NeuN and MAP-2 positive cells was associated with a decrease of ubiquitin-positive cells and an increase of TUNEL staining indicating neuronal loss. Cortical cultures infected with the same vector accumulated numerous inclusions but showed no alterations in the levels of NeuN or MAP-2 expression. Finally, co-transfection with *htt171-82Q* and lentiviral vectors encoding the neurotrophic factors CNTF or BDNF prevented striatal dysfunction and degeneration, but did not alter inclusion formation. This cellular model of HD, which displays progressive and selective striatal pathology over a period of 2 months, provides a flexible system for quantitative analysis and dissection of the intracellular pathways underlying mutant *htt*-mediated cell death.

2.2. INTRODUCTION

Huntington's disease (HD) is a progressive, fatal, autosomal dominant disorder of the nervous system characterized by uncontrolled movements, mood imbalance, depression, and dementia. The mutation leading to HD has been identified as a glutamine expansion in the N-terminal portion of htt (The Huntington's Disease Collaborative Research Group, 1993). In the brain, htt is mainly expressed in neurons (Sapp et al., 1997). The striatum is the main site of degeneration in individuals with HD, although other structures are affected at later stages (Vonsattel et al., 1995). *In vitro* models of HD have been developed using various cell lines, including those of neuronal origin (Cooper et al., 1998; Hackam et al., 1998; Liu, 1998; Lunkes and Mandel, 1998; Li et al., 1999; Rigamonti et al., 2001; Zuccato et al., 2003), transfected neuronal cultures (Saudou et al., 1998; Sanchez et al., 1999; Goffredo et al., 2002), primary neurons from HD transgenic mice and striatal cell lines derived from knock-in mice (Trettel et al., 2000; Petérsen et al., 2001a; Snider et al., 2003; Zeron et al., 2004). These models have been used to identify intracellular pathways involved in HD pathology. Studies suggest that mutant htt is cleaved by proteases (caspases, calpains, and aspartyl proteases) and that the toxic N-terminal product containing the polyglutamine tract is translocated into the nucleus where it forms inclusions, sequesters transcription factors and binds to several essential proteins (Sanchez Mejia and Friedlander, 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Bates, 2003; Sugars and Rubinsztein, 2003). In addition, mutant htt aberrantly interacts with proteins in the cytoplasm leading to caspase activation, mitochondrial toxicity, and the formation of neuritic aggregates that block axonal transport (Ross, 2002; Gunawardena et al., 2003). However, the function of wild type htt, the neuronal dysfunctions induced by the CAG expansion, and the sequence of events leading to neuronal death are still poorly understood.

To gain further insight into the pathological events induced by mutant htt, HD models suitable for analytical studies and showing a progressive but severe striatal pathology are required. Unfortunately, primary striatal cultures obtained from transgenic mice expressing mutant huntingtin display subtle changes in intracellular pathways, but not cell death (Petérsen et al., 2001a; Hermel et al., 2004; Zeron et al., 2004). In contrast, neuronal degeneration and cell death have been detected in striatal cultures transfected with mutant htt fragments, but transfection efficiency was too low to allow extensive biochemical analysis (Saudou et al., 1998). Thus, we have developed an *in vitro* model of HD by infecting E16 rat striatal neurons

with a lentiviral vector encoding the first 171 amino acids of mutant (82Q) htt. The high transduction rate and sustained transgene expression obtained with lentiviral vectors offers a new opportunity to analyze mutant htt-induced cell death with cytological, molecular and biochemical methods. In addition, the data gathered from this model can be rapidly validated *in vivo* with the same gene transfer system (de Almeida et al., 2002; Régulier et al., 2003).

2.3. MATERIALS AND METHODS

2.3.1. Cell cultures

Timed-pregnant Sprague Dawley rats (Charles Rivers Laboratories, Les Oncins; France) were killed by CO₂ inhalation and embryos (E16) were collected in a Petri Dish and placed on ice. Dissections were performed under a stereomicroscope in ice cold dissection medium (DMX) containing Ca²⁺- and Mg²⁺-free phosphate buffer saline (PBS), 0.6% D-Glucose, 1% Pen-Strep (10000 U/ml, 10000 µg/ml), and 10mM HEPES (Invitrogen AG, Basel; Switzerland). Ganglionic eminences or cerebral cortices were isolated, cut into pieces using forceps and collected in a 15 ml Falcon tube. The medium and buffer were removed by decanting on ice. The tissue was homogenized by repeated pipetting with a fire-polished Pasteur pipette in DMX containing 1% bovine serum albumin (BSA) (Fluka, Buchs; Switzerland). Cells were centrifuged at 4°C for 5 min at 1000 g and resuspended in 10 ml of glutamate-free Neurobasal medium containing 1% B27 (Invitrogen) 1% Pen-Strep (10000U/ml, 10000µg/ml), 0.5mM L-Glutamine and 15mM KCl. Cells were plated at a density of 150 000 cells/cm² in multi-well dishes coated with 20 µg/ml Poly-L-lysine (Sigma, Buchs; Switzerland). The cultures were maintained in a humid incubator (5% CO₂, 95% air at 37°C) and half of the medium was changed weekly. Cultures were kept for up to 8 weeks. CNTF and BDNF treatments were carried out by adding 50 ng/ml of the appropriate recombinant protein to the media 3 times a week starting week 4.

2.3.2. Lentiviral vector production and infection

cDNAs encoding enhanced GFP (Clontech, Palo Alto; USA), nuclear localized β-galactosidase (nls-LacZ), human CNTF (murine immunoglobulin signal peptide [nucleotides 13-60, Genbank K00608] fused to the human CNTF cDNA [nucleotides 84-684, Genbank NM000614]), human BDNF (human pre-proNGF domain [nucleotides 170-532, Genbank X52599] fused to the human BDNF [nucleotides 681-1040, Genbank X60201]) and the first 171 amino acids of mutant human huntingtin fragments containing either 19 or 82 CAG repeats (htt171-19Q/82Q) were cloned into the SIN-W-PGK transfer vector (Déglon et al., 2000; de Almeida et al., 2001; Régulier et al., 2003). The viral productions were performed in 293T cells with a four plasmid system as described previously (Déglon et al., 2000; de Almeida et al., 2001; Régulier et al., 2003). The viruses were resuspended in (PBS) with 1% BSA and matched for particle content of 1500 ng p24 antigen/ml as measurement by

ELISA (Perkin Elmer Life Sciences; USA). The cell cultures were infected with lentiviral vectors at ratio of 10 ng of p24 antigen/ 10^5 cells one day after plating (1 DIV). Co-infections were carried out using two viral vectors, each at a ratio of 10 ng p24 antigen/ 10^5 cells. At 2 DIV, half of the medium was replaced with freshly prepared culture medium.

2.3.3. Immunostaining

Cell cultures were washed with cold PBS and fixed in 4% paraformaldehyde (Fluka) for 10 min at 4°C. Cultures were subsequently washed with PBS and then incubated in a blocking solution of PBS supplemented with 10% normal goat serum (NGS) (Dakocytomation, Zug; Switzerland) and 0.03% Triton X-100 (Sigma). The cells were then incubated overnight at 4°C in blocking solution containing a primary antibody and then for 2h at room temperature with secondary antibodies coupled to fluorophores (Jackson Immunoresearch Laboratories, West Grove; USA) or biotin (Vector laboratories, Peterborough; UK). Cells incubated with biotin-coupled secondary antibodies were further processed using the Vectastain Elite ABC detection kit (Vector laboratories, Peterborough; UK) and diaminobenzidine tetrahydrochloride, according to the manufacturer's recommendations. The following antibodies and dilutions were used: mouse monoclonal 2B4: 1/1000 (gift from Yvon Trottier, CNRS/INSERM/ULP, Illkirch, France (Lunkes et al., 2002)), rabbit polyclonal EM48: 1/1000 (kindly provided by Dr. X. J. Li, Emory University School of Medicine, Atlanta, GA (Li et al., 1999)), mouse monoclonal mEM48 (Chemicon International Inc., Temecula; USA), rabbit polyclonal GFAP: 1/300 (Molecular Probes, Leiden; Netherlands), rabbit polyclonal Hsp70: 1/200 (Stress-Gene, San Diego; USA), rabbit polyclonal LacZ: 1/1500 (Promega, Madison; USA), mouse monoclonal MAP-2: 1/600, mouse monoclonal NeuN: 1/400 (Chemicon International Inc., Temecula; USA), mouse monoclonal NF160: 1/400 (Sigma Chemicals, Deisenhof; Germany) and rabbit polyclonal Ubiquitin: 1/1000 (Dakocytomation, AG, Zug; Switzerland). To detect endogenous and exogenous htt (2B4), cultures were pre-treated for 30 min with 1% cyanoborohydride (Fluka) and then incubated for 30 min in 0.4% Triton X-100 in PBS.

Confocal microscopy was performed using a Leica TCS SP2 AOB2 microscope (Heidelberg; Germany). Cell cultures stained with NeuN or MAP-2 were counted using an Olympus CK40 microscope (Alberstland; Danemark) at a magnification of x20. At least eight fields of view were counted for each of the samples stained with a given antibody and the mean number of cells were calculated and normalized using non-infected cells from the same plate. Duplicates

of three independent experiments were analyzed for each group.

2.3.4. Flow cytometry analysis of GFP expression and DNA fragmentation

GFP-infected cells (1×10^6 at 7 DIV) were treated with 1% Trypsin, 1 mM EDTA (Invitrogen) for 15 min. A solution of 10% fetal bovine serum (FBS) was added to stop the reaction. The cells were dissociated, centrifuged at 1500g for 10 min, resuspended in PBS, fixed with PBS containing 2% paraformaldehyde (Fluka) for 10 min on ice, centrifuged at 1000g for 5 min and resuspended in 500 μ l of PBS. Their GFP content was then measured.

DNA fragmentation analysis was performed using the Cell Death FITC kit (Roche Pharma, Basel; Switzerland). Trypsinization and fixation of the cultures were performed as described for above for the GFP quantification. Cells were then permeabilized by incubation with 0.1% saponin for 10 min at RT and processed for TUNEL staining according to the manufacturer's instructions. As controls, cells were processed for the TUNEL reaction in the absence of transferase (negative control) or following pre-incubation with DNase I (positive control) to determine the gate of interest for FACS analysis. Cell counts were performed on FACScalibur cell sorter on four sister cultures and analyzed using the CellQuest software (BD Bioscience Franklin Lakes; USA). Data from the TUNEL analysis were quantified by subtracting the background counts obtained from analyzing non-infected cultures from the counts obtaining with treated cultures.

2.3.5. Western Blot and ELISA

Cultures were treated with 1% Trypsin and 1 mM EDTA (Invitrogen) for 15 min and then centrifuged for 10 min at 1000g. The pellets were lysed by a 30 min incubation on ice with RIPA-modified lysis buffer (150 mM NaCl, 50 mM TRIS pH 8.0, 5 mM EDTA, 0.5% Triton and 0.5% protease inhibitor cocktail (Sigma)) and vortexing every 10 min. The lysates were centrifuged at 13,000 g for 30 min at 4°C, and the supernatants were stored at -80°C. Protein concentrations were determined using the BCA protein Assay (Pierce, Rockford; USA). For Western Blot analysis, proteins were denatured for 5 min by boiling in an SDS buffer (10% sucrose, 1.2% SDS, 5% β -mercaptoethanol and 0.025% bromophenol blue). Protein extracts were then subjected to SDS-PAGE on 15% acrylamide gels and electro-blotted onto polyvinylidene difluoride membranes (Bio-rad, Hercules, CA, USA). Membranes were incubated overnight at 4°C in 5% non-fat milk with primary antibodies (GFP: 1/1000

(Molecular Probes), Hsp70 1/1000 (Stress-Gene, San Diego; USA), myc 1/1000 (Upstate Biotech Chicago, IL) and NF160 1/1000 (Sigma)) diluted in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.05% Tween 20) with % non-fat dry milk at 4°C. After the incubation for 1 hr at room temperature with a secondary horseradish-peroxidase conjugated antibody (1/5000; Amersham), antigens were revealed by enhanced chemiluminescence using the ECL+ kit (Amersham) according to the manufacturer's instructions.

The concentrations of CNTF and BDNF were determined by ELISA (R & D, Abingdon, UK) on media samples collected at 8 weeks and stored at -20°C until use. Samples were processed accordingly to the manufacturer's instructions. The detection limits were 8 pg/ml for CNTF and 20 pg/ml for BDNF.

2.3.6. Statistical Analysis

Data are expressed as the mean +/- standard error of the mean (SEM). One-way ANOVA was used to analyze the time course of ubiquitination. For the kinetic analysis of NeuN expression, two-way ANOVA was performed with with independent variables of time factor (df = 3) and group factor (df = 2). *Post hoc* analysis with LSD test was applied as a follow-up for the significant differences shown by ANOVA for both time and dosage factors. A cut-off value of $p < 0.05$ was used for statistical significance. Statistical analysis was performed using the Statistica software version 5 (StatSoft, Tulsa, USA).

2.4. RESULTS

2.4.1. GFP-expressing striatal neurons can be cultured for up to 8 weeks

We constructed a recombinant lentiviral vector expressing the GFP under the phosphoglycerate kinase (PGK) promoter to assess the transfection efficiency of primary striatal cultures. One week after infection, FACS analysis revealed that 92-94% of the cells were GFP-positive (Figure 13A). Stable transgene expression (Figure 13B) and high neuronal survival (Figure 13C) were observed for up to 8 weeks, allowing long-term analysis. Co-infection of striatal cultures with two different vectors expressing GFP or nls-LacZ revealed that most of the cells integrated at least two proviruses and expressed both reporter genes (Figure 13E). These results show that this system can be used to co-express mutant htt and either reporter or therapeutic genes with the aim of dissecting the intracellular pathways involved in mutant htt toxicity.

The phenotype of the striatal cells was analyzed using the neuronal and astrocytic markers NeuN and GFAP, respectively. At 2 weeks, $84\pm 2\%$ of the cell in the culture were NeuN-positive and $8\pm 2\%$ were GFAP-positive. Figure 13D shows that in infected cultures, GFP-expressing cells had a neuronal morphology and expressed NeuN. More than 95% of the NeuN-positive cells expressed GFP, whereas GFP-expressing astrocytes were rarely observed (Figure 13D). Confocal microscopy demonstrated the segregation between GFAP and GFP labeling (Figure 13D). However, transgene expression in astrocytes was observed when the cultures were infected with a vector containing the GFAP promoter (Figure 14). The PGK promoter was chosen for all subsequent experiments to recapitulate the preferential expression of huntingtin in neurons (The Huntington's Disease Collaborative Research Group, 1993; DiFiglia et al., 1995; Landwehrmeyer et al., 1995; Ferrante et al., 1997).

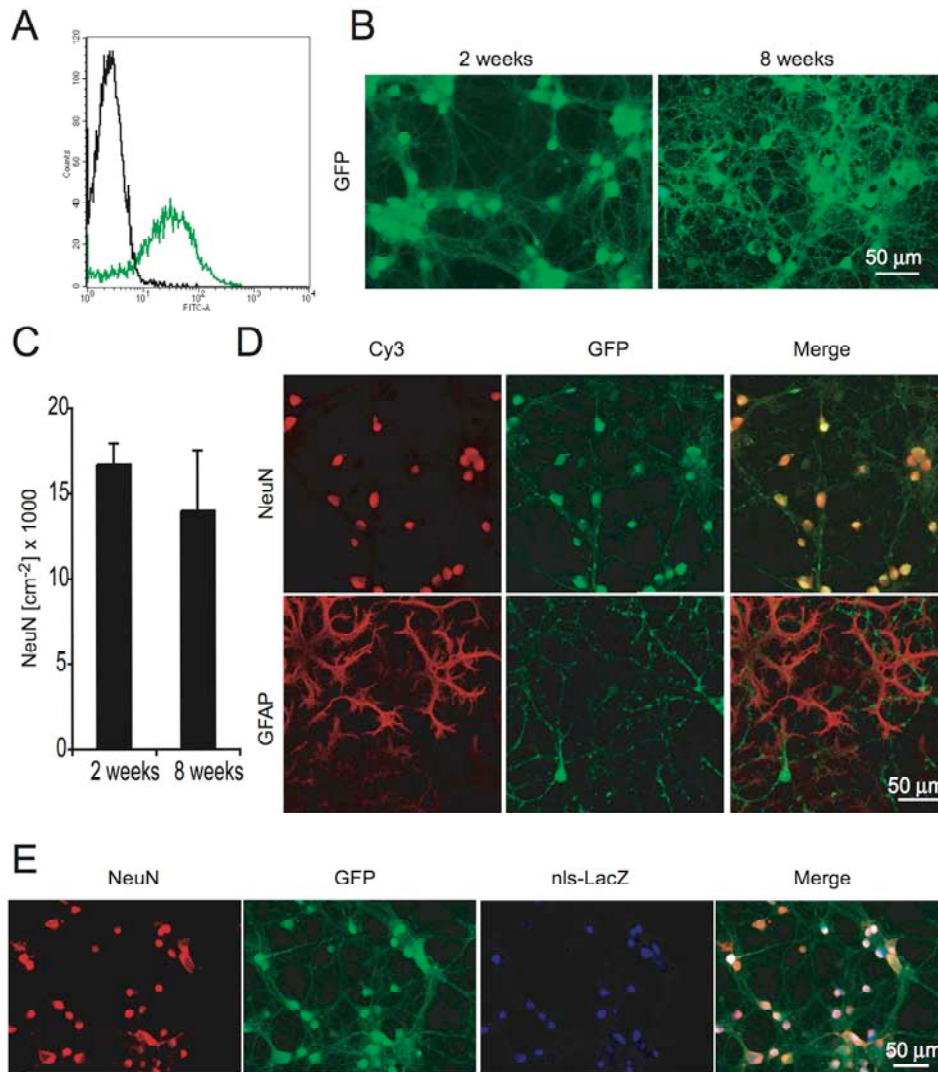


Figure 13: High transduction efficiency and long-term expression of the GFP transgene in striatal neurons

(A) The transduction efficiency of primary striatal neurons was quantified by FACS at one week post-infection using a GFP-expressing vector. A shift in FITC-A fluorescence (relative to controls) was observed in 92-94% of the GFP-infected cells. (B) Cultures infected at DIV 1 with a GFP-expressing vector were fixed 2 and 8 weeks later to assess the expression of the reporter gene. (C) The long-term survival of striatal cultures was assessed using the neuronal marker NeuN. No significant decreases in NeuN density were observed between 2 and 8 weeks post-infection (mean \pm SEM; $p = 0.43$) (D) The phenotype of GFP-infected cultures was analyzed using the NeuN and GFAP markers. Most of the green fluorescent cells have neuronal morphology and express the neuronal marker, NeuN. Confocal microscopy revealed that the GFP and GFAP markers were segregated, indicating that astrocytic expression did not occur. (E) Striatal cultures were co-infected at DIV 1 with two independent vectors expressing the GFP and nls-LacZ reporter genes and fixed 2 weeks later. NeuN and LacZ were detected by immunostaining and fluorescence was detected directly. The merged picture shows that a high proportion of infected neurons express both transgenes.

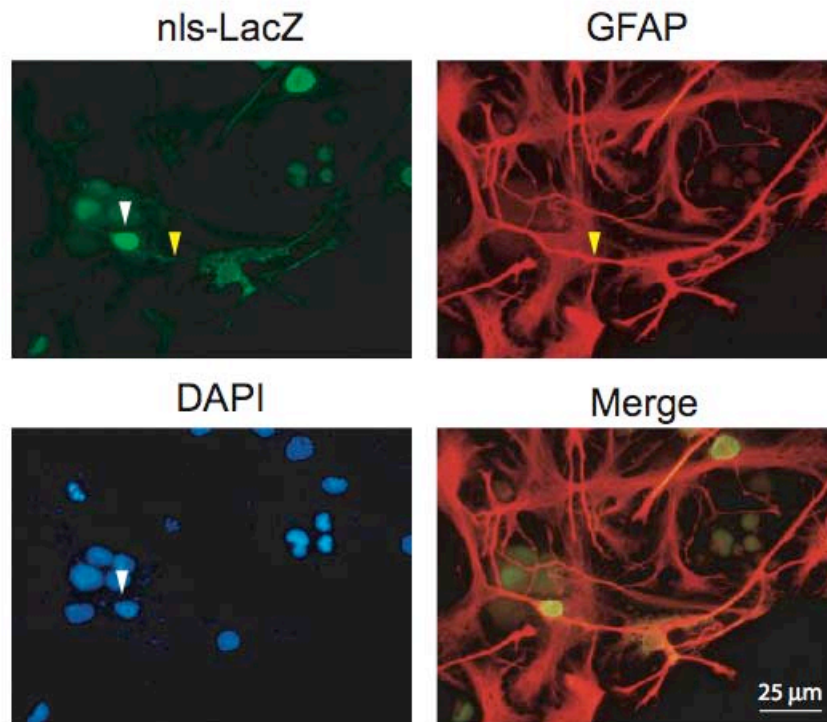


Figure 14: Lentiviral-mediated expression in astrocytes

Primary striatal cultures were infected at DIV 1 with a lentiviral vector encoding nuclear LacZ (nls-LacZ) under the control of the GFAP promoter. Cultures were fixed at weeks 2 and stained for LacZ, GFAP and DAPI. The merged picture shows LacZ staining in a GFAP-positive cell.

2.4.2. Mutant htt accumulates in neurons and forms nuclear and neuritic inclusions

To model HD *in vitro*, we infected striatal cultures with a lentiviral vector encoding htt171-82Q. Non-infected cultures or cultures infected with htt171-19Q, GFP were used as controls. Expression of mutant htt was detected using the 2B4 antibody, which recognizes the N-terminal part of both endogenous rat htt and human htt171-19/82Q (Lunkes et al., 2002) (Figure 15A). The transgene was expressed in most neurons and progressively accumulated over time (Figure 15A). At 8 weeks post-infection, nuclear and neuritic inclusions were present in the majority of the htt171-82Q-infected neurons (Figure 15A). Human htt171-19/82Q expression was confirmed by Western blot analysis using an antibody recognizing the myc epitope located at the N-terminus of the htt-171-19/82Q fragments. Fragments with molecular weights corresponding to those predicted for htt-171-19/82Q fragments, were present at 6 weeks post-infection (Figure 15B). Interestingly, mutant htt171-82Q was more abundant than the wild type htt171-19Q protein. The progressive accumulation of the mutant htt protein and the relative abundance of this protein compared to wild type suggest that

proteosomal degradation of mutant htt is impaired (Jana et al., 2001).

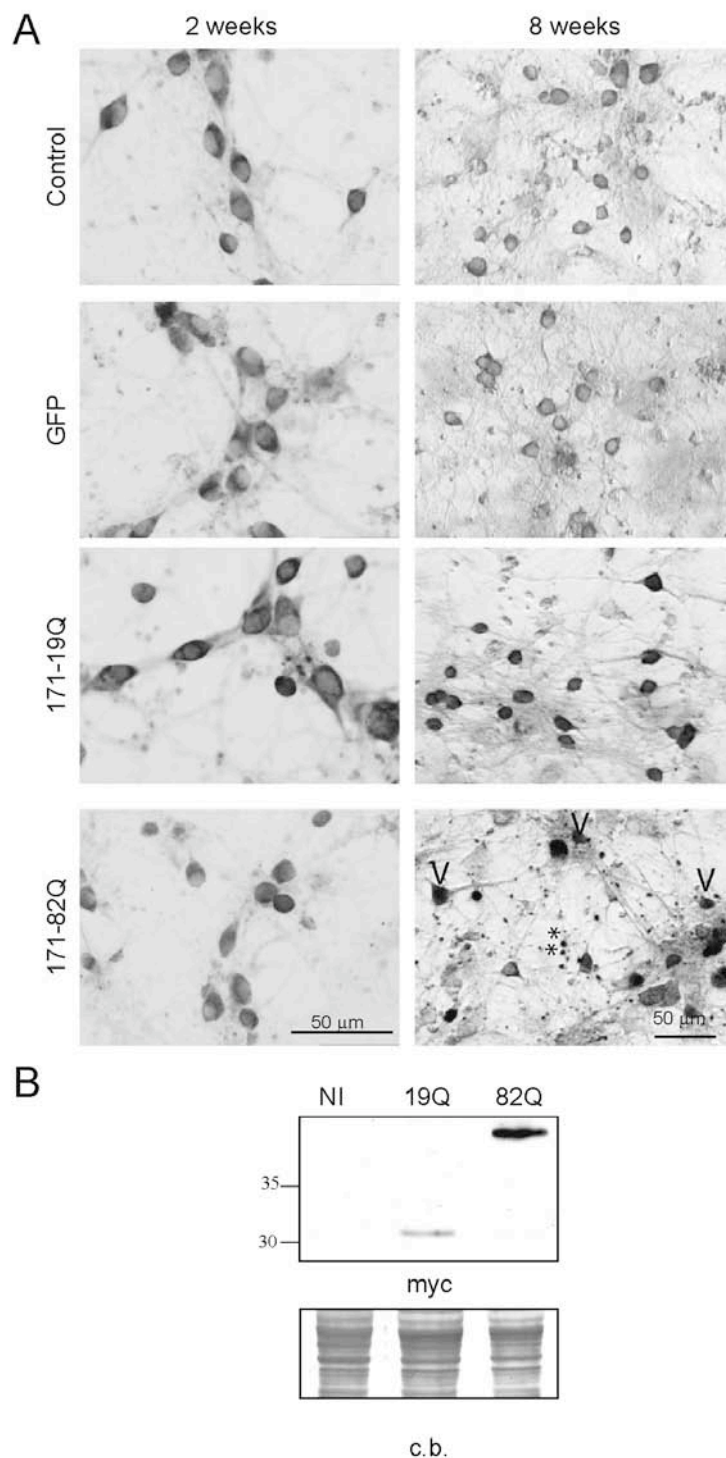


Figure 15: Expression of htt in striatal cultures and formation of htt inclusions

(A) Control or infected (GFP, htt171-19/82Q) striatal cultures were fixed at 2 and 8 weeks post-infection and stained with the 2B4 antibody recognizing the N-terminal part of htt. Note the level of endogenous rat huntingtin staining in control and GFP-infected cells. Wild type human htt171-19Q fragment is present in the cytoplasm of neurons and does not lead to the formation of aggregates, whereas mutant htt171-82Q progressively forms nuclear (V) and neuritic aggregates (*). (B) Western blot analysis using the myc antibody showed that htt171-19/82Q was present at 6 weeks post-infection and that mutant htt fragment accumulated in the cells. Coomassie Blue (c.b.) staining is shown as a control to assess protein loading.

2.4.3. Mutant htt leads to the progressive formation of ubiquitinated nuclear inclusions

The presence of ubiquitinated nuclear inclusions in neurons is a typical feature of HD. We therefore monitored the appearance of these aggregates over time. As expected, ubiquitin-positive inclusions were not detected in the GFP- or htt171-19Q-infected cultures. No inclusions were present in the htt171-82Q-infected cultures up to 2 weeks post-infection (Figure 16B). At 4 weeks, $31\pm 3\%$ of neurons had at least one ubiquitinated nuclear inclusion. Figures 3A and C show the distribution and variability in the number and size of the nuclear inclusions in mutant htt-infected cells. After 6 weeks, the proportion of ubiquitin-positive inclusions reached $56\pm 3\%$ (Figure 16B). Between 6 and 8 weeks, this percentage declined to $46\pm 2\%$, probably as a result of neuronal death. A subset of mEM48-positive nuclear inclusions were not labeled with the ubiquitin antibody at 6 weeks (Figure 16B; $63\pm 3\%$ of neurons had mEM48-positive inclusions vs. $56\pm 3\%$ with ubiquitin-positive inclusions). Similar results have been obtained previously (Gutkunst et al., 1999).

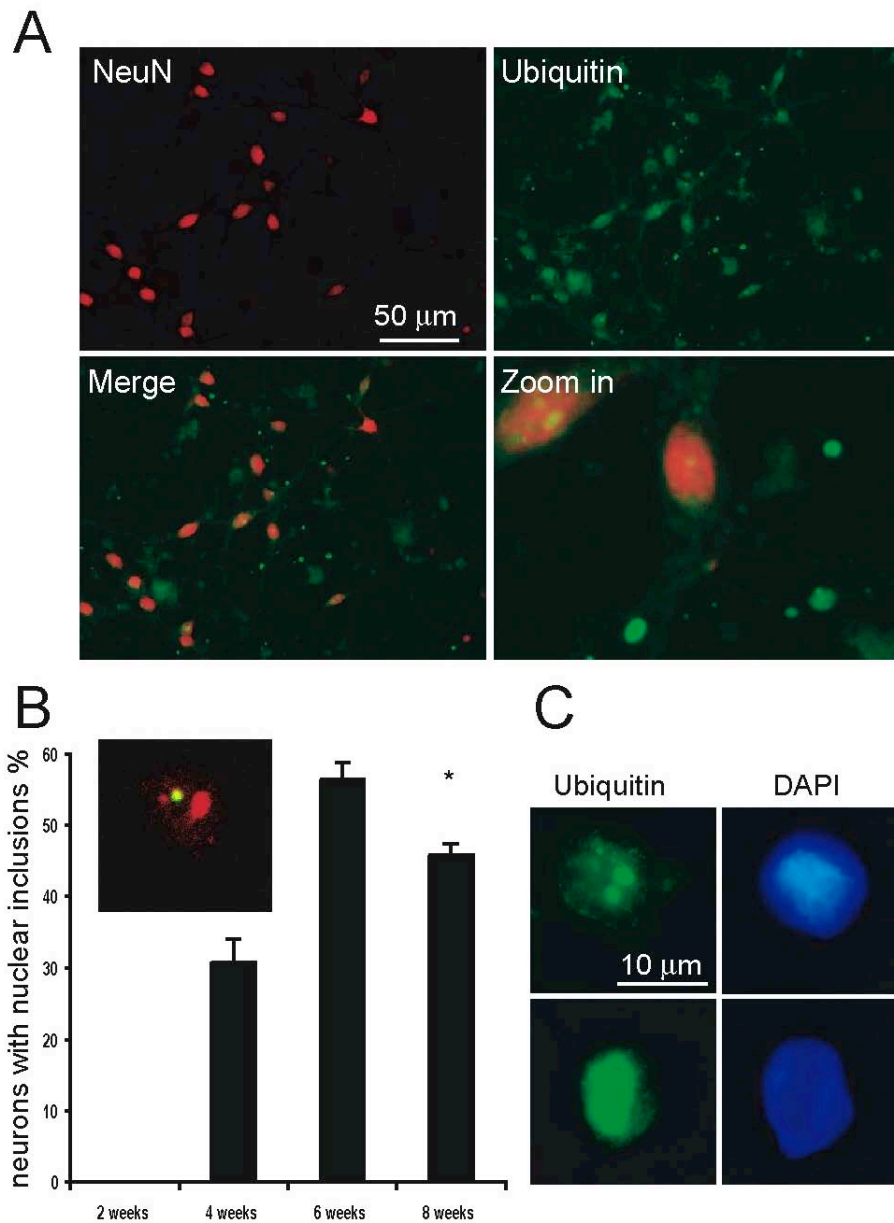


Figure 16: Progressive appearance of ubiquitinated inclusions

(A) Striatal cultures infected with htt171-82Q were fixed at 6 weeks post-infection and immunostained for NeuN and ubiquitin. The merged picture at high magnification shows the nuclear inclusions and the abundant of neuritic aggregates in the neurons. (B) Striatal cultures (n=6 per time point) infected with htt171-82Q were immunostained for ubiquitin and the number of neurons containing ubiquitin-positive nuclear inclusions was quantified. The graph shows that the appearance of nuclear aggregates in the striatal cultures was progressive. Note: inclusions were not present at 2 weeks, ubiquitin-positive inclusions then appeared gradually and reached $56 \pm 3\%$ at 6 weeks, after which the number declined to reach $46 \pm 2\%$ at 8 weeks. This decline was probably a consequence of neuronal degeneration. Values are the mean \pm SEM and * represents values for which $p < 0.05$). The insert shows a confocal image of nuclear inclusions labeled using the 2B4 (red) and ubiquitin (green) antibodies and fluorescent-marker conjugated secondary antibodies. (C) Representative pictures showing the heterogeneity of nuclear ubiquitinated inclusions co-localized with DAPI-stained chromatin.

2.4.4. Presence of dystrophic neurites and induction of a stress response

Nuclear and neuritic htt inclusions have been reported in the brains of humans with HD (DiFiglia et al., 1997). To analyze the appearance of aggregates in neuronal processes, htt171-82Q-infected cultures were double-labeled with htt and neurofilament antibodies. The first neuritic inclusions were visible at 4 weeks post-infection and were often associated with a decrease in neurofilament immunoreactivity, 4 weeks post-infection (Figure 17A). Immunostaining and Western blot analysis revealed that this reduction in neurofilament expression was more pronounced at 6 weeks post-infection (Figure 17A). This down-regulation appeared to be specific to the neurofilament class of structural proteins, as levels of α - and β - tubulin staining were not modified by the expression of htt171-82Q (Figure 18). As previously reported (Parker et al., 2001), the neuritic inclusions led to aberrant enlargement and swelling of the neurites in which they occurred (Figure 17C). This phenomenon was also observed in cultures co-infected with vectors encoding GFP and htt171-82Q (Figure 17B).

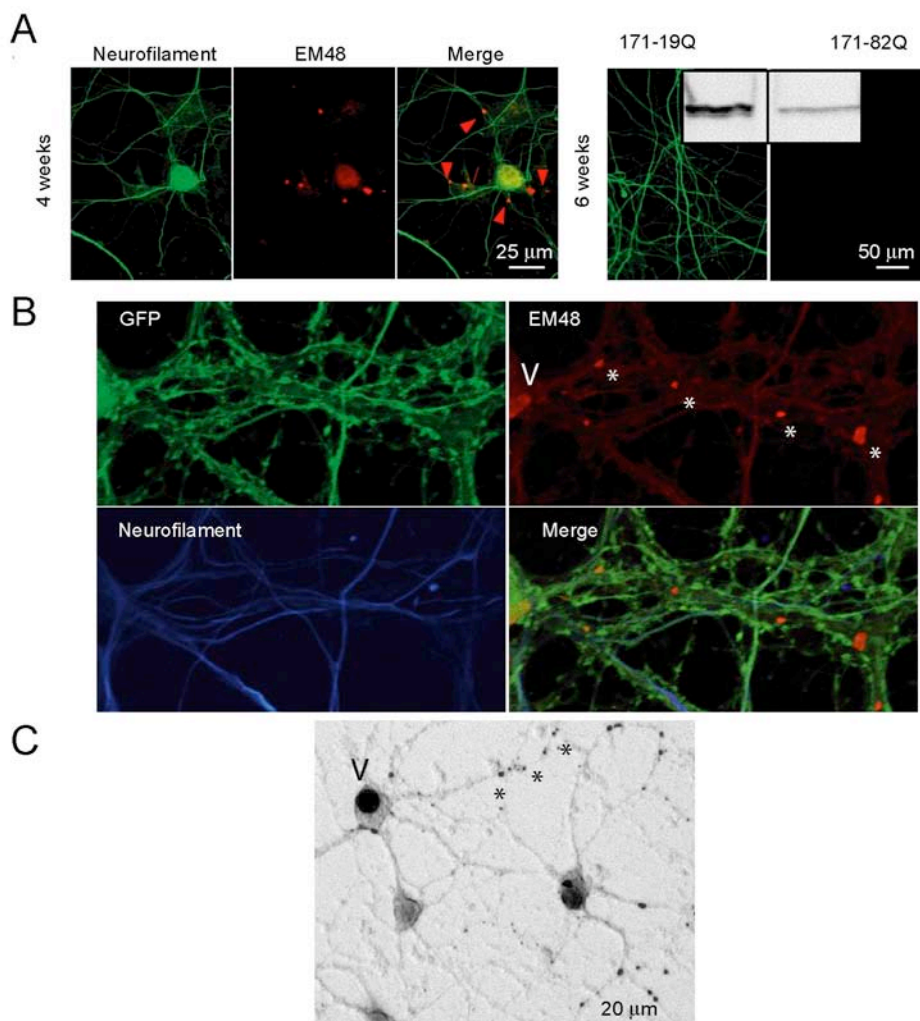


Figure 17: Presence of dystrophic neurites

(A) Striatal cultures infected with htt171-82Q were fixed at 4 weeks post-infection and stained for neurofilament (NF antibody) and huntingtin (EM48 antibody). The merged image shows that NF immunoreactivity is reduced in neurites with htt aggregates (see arrows). Immunostaining and Western blot analysis show that this reduction in NF expression is more pronounced at 6 weeks. Pictures taken at the same exposure reveal the drastic down-regulation of NF expression in htt171-82Q infected cells compared to that in control cultures. Western blot analysis using antibodies raised against NF confirmed this reduction at 6 weeks post-infection. (B) Confocal images of GFP fluorescence in neurites stained for ubiquitin (red), for NF (blue). Striatal cultures co-infected with lentiviral vectors expressing htt 171-82Q or GFP were fixed at 6 weeks post-infection to illustrate the segregation between neuritic inclusions (*) and the GFP-compartment. (C) Striatal cultures infected with htt171-82Q were fixed at 8 weeks post-infection and immunostained for htt with the 2B4 antibody. Analysis revealed swelling induced by the formation of neuritic inclusions. (*) inclusions in htt171-82Q neurons while the over-expression of wild-type htt fragment was not associated with the formation of inclusions as was illustrated in Fig. 2A (V = nuclear inclusions).

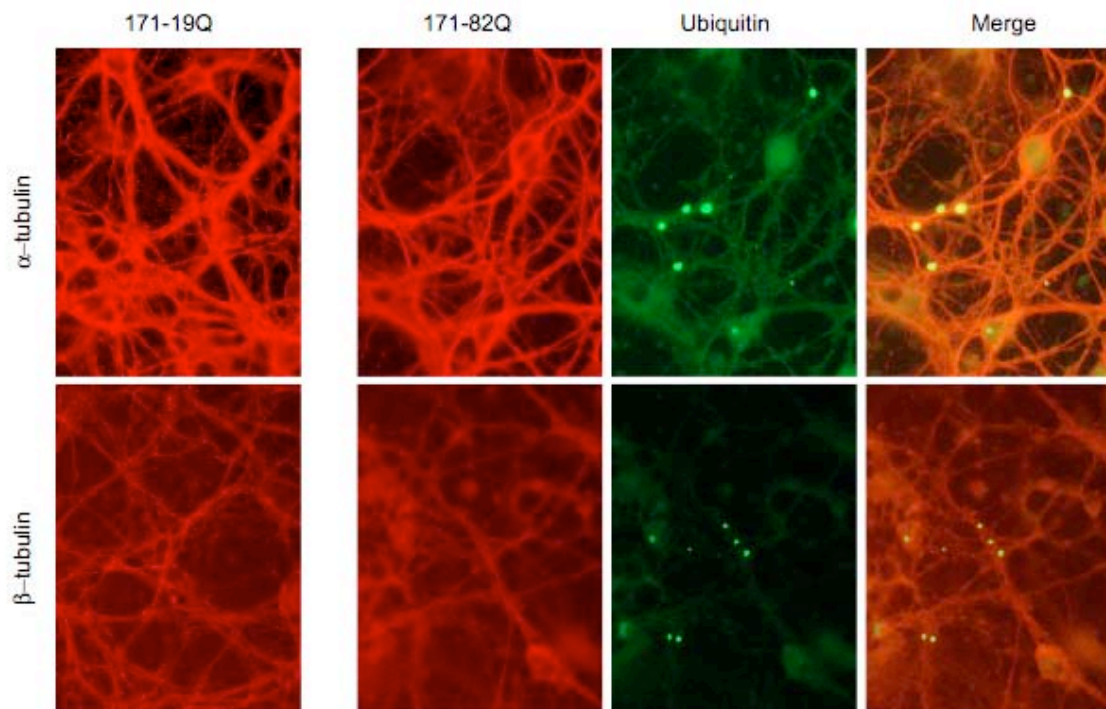


Figure 18: Mutant htt has no effect on α - and β - tubulin expression

Primary striatal cultures were infected at DIV 1 with a lentiviral vector encoding htt 171-82Q or htt 171-19Q. Cultures were fixed at 6 weeks post-infection and stained for α - or β -tubulin and ubiquitin. The photomicrographs show that mutant htt inclusions do not alter α - or β -tubulin expression.

Accumulation of misfolded proteins induces a stress response and the co-localization of chaperones with inclusions has been reported in autopsy material from the brains of humans with polyglutamine diseases and in HD transgenic mice (Cummings et al., 1998). In striatal neurons expressing mutant htt, the appearance of the first inclusions at 4 weeks was

associated with an increase in Hsp70 staining.

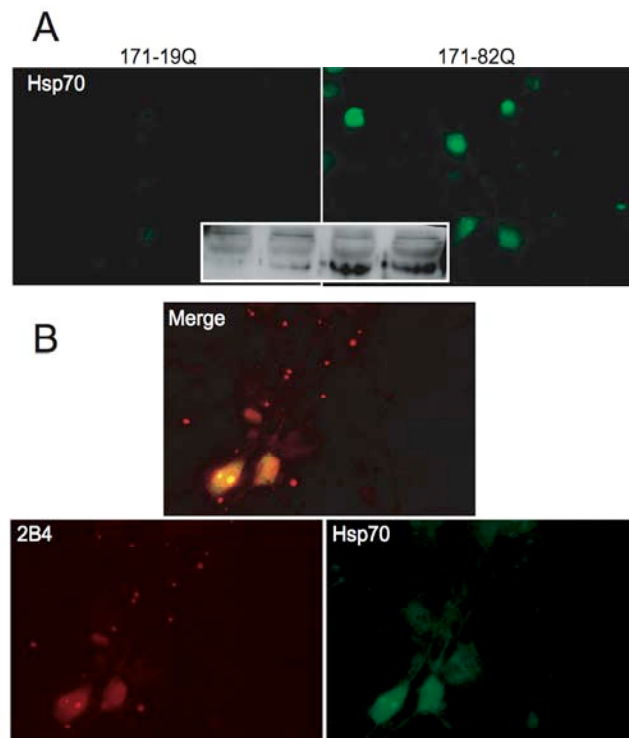


Figure 19: Stress response to mutant htt

(A) Striatal cultures infected with htt171-19Q and htt171-82Q were fixed at 4 weeks post-infection and stained for Hsp70. The up-regulation of Hsp70 in striatal cultures infected with htt171- 82Q suggested production of the mutant protein induced a stress response. No alterations in Hsp70 levels were observed in cultures infected with the wild type htt fragment. This increase in of Hsp70 chaperone expression was confirmed by western blot. (B) Hsp70 to co-localizes with nuclear inclusions (2B4 staining) but not with neuritic aggregates at 6 weeks post-infection.

This up-regulation was confirmed by Western blot analysis (Figure 19A). Double labeling of Hsp70 and htt revealed co-localization of the two proteins in nuclear, but not neuritic, inclusions (Figure 19B). Interestingly Hsp27 and Hsp40 were not detected in aggregates of either type (Figure 20).

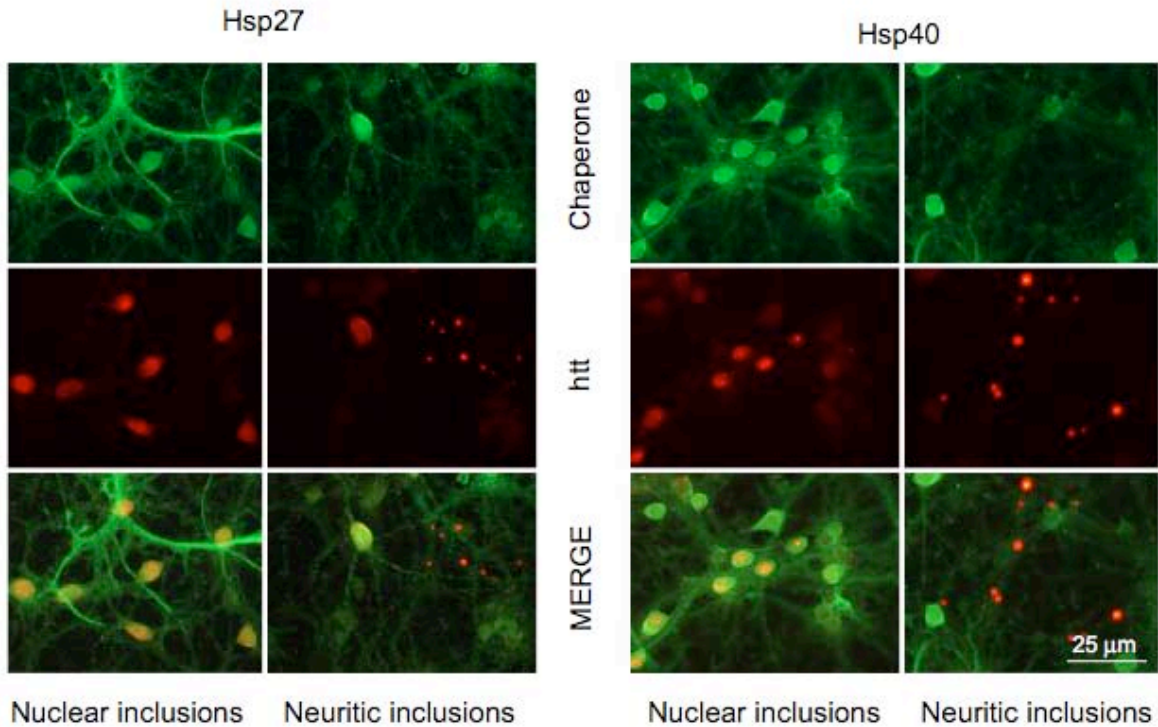


Figure 20: Hsp27 and Hsp40 do not co-localize with htt inclusions

Primary striatal cultures were infected at DIV 1 with a lentiviral vector encoding htt171-82Q. The cultures were fixed at 6 weeks post-infection and stained for htt, Hsp27 or Hsp40. The photomicrograph shows that the chaperones do not co-localize with the nuclear and neuritic htt inclusions (2B4-labeled).

2.4.5. Chronic expression of mutant htt induced a slow neurodegenerative process leading to cell death

We evaluated the neuronal dysfunction induced by the expression of mutant htt in the striatal cultures by staining with a neuronal marker (NeuN) and then counting the number of NeuN-positive cells (Figure 21A). No differences were detected between control (GFP) and infected (htt171-19/82Q) cultures at 2 and 4 weeks post-infection, demonstrating that viral vector infection was not toxic. A $21 \pm 4\%$ reduction in the number of NeuN-positive neurons was observed for cultures expressing mutant htt at 6 weeks post-infection. This decline continued during the following two weeks, leading to a $48 \pm 3\%$ reduction in NeuN-positive cells. This neuronal pathology was confirmed using a second neuronal marker, MAP-2 (Figure 21B): a $33 \pm 1\%$ reduction in the number of MAP-2-positive cells was observed at 8 weeks. To discriminate between neuronal dysfunction and cell death, striatal cultures were harvested at 8 weeks and stained using the TUNEL method. DNA fragmentation was analyzed *in situ* by

FACS. The number of cells containing fragmented chromatin was significantly higher in htt171-82Q-infected cultures than in htt171-19Q-infected cultures (Figure 21C). Our data indicate that the lentiviral-based expression of htt171-82Q in primary striatal cultures induced a slow and progressive neuropathology, characterized by the presence of neuritic and nuclear inclusions, and alterations in the expression of neuronal markers. Cell death occurred between 6 and 8 weeks after infection.

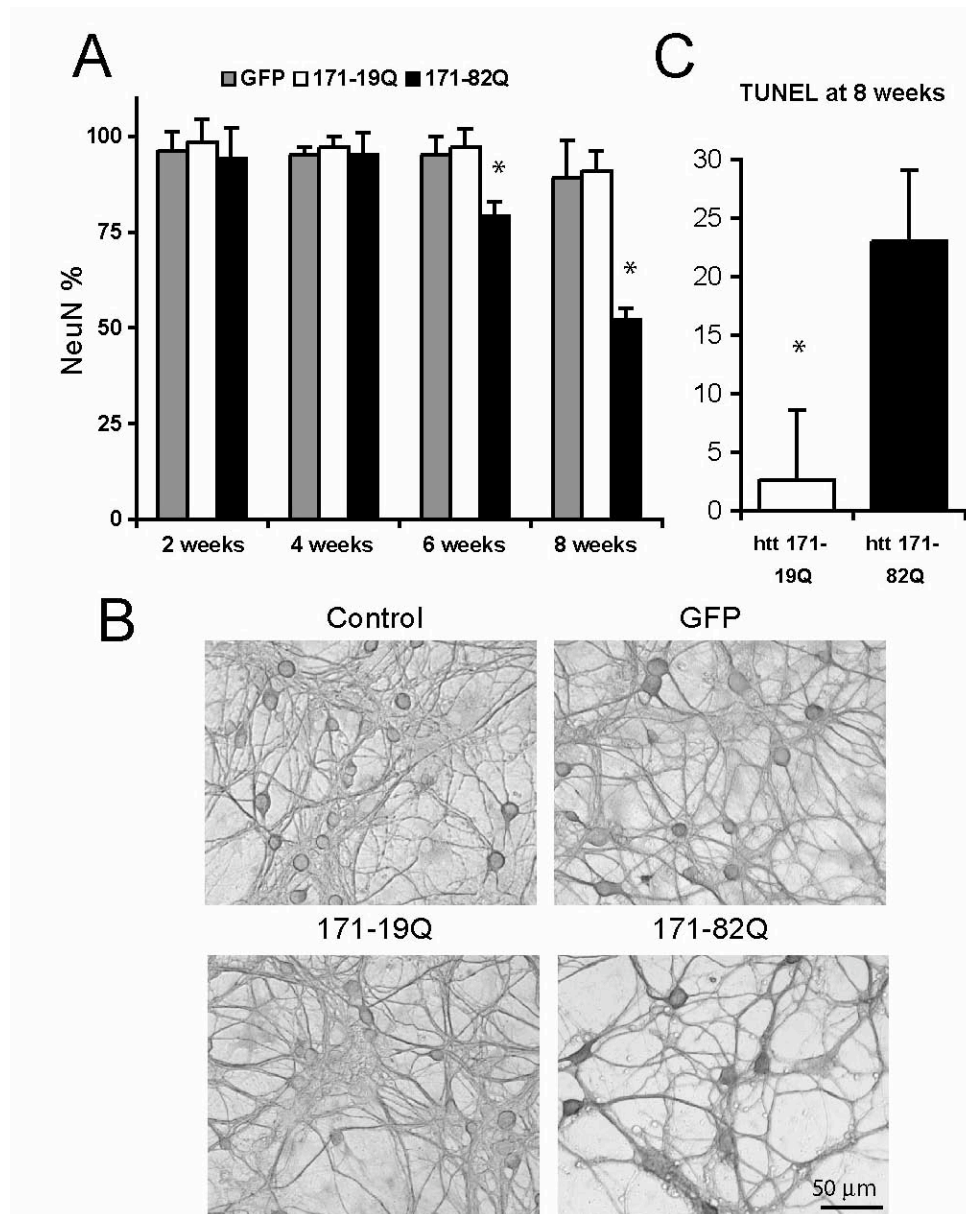


Figure 21: Kinetics of neuronal dysfunction and cell death

(A) Striatal cultures (n=6) infected with GFP, htt171-19/82Q and non-infected cultures were fixed at 2, 4, 6, 8 weeks post-infection and stained for NeuN. Neuronal dysfunction was assessed by counting the number of NeuN-positive cells and values were normalized against those obtained for control cultures. A progressive decrease in the number of NeuN-positive cells started at 6 weeks post-infection in the htt171-82Q-infected cultures. (B) The neuronal dysfunction in striatal neurons expressing mutant htt was confirmed by MAP-2 immunostaining (8 weeks post-infection). (C) Cell death was quantified at 8 weeks using TUNEL-stained

samples. The number of TUNEL-positive cells was significantly higher in the htt171-82Q expressing cultures.

2.4.6. Cortical neurons are resistant to mutant htt-induced cell death

The striatum is the main site of HD pathology but other structures are affected during later stages of the disease. Thus, striatal neurons would be expected to be more sensitive to mutant htt infection than neurons from other brain structures. We addressed this issue by dissecting cortical cultures and infecting them with the htt171-82Q vector. The GFP and htt171-19Q vectors were used as controls and results were compared those obtained with the infected striatal cultures. As described above for the striatal neurons, the majority of NeuN-positive cortical cells expressed the GFP reporter gene (Figure 22A; Figure 13A). In addition, nuclear and neuritic htt inclusions were observed in the cortical neurons at 6 weeks (Figure 22B). However, in contrast to the result obtained with the striatal cultures, no alteration in the number of NeuN and MAP-2 cells was observed at 8 weeks in the cortical cultures (Figure 22C-D), despite the presence of nuclear aggregates in $63\pm 1\%$ of the cortical neurons (Figure 22B).

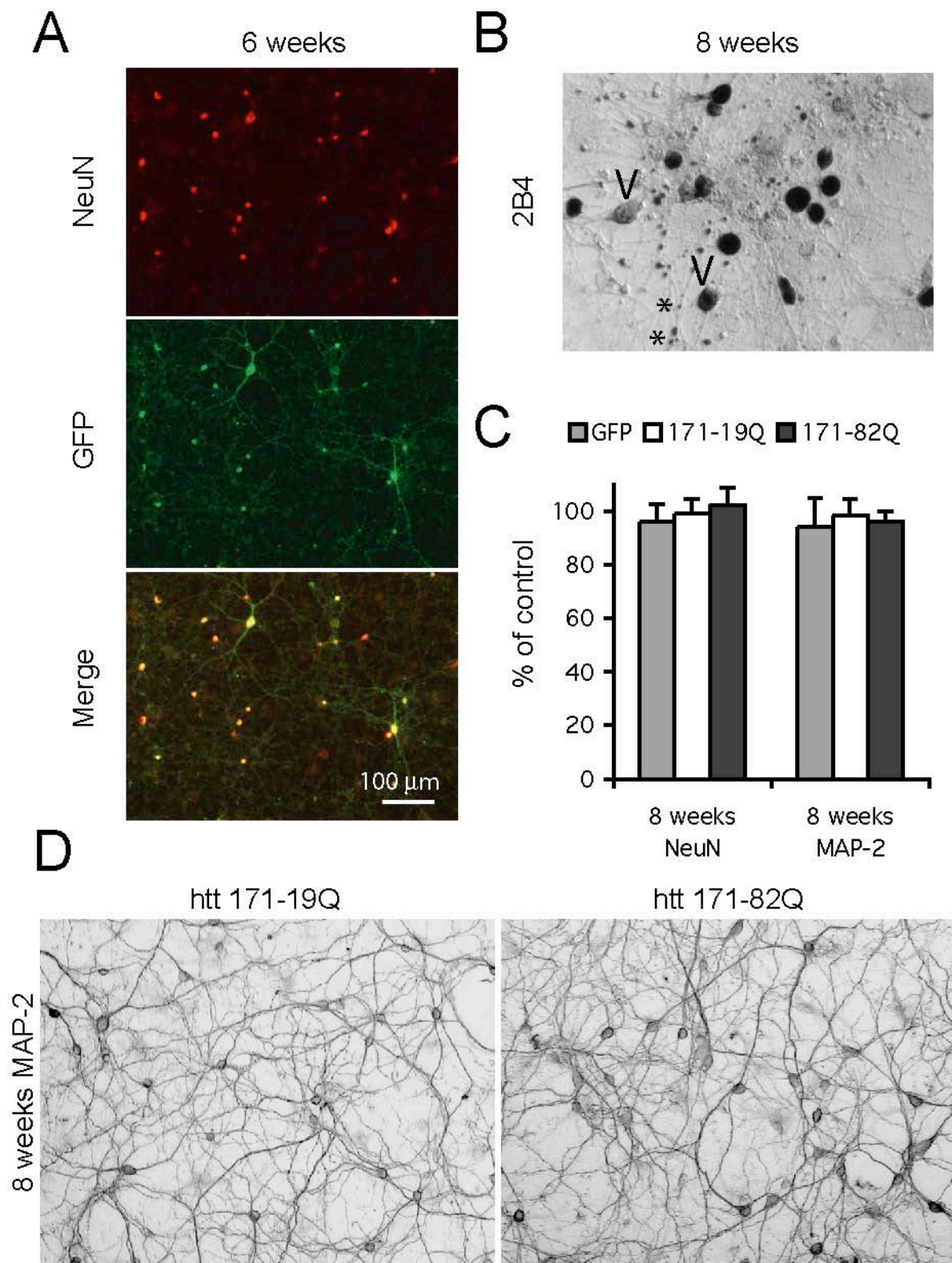


Figure 22: Study htt toxicity in cortical neurons

(A) Cortical cultures infected with a lentiviral vector expressing the GFP were fixed at 6 weeks post-infection and stained for NeuN and GFP. The transduction rate was high and the transgene expression stable in NeuN-positive cortical cells. (B) Cortical cultures infected with htt171-82Q were fixed at 8 weeks post-infection and the formation of nuclear (V) and neuritic (*) inclusions was revealed by huntingtin immunostaining. (C) Htt-induced cortical dysfunction was assessed using NeuN and MAP-2 immunostaining (n=6). (D) Infection with htt171-82Q did not effect the expression of these neuronal markers in the cortical cultures, despite the presence of numerous htt aggregates.

2.4.7. Neuroprotective effects of CNTF and BDNF

To further validate our model, two trophic factors that prevent mutant htt-induced toxicity in an acute *in vitro* model (Saudou et al., 1998) and in animal models of HD (Emerich et al., 1997a; Bemelmans et al., 1999; de Almeida et al., 2001) were tested. Two delivery systems were compared: the addition of recombinant proteins and lentiviral-mediated administration. In the first set of experiments, recombinant CNTF and BDNF (50ng/ml) were added to control and htt171-19/82Q cultures several times per week between weeks 4 and 8. At 4 weeks post-infection, the culture is at the dysfunctional stage, with an accumulation of htt inclusions and a reduction in neurofilament expression but no cell death. No alteration in the number of NeuN-positive cells was found 8 weeks, indicating that both CNTF and BDNF prevented striatal degeneration (Figure 23A). In the second set of experiments, cultures were first infected with lentiviral vectors encoding either wild type or mutant htt and then, 4 weeks later, with vectors encoding CNTF, BDNF or GFP. NeuN quantification indicated that the CNTF and BDNF delivered by the lentiviral vectors rescued neurons from mutant htt-induced toxicity (Figure 23B). Trophic factor secretion was analyzed by ELISA in media collected from 8-week old cultures. The supernatant of the htt177-19/82Q-infected neurons contained 140-270 pg/ml of BDNF and 25-30 ng/ml of CNTF (Table 1). The percentage of neurons with ubiquitinated nuclear inclusions was higher in CNTF- and BDNF-treated cultures than in controls (38±2% htt171-82Q/GFP; 68±2% htt171-82Q/CNTF; 82Q/CNTF; 64±4% htt171-82Q/BDNF; $p < 0,005$ GFP vs CNTF and BDNF) (Figure 23C). These results are consistent with those of Saudou et al. 1998.

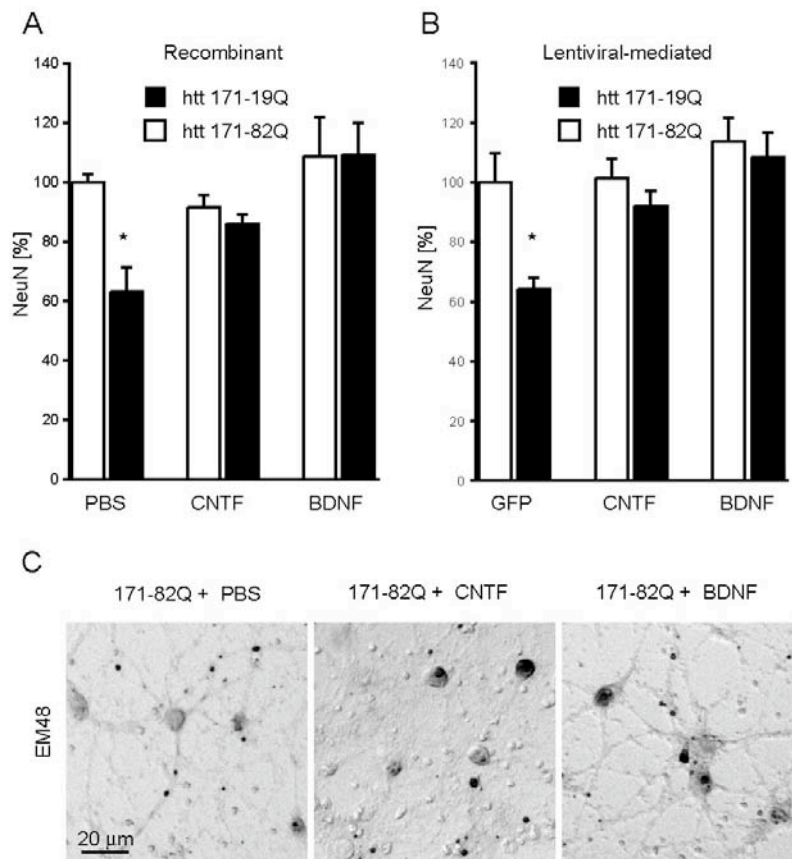


Figure 23: Protection of CNTF and BDNF

(A) Primary striatal cultures (n=6) infected either with htt171-19Q or htt171-82Q were treated every other day, starting from week 4, with 50 ng/ml of recombinant CNTF, BDNF or PBS (vehicle control). (B) In a second set of experiments, CNTF and BDNF were produced from lentiviral vectors. Cultures (n=6) were infected at 4 weeks with lentiviral vectors encoding GFP, CNTF or BDNF. BDNF and CNTF prevented the down-regulation of NeuN at 8 weeks post-infection in both sets experiments. (C) The formation of htt inclusions was assessed using the EM48 antibody. Inclusion formation was not affected by these trophic factors.

Group	BDNF [pg/ml]	CNTF [ng/ml]
htt 171-19Q + GFP	BL	BL
htt 171-82Q + GFP	BL	BL
htt 171-19Q + BDNF	270 ± 37	BL
htt 171-82Q + BDNF	149 ± 26	BL
htt 171-19Q + CNTF	BL	30 ± 15
htt 171-82Q + CNTF	BL	26 ± 10

Table 3: Lentiviral mediated release of CNTF and BDNF

Primary striatal cultures were infected at DIV1 with lentiviral vectors expressing htt171-19Q or htt171-82Q. Four weeks later, the cultures were infected with lentiviral vectors expressing CNTF, BDNF or GFP (control). Media were collected (n=10) at 8 weeks post-infection and the concentration of trophic factors was measured by ELISA [pg/ml] (BL: background level; * p< 0.05, ϕ p = 0.5)

2.5.DISCUSSION

To recapitulate the sequence of events underlying mutant htt cell death, we have developed a chronic model of HD in primary striatal cells, the neuronal subpopulation predominantly affected in HD. The pathology associated with lentiviral-mediated over-expression of mutant htt was characterized by three distinct phases: i) mutant htt progressively accumulated during the first three week post-infection but cells showed no sign of neuropathology; ii) htt inclusions and neuronal dysfunctions appeared between weeks 4 and 6; iii) finally cell death occurred between weeks 6 and 8. The slow progression of the pathology, compared to existing *in vitro* models of HD, provides an extended window of time for dissecting the pathology of htt-induced toxicity and for discriminating the cascade of events leading to striatal cell death. In addition, the high transduction efficiency obtained with lentiviral vectors allows unlimited amounts of material to be generated for quantitative analysis of HD pathology using molecular and biochemical approaches.

The htt-induced toxicity observed in our model system is consistent with that found in an *in vivo* study using the same lentiviral-mediated gene transfer system in adult rats. In both cases, striatal degeneration was slow and progressive (de Almeida et al., 2002). The chronic and severe neuropathology observed in the htt 171-82Q-infected cells in our study may be related to preferential integration of lentiviral vectors into transcriptionally active chromosome sites (Schroder et al., 2002; Wu and Marsh, 2003) and the use of the PGK promoter. Previous studies demonstrate that the level of htt expression is an important factor in the generation of *in vitro* and *in vivo* HD models. For example, studies in transgenic mice found no differences in survival rates between striatal cultures derived from wild type and mutant (R6/2 and YAC72) littermates (Petersen et al., 2001; Zeron et al., 2004). In another study, primary neurons transfected with a plasmid expressing mutant htt using the calcium phosphate method degenerated after a few days as a result of very high transgene expression (Saudou et al., 1998).

Neuritic and nuclear inclusions appeared 4 weeks after infection of striatal neurons with mutant htt. The presence of htt aggregates in processes was associated with a dramatic reduction in neurofilament expression, whereas α - and β -tubulin levels were unaffected. These data support the findings that axonal transport and synaptic function are disrupted in HD patients and the suggestion that this disruption may contribute to neuronal dysfunction and cell death (Gunawardena et al., 2003). Cytoplasmic htt aggregates mainly accumulate in the deep layers of the cortex and GABAergic neurons of the striatum in HD patients and

the appearance of neuritic aggregates correlates with the development of neuropathological symptoms (DiFiglia et al., 1997; Gutekunst et al., 1999; Kuemmerle et al., 1999). According to Gunawardena et al. (2003), htt toxicity contributes to early HD neuropathology by one of two non-exclusive mechanisms: through its effects on axonal transport or as a result of nuclear accumulation of mutant htt. We showed that mutant htt was present in both compartments in this lentiviral-based model of HD and our results suggested that over-expression of this protein activated various intracellular pathways that led to the degeneration of striatal neurons. NeuN, MAP-2 and neurofilament expression was down-regulated by 6 weeks post-infection, whereas that of Hsp70 was unregulated in the striatal neurons expressing mutant htt. Several studies have demonstrated that translocation of mutant huntingtin to the nucleus alters the activity of transcription factors (Nucifora et al., 2001; Chai et al., 2002; Schaffar et al., 2004) and disrupts gene expression patterns (Luthi-Carter et al., 2000; Steffan et al., 2000; Wyttenbach et al., 2001; Chan et al., 2002; Dunah et al., 2002; Sipione et al., 2002). Preliminary DNA-microarray data on 6-week-old cultures indicate that changes in the level of gene expression occur prior to striatal cell death and confirm the alterations in neurofilament and Hsp70 expression reported in this study (unpublished data). Previous studies have shown that Hsp production is also stimulated in cells expressing mutant htt or an androgen receptor (Stenoien et al., 1999; Kobayashi et al., 2000) and in 30-35-weeks-old R6/1 HD transgenic mice (Jana et al., 2000). This stress response may affect polyglutamine pathogenesis and relieve neuronal dysfunction. Consistent with this view, the over-expression of various chaperones reduces aggregate formation and cell death *in vitro* (Cummings et al., 1998; Chai et al., 1999; Stenoien et al., 1999; Jana et al., 2000; Kobayashi et al., 2000; Wyttenbach et al., 2000; Wyttenbach et al., 2002). We found that Hsp70 co-localized with the htt nuclear inclusions, whereas no co-localization was observed with neuritic inclusions. The biological relevance of this localization pattern is unknown (Jana et al., 2000; Li et al., 2001).

The number of nuclear inclusions was highest in the htt 171-82Q-infected striatal neurons at 6 weeks post-infection, with $56\pm 3\%$ ubiquitin-positive and $63\pm 3\%$ mEM48-positive striatal neurons. As more than 95% of the neurons expressed htt171-82Q, these data suggest that some infected striatal neurons did not develop nuclear inclusions. This could be due to variability in the levels of htt expression between cells. We have previously shown that inclusion distribution is dose-dependent: neuropil aggregates were the most frequently observed form of inclusion in the striatum of rats injected with a vector expressing low levels of htt171-82Q, whereas nuclear inclusions predominated in neurons expressing high levels of

mutant htt (de Almeida et al., 2002). Thus, infected striatal neurons expressing low levels of mutant htt would be expected to develop neuritic aggregates. The high proportion of neuritic aggregates obtained in primary striatal neurons infected with the htt171-82Q vector compared to the *in vivo* data in adult rats (de Almeida et al. 2002) suggest that the PGK promoter is less potent *in vitro* or that striatal cultured neurons are more prone to develop neuritic inclusions *in vitro*. Detailed analysis of aggregate formation between 3 and 4 weeks post-infection and quantitative analysis of htt expression in neurons with nuclear or neuritic aggregates should provide additional information. Alternatively, sensitivity to htt aggregation and htt toxicity may differ between neuronal subpopulations. In HD brains, htt aggregates are abundant in cortical neurons but remain relatively rare in the striatum despite a severe degeneration in this structure (Heinsen et al., 1994). We addressed this issue by investigating the effects of mutant htt in cortical cultures. Mutant htt over-expression had no effect on cortical neuron survival rates up to 8 weeks post-infection, despite the fact that the percentage of cells with inclusions was higher for cortical neurons than for striatal neurons ($63\pm 1\%$ vs. $46\pm 2\%$; $p < 0.05$). The presence of inclusions in 64-68% of the CNTF/BDNF-treated striatal neurons at 8 weeks post-infection, however, suggests that the kinetics of aggregate formation are similar in both subpopulations. Thus, these data suggest that the selective degeneration of striatal neurons is an intrinsic property of these neurons and that this vulnerability is independent of corticostriatal input. Differences in htt protein processing or to cell-type specific factors may account for this differential susceptibility. Lentiviral-mediated delivery of mutant htt may therefore be a useful tool for *in vitro* and *in vivo* studies aiming to determine the relative contribution of striatal and cortical compartments in HD pathogenesis. Similarly, this method may also be useful for studies investigating the molecular basis of the selective sparing of NADPH-d- and choline-acetyltransferase- choline-acetyltransferase-positive interneurons and the differential susceptibility of projecting striatal neurons expressing enkephalin and those expressing substance P (Ferrante et al., 1985; Graveland et al., 1985; Behrens et al., 1996).

Interest in the therapeutic potential of neurotrophic factors for HD patients had been renewed by recent evidence suggesting a link between the production of BDNF and htt (Zuccato et al., 2001; Alberch et al., 2004). Huntingtin was shown to regulate BDNF transcription and vesicular transport (Ferrer et al., 2000; Zuccato et al., 2001; Gauthier et al., 2004). Evidence that BDNF influences HD pathology was also obtained from studies of the offspring generated by cross-mating HD transgenic mice (R6/1) and BDNF^{+/-} mice: an early onset of motor deficits and a lack of DARPP-32 expression in the striatum were observed in the

double transgenic mice (Canals et al., 2004). The administration of BDNF as well as other neurotrophic factors has been shown to enhance the survival rate of GABAergic neurons in drug-induced models of HD (Alberch et al., 2004). However, data on the potential therapeutic benefits of this type of treatment in genetic models of the disease and in HD patients is limited (Saudou et al., 1998; Bachoud-Levi et al., 2000; Zala et al., 2004). In addition, the intracellular signaling cascades mediating these neuroprotective effects are still poorly defined. Recent data suggest that CNTF-mediated activation of astrocytes may alter energy metabolism and neuronal function and thus contribute to the neuroprotective effect of this molecule (Escartin et al., 2004). It still needs to be established whether the administration of these molecules in the striatum of HD patients will delay the progression of the disease.

In summary, this study demonstrates that infection of striatal cultures with a lentiviral vector encoding mutant htt induces a pathology replicating the prominent features of HD. The pathology observed was chronic, progressive and striatal neuron-specific. This cellular model provides a versatile and unique tool for quantitative and mechanistic analysis that will be particularly useful for further dissecting HD pathology, screening therapeutic candidates and analyzing the intracellular mechanisms associated with the neuroprotective effects of various agents.

2.6. ACKNOWLEDGEMENTS

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3. LONG-TERM EXPRESSION OF CNTF IN THE STRIATUM OF HD MICE

Published as:

Evaluation of long-term effects of lentiviral-mediated CNTF delivery in the striatum of wild-type and HD transgenic mice.

Zala, D., Bensadoun, J.C., de Almeida, L.P., Leavitt, B.R., Gutekunst, C.A., Aebischer P., Hayden, M., and Déglon N. (2004). *Exp Neurol* 185: 26-35

3.1.ABSTRACT

Ciliary Neurotrophic Factor (CNTF) has been shown to prevent behavioral deficits and striatal degeneration in neurotoxic models of Huntington's disease (HD) but its effect in a genetic model has not been evaluated. Lentiviral vectors expressing the human CNTF or LacZ reporter gene were therefore injected in the striatum of Wild-type (WT) and transgenic mice expressing full length huntingtin with 72 CAG repeats (YAC72). Behavioral analysis showed increased locomotor activity in 5-6 month old YAC72-LacZ mice compared to WT-LacZ animals. Interestingly, CNTF expression reduced the activity levels of YAC72 mice compared to control animals. In both WT and YAC72 mice, CNTF expression was demonstrated in striatal punches up to a year after lentiviral injection. Stereological analysis revealed that the number of LacZ and DARPP-32-positive neurons were decreased in YAC72-LacZ mice compared to WT-LacZ animals. Assessment of the benefit of CNTF expression in the YAC72 mice was, however, complicated by a down-regulation of DARPP-32 and to a lesser extent of NeuN in all mice treated with CNTF. The expression of the neuronal marker NADPH-d was unaffected by CNTF, but expression of the astrocytic marker GFAP was increased. Finally, a reduction of the number of striatal dark cells was observed in YAC mice treated with CNTF compared to LacZ. These data indicate that sustained striatal expression of CNTF can be achieved with lentiviruses. Further studies are, however, needed to investigate the intracellular signaling pathways mediating the long-term effects of CNTF expression on dopamine signaling and glial cell activation and how these changes may affect HD pathology.

3.2. INTRODUCTION

Huntington's disease is a fatal dominant disorder characterized by a selective neuronal loss in the striatum and to a lesser extent in the cortex (Vonsattel and DiFiglia, 1998). The disorder is caused by an expansion of a CAG repeat tract in the HD gene (The Huntington's Disease Collaborative Research Group, 1993) which can be used to genetically identify pre-symptomatic individuals who are at-risk for developing the disease (Andrew et al., 1993). Initiation of therapeutic interventions prior to the onset of clinical symptoms and neuronal degeneration can therefore be envisaged. Over the last few years, neuroprotective strategies based on the use of neurotrophic factors have been developed, and significant rescue of striatal neurons associated with behavioral improvement were observed in drug-induced models of HD (Frim et al., 1993; Anderson et al., 1996; Emerich et al., 1996; Araujo and Hilt, 1998; Volpe et al., 1998; Bemelmans et al., 1999; Mittoux et al., 2000; Perez-Navarro et al., 2000a).

CNTF (Ip and Yancopoulos, 1996), a member of the cytokine family, has been shown to prevent behavioral deficits and striatal degeneration in quinolinic acid-lesioned rats and primates (Anderson et al., 1996; Emerich et al., 1996; Emerich et al., 1997a; de Almeida et al., 2001). Recently, we have further investigated whether CNTF could reduce neuronal dysfunction and neurodegeneration in monkeys chronically treated with 3-nitropropionic acid (3-NP) (Mittoux et al., 2000). This study demonstrated that the intracerebral and continuous delivery of CNTF at the time of appearance of striatal dysfunction and motor/cognitive symptoms, not only prevents neuronal degeneration, but also alleviates functional deficits. Moreover, Saudou and collaborators have demonstrated that CNTF rescues primary rat striatal cultures from cell death caused by the expression of a mutant htt fragment (Saudou et al., 1998). This *in vitro* study suggests that CNTF might alter the degeneration of GABAergic neurons in a genetic model of the disease.

In the present study, we have assessed the effect of long-term CNTF delivery in wild-type mice and HD YAC transgenic mice expressing full-length htt with 72 CAG repeats (Hodgson et al., 1999). These animals show electrophysiological abnormalities at 6 months, a mild hyperkinetic movement disorder with a significantly increased activity between 5 and 9 months. By 12 months of age, these mice display an increase in the number of striatal dark cells as measured by toluidine blue staining (Hodgson et al., 1999). Lentiviral vectors were used to achieve local and long-term delivery of CNTF in the brain while avoiding side effects

associated with systemic administration (Abicht and Lochmuller, 1999). Lentiviruses expressing LacZ or human CNTF were bilaterally injected into the corpus striata of 4 month old YAC72 mice and wild-type littermates. Behavioral analyses were performed during the entire experiment to monitor the progression of the disease. Finally, at 13 months of age, the animals were sacrificed and their brains processed to assess the effects of long-term CNTF expression.

3.3.MATERIAL AND METHODS

3.3.1. Lentiviral vector production

The cDNA coding for a nuclear-localized β -galactosidase (LacZ) and the human CNTF (Aebischer et al., 1996) were cloned in the SIN-W-PGK transfer vector (Déglon et al., 2000; de Almeida et al., 2001). The packaging construct and the vesicular stomatitis virus G protein (VSV-G) envelope used in this study were the pCMV Δ R-8.92, pRSV-Rev and pMD.G plasmids (Dull et al., 1998; Hottinger et al., 2000; Naldini et al., 1996). The viral particles were produced by transient transfection of 293T cells as previously described (Hottinger et al., 2000). LacZ- and CNTF-expressing viruses were resuspended in phosphate buffered saline (PBS) with 1% bovine serum albumin and matched for particle content of 100'000 ng p24 antigen /ml as measurement by ELISA (Perkin Elmer Life Sciences, MA, USA).

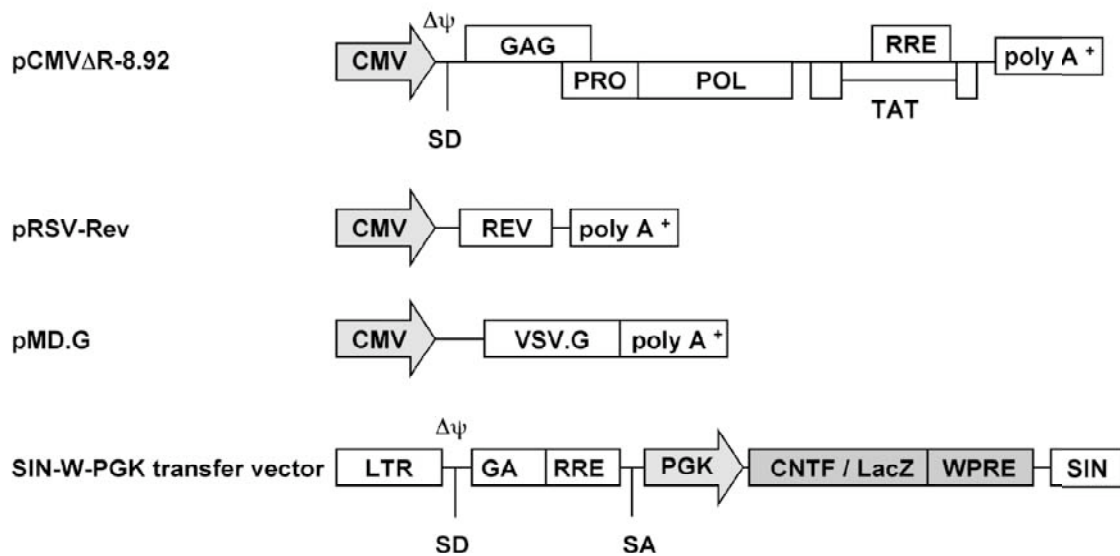


Figure 24: Schematic representation of the four plasmids used for the production of lentiviral vectors in 293T cells.

pMD.G (VSV-G envelope), pCMV Δ R-8.92 (Packaging construct), pRSV-Rev (Rev protein) and SIN-W-PGK (transfer vector). CMV: cytomegalovirus promoter. GAG: structural precursor protein producing the matrix, capsid and nucleocapsid. LTR: long-terminal repeat. PGK: mouse phosphoglycerate kinase 1 promoter. RSV: Rous sarcoma virus promoter. RRE: Rev-responsive element. SA: splice acceptor. SD: splice donor. SIN: self-inactivating (deletion of the U3 region of the 3' LTR). POL: sequence coding for the reverse transcriptase and integrase. PolyA⁺: polyadenylation signal. PRO: protease. ψ : packaging signal. VSVG: vesicular stomatitis virus G glycoprotein. WPRE: post-transcriptional regulatory element from the woodchuk hepatitis virus.

3.3.2. In vivo experiments

YAC72 mice (mouse line FVB-TgN (353G6-72) 2511, YAC72) and wild-type FVB/N (Iffa-Credo, France) mice (Hodgson et al., 1999) were used in these studies. The animals were housed in a controlled temperature room that was maintained on a 12 hr light/dark cycle. Food and water were available *ad libitum*. The animals injected with lentiviral vectors were maintained in ventilated cabinets in biosafety level 2 laboratories. The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for care and use of laboratory animals.

3.3.3. Injection of the lentiviruses

The concentrated viral stocks were defrosted and re-suspended by repeated pipetting. CNTF or LacZ-expressing lentiviral vectors were stereotaxically injected in the left (1 μ l) and right (1 μ l) striatum of 4 months old pentobarbital anesthetized (75 mg/kg, i.p.) FVB/N and YAC72 mice using a Hamilton syringe with a 33 gauge blunt tip needle (Hamilton, Reno, NV, USA). The stereotaxic coordinates for the injection were: 0.4 mm rostral to bregma; 1.8 mm lateral to midline (LM); 3.5 mm ventral from the dural surface. The suspension was injected at 0.2 μ l/min by means of an automatic injector (Stoelting Co., IL, USA) and the needle was left in place for 5 min. The skin was closed using a 6-0 Vicryl® suture (Ethicon, Johnson and Johnson, Brussels).

3.3.4. Behavioral Analysis

Mice were placed in a monitoring box (10 x 10 x 30 cm) (Digiscan Animal Activity Monitor, AccuScan Instruments, OH, USA) and their activity recorded for 2 hr. Infra-red beam breaks were converted into total distance traveled. The activity was tested every other week at a fixed time corresponding to the dark phase period for the animals (inverted light cycle). The test was performed during the entire experimental period.

3.3.5. CNTF measurements

The animals were sacrificed, perfused in ice-cold BPS containing 0.02% ascorbic acid (Sigma, MO, USA) and 5'000U of heparin (Liquemin, Roche Pharma, Reinach, Switzerland).

The *in vivo* synthesis of CNTF was determined from 2 mm long striatal punches taken around the injection sites and from corresponding non-injected hemispheres. The samples were rapidly frozen on dry ice and kept at -80°C until processing. The samples were sonicated in 500 µl PBS, containing a cocktail of protease inhibitors (pronase, thermolysin, chymotrypsin, trypsin, papain; Roche Pharma, Reinach, Switzerland). Quantitative analysis of the production of CNTF was performed by an ELISA assay according to the supplier's manual (R&D system Abington, UK).

3.3.6. Histological processing

The mice were sacrificed by a sodium pentobarbital overdose and then transcardially perfused with a 4% paraformaldehyde containing saline solution. The brains were removed, weighed and one hemisphere was post-fixed in 4% paraformaldehyde for approximately 24 hr and cryoprotected in 25 % sucrose / 0.1M phosphate buffer (PBS) for 48 hr. These samples were frozen in dry ice and coronal sections were cut on a sliding microtome cryostat (Cryocut 1800, Leica Microsystems, Nußloch, Germany) at a temperature of -20°C and a thickness of 20 µm. Slices throughout the entire striatum were collected and stored in 48 well trays (Costar, Cambridge, MA, USA) as free floating sections in PBS containing 0.12 µM sodium azide. The trays were stored at 4°C until immunohistochemical processing.

The second brain hemisphere was post-fixed in 0.15% glutaraldehyde in 0.1M PBS pH7.2. Fifty micrometers sections were cut with a Leica Ultracut ultramicrotome and cresyl violet stained to assess the appearance of dark cells. Sections were put for 2 min in sodium acetate solution containing 0.5% cresyl violet (Sigma, MO, USA) and then rinsed in the acetate solution. The sections were dehydrated in ethanol, passed in toluene and coverslipped with Merckoglas®.

3.3.7. Immunohistochemical analysis

Enzymatic staining for nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) was performed as previously described (Ellison et al., 1987). For immunohistochemical stainings, endogenous peroxidase activity was quenched with 0.1% diphenylhydrazine/PBS (37°C for 30 min) and washed 3 times in PBS. Free floating sections were incubated overnight in 5% Normal Goat Serum (NGS, Dako Diagnostics, Untermüli, Switzerland)/0.1M phosphate buffered saline at 4°C, followed by an overnight reaction in a PBS/1%NGS

solution, with the respective antibodies: DARPP-32 (1:5000, Chemicon, CA, USA), LacZ (1:500, 5 Prime 3 Prime Inc., USA), GFAP (1:300, Clontech, CA, USA) and neuronal nuclei (NeuN, 1:400, Chemicon, CA, USA). After 3 washes, the sections were incubated with the corresponding biotinylated secondary antibodies (1:200, Vector laboratories, CA, USA) for 2 hr at room temperature. Bound antibodies were visualized with the ABC system (Vectastain ABC Kit, Vector Laboratories) and 3,3' diaminobenzidine as chromogen (DAB Metal Concentrate, Pierce, IL, USA). The sections were dehydrated in ethanol, passed in toluene and coverslipped with Merckoglas®. Secondary antibodies coupled to fluorescent chromophores (Jackson ImmunoResearch Laboratories, West Grove, USA) were used for LacZ (FITC-Goat Anti rabbit, 1:200). The sections were mounted on glass slides with Fluorsave™ Reagent (Calbiochem-Novabiochem Corporation, CA, USA).

3.3.8. Quantification of NADPH-d and LacZ-positive cells

Counts were performed on 9 NADPH-d stained sections per animal (240 µm apart) and 18 LacZ-stained sections per animal (120 µm apart). The number of cells were extrapolated with the Abercrombie's equation with the following parameters: section thickness, 20 microns; nuclear diameter, 5 microns; section selection, 1/8 for LacZ and 1/16 for NADPH-d.

3.3.9. Stereological analysis

The quantification of the DARPP-32 and NeuN cells was performed on 18 sections (120 µm apart) per animal with an Olympus microscope and analyzed with the stereological software C.A.S.T. Grid (The International Stereology Center Olympus Albertslund, Danmark) using the optical dissector method. Briefly, the striatum area (ROI) was defined at low magnification (1.25x) and striatum volume was integrated using the method of Cavalieri. Neuronal count was performed at a magnification of 100x on grid of 500 µm x 300 µm and the optical dissector was set at 10 µm. An average of 20 grids were used per section analyzed.

3.3.10. Data analysis

Data are expressed as mean ± SEM and evaluated for analysis of variance (ANOVA) followed by a Scheffe's PLSD post-hoc test (JMP 3.0, SAS Institute Inc., USA). The significance level was set at $p < 0.05$.

3.4. RESULTS

3.4.1. Stable and long-term transgene expression in the striatum

Figure 24 shows the lentiviral vectors used for the assessment of long-term *in vivo* expression of CNTF in wild type and YAC72 mice. Striatal punches, collected up to a year post-lentiviral injection, were processed for ELISA. Average values of approximately 500 pg CNTF/punch were measured, whereas no CNTF was detected in control animals or in animals injected with LacZ lentiviral vectors (Figure 25). CNTF expression was sustained and no significant differences were observed over time, or between wild type and YAC72 mice (Figure 25). These results further demonstrate that lentiviral vectors lead to long-term and sustained transgene expression in the CNS (Dégion et al., 2000).

Interestingly, the local and continuous delivery of CNTF in the striatum of these mice was not associated with a loss in body weight (26 ± 1 g WT-LacZ vs 26 ± 4 g WT-CNTF at 13 months), a side effect previously observed with systemic administration of this neurotrophic factor (Penn et al., 1997).

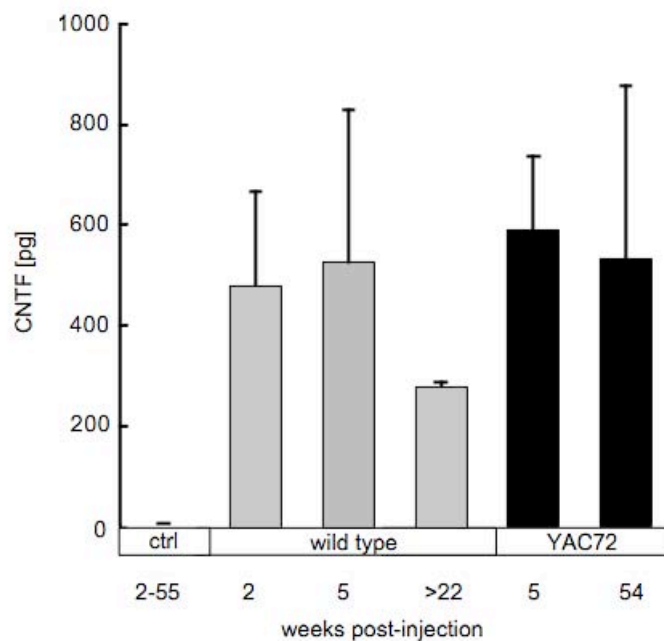


Figure 25: Sustained lentiviral-mediated expression of CNTF in the striatum of mice.

Striatal punches from wild-type and YAC72 transgenic mice were taken 2 to 54 weeks post-injection of CNTF- or LacZ-expressing lentiviral vectors and processed for ELISA assay. Values below the detection level (20 pg) were measured on non-injected hemispheres (ctrl) or LacZ-infected striata (LacZ). Values are expressed as mean \pm SEM.

3.4.2. CNTF delivery decreases the behavioral deficit of YAC72 mice

The appearance of behavioral abnormalities, in particular hyperactivity, was assessed by measuring locomotor activity with the open field test (Figure 26). A progressive spontaneous hyperactivity, starting at 5 months of age, was observed in the YAC72-LacZ mice (Figure 26). Although a large inter- and intra-individual variability was observed, the total distance traveled was higher in YAC72-LacZ mice than in wild type animals. At 7 and 8 months a significant hyperactivity was recorded in the YAC72-LacZ mice compared to the WT group. Importantly, CNTF-treated mice displayed lower spontaneous activities compared to YAC72-LacZ mice and statistically significant differences were observed at 5 ($p=0.04$) and 8 months ($p=0.03$) (Figure 24). Performances in other behavioral tests such as foot-clasping and rotarod did not differ significantly between groups (data not shown).

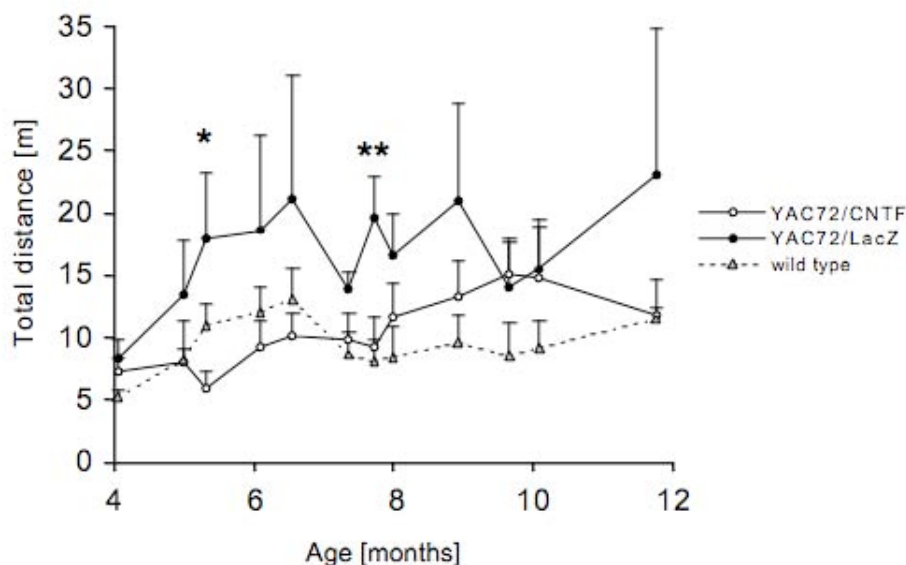


Figure 26: Decreased hyperactivity in CNTF-treated YAC72 mice.

Values represent the mean of total distance in meters traveled in the activity box during 2 hr. The general activity of WT/LacZ and WT/CNTF mice were similar and have been plotted together in the graph. LacZ-injected transgenic mice ($n=6$) displayed an increased activity starting at 5 months of age, whereas YAC/CNTF mice ($n=7$) displayed a normal activity. Significant differences between the YAC/LacZ and the YAC/CNTF was found at 5 and 8 months (* $p=0.04$, ** $p=0.03$). Values are expressed as mean \pm SEM.

3.4.3. Effect of long-term CNTF delivery

a) Brain weight and LacZ expression

Mouse brains were weighed to assess for global brain weight loss. At 13 months of age, no significant differences were observed between wild type and YAC72 mice (WT-LacZ (n=3): $0.610 \pm 0.058\text{g}$, WT-CNTF (n=3): $0.581 \pm 0.038\text{g}$, YAC72-LacZ (n=6): $0.576 \pm 0.006\text{g}$, YAC72-CNTF (n=6): $0.599 \pm 0.013\text{g}$).

In wild type mice, $35'629 \pm 6'662$ LacZ-infected cells covering a large area of the striatum were detected 9 months post injection (Figure 27A). These results correspond to the typical infected area observed 2 weeks post-infection (data not shown). Interestingly, a smaller number of LacZ-positive cells ($12'709 \pm 464$) was observed in YAC72-LacZ injected animals probably due to the loss of infected cells (Figure 27B). Large nuclear structures with intense LacZ staining (of unknown significance) were observed in the striatum of YAC72-LacZ mice but were not seen in wild type-LacZ animals mice (Figure 27A). These differences in the observed pattern of LacZ expression may reflect the ongoing neurodegenerative process occurring in the YAC72 mice.

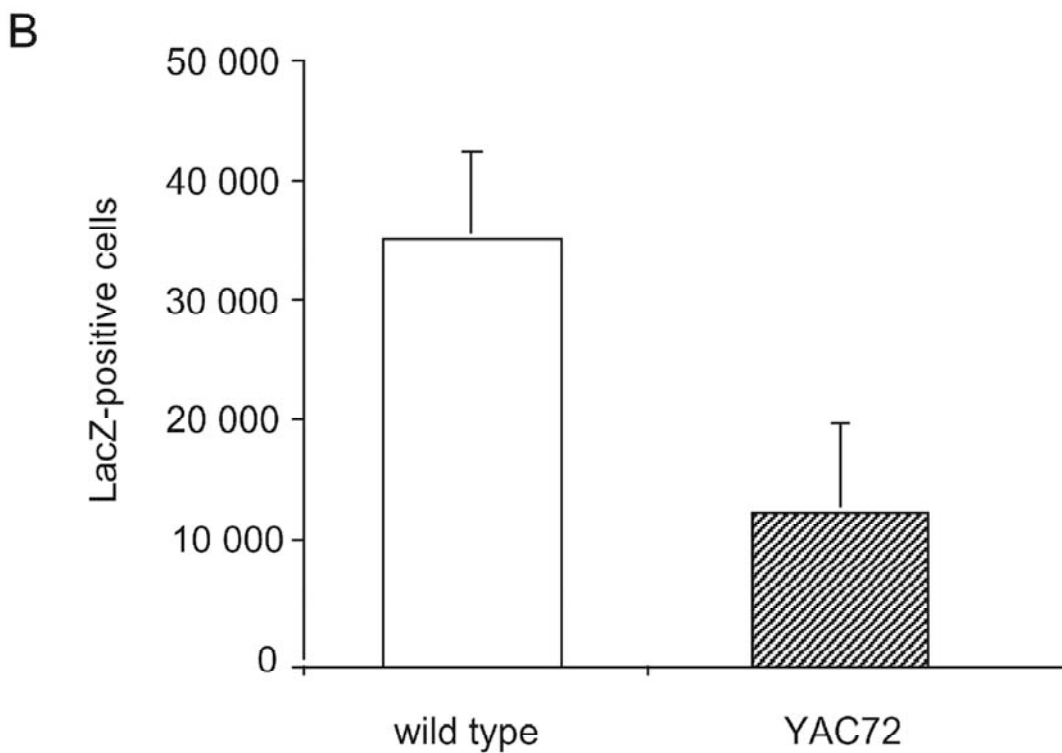
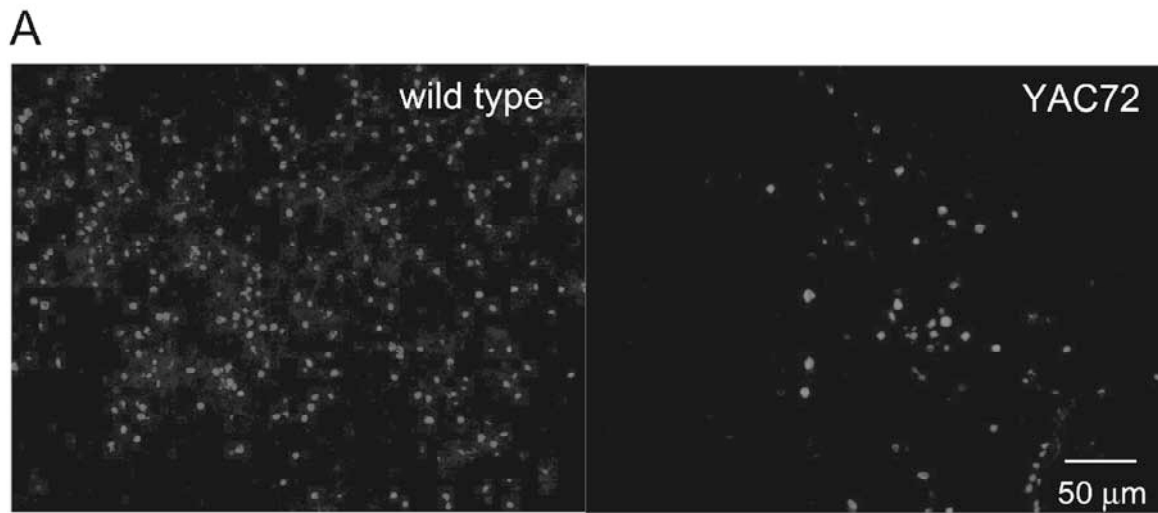


Figure 27: Loss of LacZ-positive cells in the striatum of YAC72 mice at 9 month post-injection.

(A) Photomicrograph of LacZ-infected cells in the striatum of a wild type and YAC72 mice. (B) Histogram showing the number of LacZ-infected cells in wild type (n=2) and YAC72 (n=6) mice. Values are expressed as mean \pm SEM

b) Dark cells

Cresyl violet stained sections were used to reveal the presence of striatal dark cells and assesses the effect of CNTF on the incidence of these cells. Dark hyperchromatic neurons were visible in YAC72-LacZ mice (Figure 28A), whereas no sign of these cells was found in

WT-LacZ and WT-CNTF animals. The intra-striatal administration of CNTF significantly reduced the number of dark cells: in YAC72-CNTF mice (60 ± 16) compared to YAC72-LacZ mice (129 ± 12 ; $p < 0.001$) (Figure 28 A and B). This effect was not limited to the injection site but was observed throughout the striatum (data not shown).

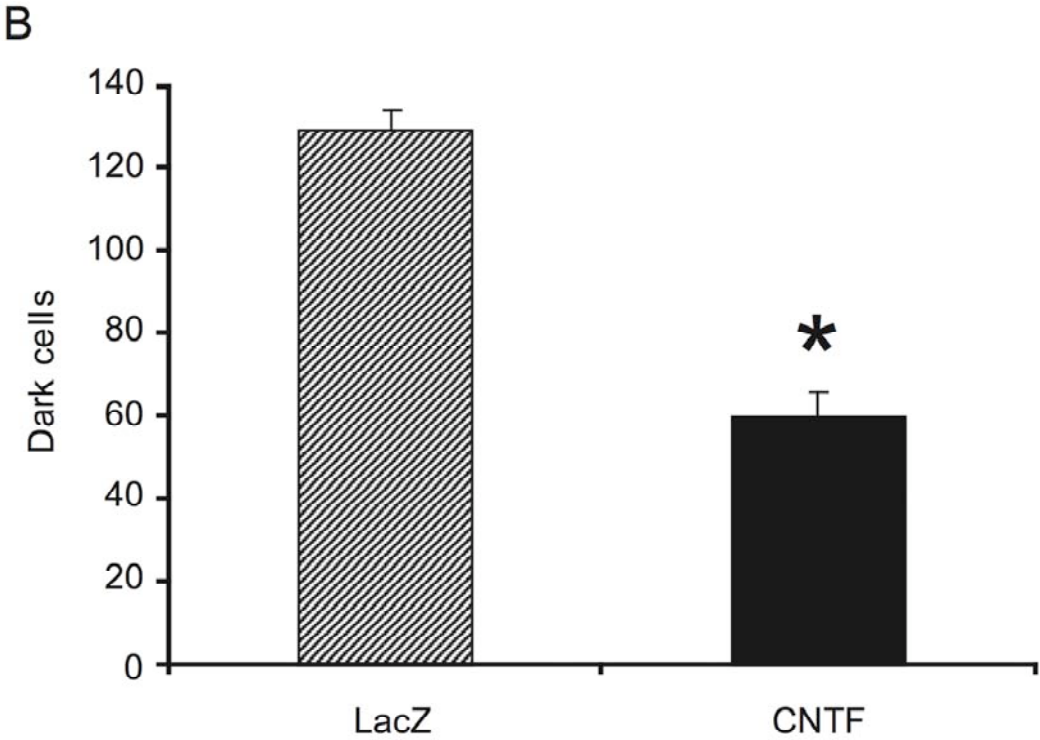
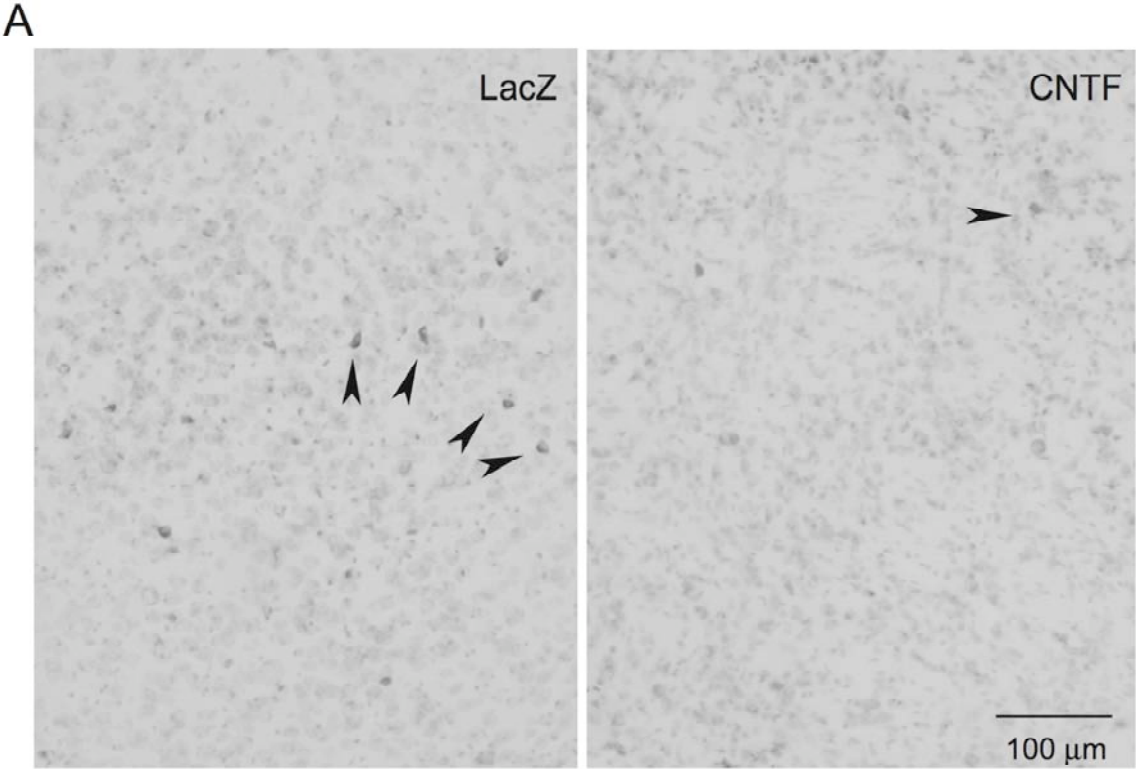


Figure 28: Significant reduction of cresyl violet-stained dark cells in YAC72-CNTF mice.

A) Photomicrographs of dark cells in the striatum of YAC72 mice injected with a LacZ- or CNTF-expressing lentivirus. Arrow shows some hyperchromatic and shrunken cells. (B) Histogram showing the total number of degenerating cells in the striatum of YAC72-CNTF (n=7) and YAC72-LacZ (n=6) (*p<0.001). Values are expressed as mean \pm SEM.

c) DARPP-32 and NeuN immunoreactivity

To further assess the effect of CNTF, stereological counts of DARPP-32 stained sections were performed. This marker of the striatal GABAergic spiny neurons (Ouimet et al., 1998) is decreased in several mouse models of HD (Bibb et al., 2000; Luthi-Carter et al., 2000; van Dellen et al., 2000). Interestingly, the long-term expression of CNTF in WT mice led to a decrease in DARPP-32 labeling as shown in Figure 6A. The neuritic staining and the number of DARPP-32 positive neurons were reduced ($1.10 \times 10^6 \pm 0.15$) compared with WT-LacZ animals ($1.44 \times 10^6 \pm 0.15$; $p = 0.13$) (Figure 29B). Unfortunately, this result precludes drawing any conclusions from the YAC72 mice, on any potential protective effect of CNTF on medium spiny neurons (YAC72-LacZ: $1.20 \times 10^6 \pm 0.07$; YAC72-CNTF: $1.15 \times 10^6 \pm 0.11$). To further investigate the pathology of 13 months old YAC72 mice and the effect of CNTF administration, striatal sections were stained with the neuronal marker NeuN. No significant change in the staining and number of NeuN-positive cells was observed between WT-LacZ ($1.52 \times 10^6 \pm 0.06$) and YAC72-LacZ mice ($1.42 \times 10^6 \pm 0.10$; $p = 0.2$) whereas a reduction in the number of NeuN-positive neurons was observed in the CNTF-treated mice (WT-CNTF: $1.35 \times 10^6 \pm 0.07$; YAC72-CNTF: $1.04 \times 10^6 \pm 0.07$; $p = 0.01$) (Figure 30A). Finally, immunohistochemical analysis of NADPH-d neurons, which are spared in HD patients, failed to reveal any alterations in the brain of WT and YAC72 mice treated or not with CNTF (data not shown).

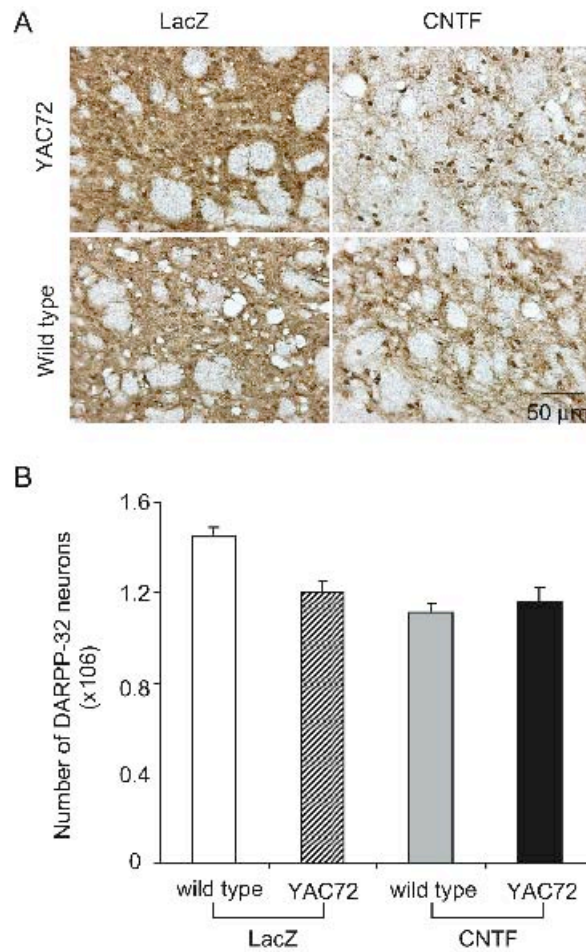


Figure 29: Stereological counts of DARPP-32 positive neurons.

(A) DARPP-32-stained striatal sections from WT-LacZ, WT-CNTF, YAC72-LacZ and YAC72-CNTF. (B) Quantification of the total number of the DARPP-32-positive cells in the striatum WT-LacZ (n=3), WT-CNTF (n=3), YAC72-LacZ (n=6) and YAC72-CNTF (n=7). Values are expressed as mean \pm SEM.

d) GFAP staining

Previous *in vitro* and *in vivo* studies have shown that CNTF induces astrocyte differentiation (Kahn et al., 1995; Mayer et al., 1994; Winter et al., 1995). We have therefore performed a glial fibrillary acidic protein (GFAP) immunostaining to reveal the presence of activated astrocytes (Figure 30B). Both WT-CNTF and YAC72-YAC72-CNTF-injected animals showed a substantial GFAP immunoreactivity (Figure 307), whereas only few GFAP-positive astrocytes were visible in WT-LacZ- and YAC72-LacZ-injected mice (Figure 30).

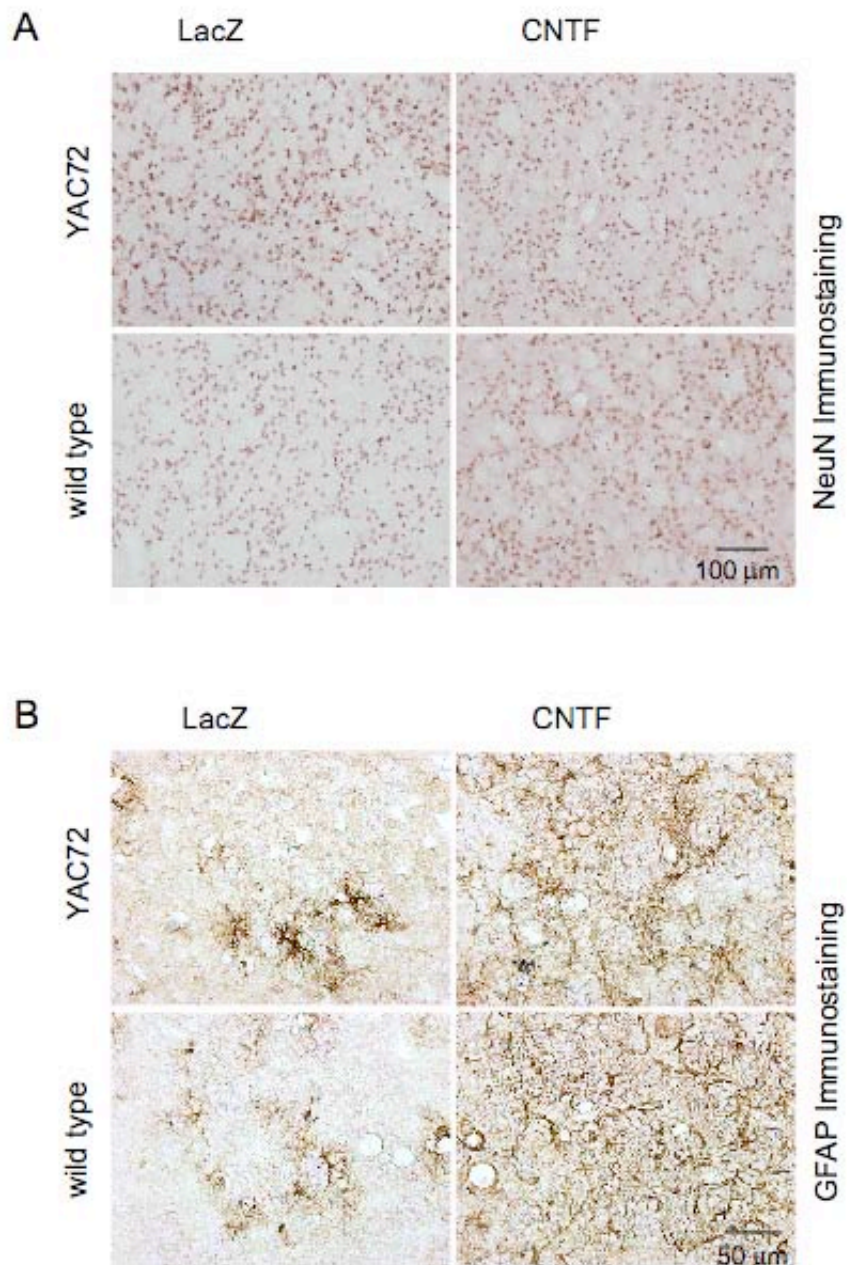


Figure 30: Analysis of NeuN stained striatal sections.

(A) NeuN stained striatal sections from WT-LacZ, WT-CNTF, YAC72-LacZ and YAC72-CNTF. (B) Evaluation of the astrocytic response in WT and YAC72 mice.

Photomicrographs showing GFAP-stained striatal sections in WT-LacZ, WT-CNTF, YAC72-LacZ and YAC72-CNTF injected animals. The administration of CNTF for 9 months leads to a significant increase in GFAP immunoreactivity. In YAC72-lacZ mice the staining did not differ from WT-LacZ animals.

3.5.DISCUSSION

In the present study, we assessed the effect of local and long-term lentiviral-mediated CNTF expression in the striatum of wild-type and HD transgenic mice. Lentiviral vectors led to robust transgene expression up to 9 months post-injection in the striatum of mice (Déglon et al., 2000) and lentiviral-mediated CNTF delivery was shown to protect striatal neurons in a drug-induced rat model of HD (de Almeida et al., 2001). Lentiviruses expressing the LacZ reporter gene or human CNTF gene were therefore injected bilaterally in 4 months old wild-type and YAC72 mice. Nine months post-injection, approximately 35'000 LacZ-infected cells were counted in wild type mice, whereas a reduction in the number of β -galactosidase-positive cells was observed in 5 out of the 6 HD transgenic mice. The loss of LacZ expression may result from the ongoing pathological process occurring in YAC72 mice and suggests that this reporter gene could be used as a surrogate marker to follow the appearance of HD pathology in animal models lacking severe neurodegeneration. Whether this loss of LacZ staining is due to an aberrant transcriptional regulation of the reporter gene, as reported in various experimental paradigms of HD, still needs to be investigated . The cell bodies of some spared LacZ-positive neurons appear larger with an intense staining. This phenotype is reminiscent of the results published by Bence and co-authors. They showed that cells co-expressing a mutated huntingtin fragment and a destabilized green fluorescent protein (GFP) have a 4-fold increase in GFP fluorescence compared to controls and that this phenomenon was due to an impairment of the ubiquitin-proteasome pathway (Bence et al., 2001). Interestingly, no significant differences in CNTF expression were observed over time or between wild type and YAC72 mice. This suggests that CNTF expression, contrary to LacZ, is not affected by mutated huntingtin or that CNTF is altering the ongoing pathology.

During the time-course examined in our experiments, the phenotype of YAC72 transgenic mice appears to correspond to early stages of the disease, with hyperactivity and neuronal dysfunction associated with the appearance of dark cells. While nuclear huntingtin staining increases around 12 months of age very few huntingtin inclusions are identified (Hodgson et al., 1999). This experimental paradigm was used to assess the impact of a local and continuous expression of approximately 0.5 ng human CNTF in the striatum. Wild- type FVB/N littermates were used as control. The development of hyperactivity on open-field testing was the first parameter examined to assess the disease progression and the impact of CNTF expression. Large inter- and intra-individual variability of spontaneous explorative behavior was observed, but significant differences were recorded between wild-type and

transgenic mice at 5 and 8 months. This heterogeneity and the early stage of the pathology in YAC72 mice characterized by subtle changes, limits the number of validated outcome measures, which can reliably be used to monitor the effects of potential therapeutic candidates. Nuclear inclusions are, for example, very rare in these animals and can not be used as parameter. Recently a new YAC128 transgenic mouse model of HD has been developed which has increased levels of transgene expression and an accelerated neurodegenerative phenotype that is more adequate for therapeutic trials (Slow et al., 2003). Scattered atrophic neurons with condensed chromatin and a “dark” cytoplasm are present in affected areas of HD brains (Vonsattel and DiFiglia, 1998). In 17 week-old R6/2 transgenic mice, degenerating neurons surrounded by neurons with normal appearance were detected in different brain regions. These dying neurons have a higher affinity for toluidine blue and exhibit nuclear inclusions and ruffling of the plasma membrane (Turmaine et al., 2000). Dark neurons were also observed in 5-7 months old R6/1 mice (Iannicola et al., 2000) and in 8-12 months old YAC72 mice (Hodgson et al., 1999). In our study, hyperchromatic and shrunken neurons were visible in YAC72-LacZ mice and CNTF expression significantly decreases the number of dark neurons. Whether these dark cells represent a degenerating subpopulation of neurons is, however, unclear.

Mutant huntingtin expression is associated with regional and temporal alterations in gene expression (Sugars and Rubinsztein, 2003). Changes in the expression of transcription factors, neuronal markers, genes implicated in cellular metabolism or neurotransmitter receptors should, not only, provide important information related to polyQ-mediated neurodegeneration but also quantitative tools for the evaluation of therapeutic strategies. Histological studies on R6/2 animals has, for example, revealed a reduction in DARPP-32 and other dopamine-regulated proteins expressed in medium spiny neurons at 4 to 8 weeks (Bibb et al., 2000; van Dellen et al., 2000). DARPP-32 is a pivotal regulator of dopamine receptor signaling in the striatum and is therefore considered as a good marker of the pathology occurring in presymptomatic mice or early stage of the disease. Interestingly, CNTF expression induced a drastic down-regulation of DARPP-32 in wild-type mice 9 months post-lentiviral injection. This effect has not been observed when the same CNTF-expressing lentiviral vector was injected for 5 to 12 weeks in QA-lesioned rats (de Almeida et al. 2001; unpublished data) or when CNTF was delivered in 3-NP lesioned primates by encapsulated genetically engineered BHK cells (Mittoux et al., 2000). How the long-term striatal expression of CNTF leads to a decrease in DARPP-32 immunoreactivity, and how this change might affect striatal function still needs to be established. The continuous delivery of nanograms of CNTF was previously

shown to drastically decrease the extent of striatal damages in drug-induced models of HD (Emerich et al., 1996; Emerich et al., 1997a; Mittoux et al., 2000; de Almeida et al., 2001). Differences in the pathological processes between drug-induced and genetic models of HD may lead a differential effect of CNTF. The mode of action of this cytokine is still poorly understood and various intracellular pathways might be activated in various animal models of HD. The binding of the CNTF to the gp130/LIFR β /CNTFR α receptor complex has been shown to activate the Jack/STAT and PI3K/Akt signaling pathways (Alonzi et al., 2001). CNTF, in particular, induces the phosphorylation and nuclear translocation of STA3, a protein inducing the transcription of Bcl-2 and Bcl-xL genes (Catlett-Falcone et al., 1999; Karni et al., 1999) and regulating the activity of several transcription factors such as Forkhead 1, CREB and NF-kB (Du and Montminy, 1998; Brunet et al., 1999; Kane et al., 1999; Ozes et al., 1999). Long-term expression of CNTF may affect striatal functions either directly or indirectly. It is well established that CNTF induces a phenotypic alteration of astrocytes *in vivo* (Winter et al., 1995; Kahn et al., 1997; Lisovoski et al., 1997; Levison et al., 1998; Gomes et al., 1999). Consistent with this, we observed an up-regulation of the astrocytic marker GFAP in the striatum of Lenti-CNTF injected mice. This astrocytic response covers the entire striatum and is probably due to diffusion of CNTF throughout this structure. Recently, Albrecht and co-authors showed that CNTF treatment lead to an activation of spinal cord astrocytes, the production of various growth factors and to an increased survival of CNS neurons compared to untreated astrocytes (Albrecht et al., 2002). They propose that cytokine-activated astrocytes have an enhanced buffering capacity leading to decreased levels of harmful energy metabolites, which may indirectly protect neurons. This same indirect CNTF-mediated mechanism could be activated in the striatum and therefore protect neurons undergoing degeneration. Chronic impairments of mitochondrial energy metabolism are indeed thought to be involved in neurodegenerative diseases, including HD. Studies are needed to further assess CNTF's effect on striatal functions and its impact on HD pathology corresponding not only to an early stage of the disease but also to grade 2-4 pathologies.

3.6. ACKNOWLEDGEMENTS

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4. BDNF DELIVERY IN GENETIC MODELS OF HUNTINGTON'S DISEASE

4.1. ABSTRACT

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is necessary for normal striatal neuron function and for long-term survival of MSNs. The possible involvement of this factor in HD pathology is supported by recent data showing that mutant huntingtin interferes with BDNF expression in the cortex and impairs BDNF vesicular transport. The loss of trophic support may therefore contribute to the pathogenesis. Beside its survival property for MSNs, BDNF was also shown to be neuroprotective in a toxin-induced striatal lesion model of HD, as well as in primary striatal neurons expressing mutant htt fragments. Therefore, BDNF might prevent striatal degeneration in Huntington's disease. In the present study, we have further assessed the protective effect of BDNF in two genetic models of HD. BDNF was delivered in the striatum of YAC128 mice and in a lentiviral-based rat model of HD. The expression of BDNF was achieved without side effects, but motor deficits, down-regulation of DARPP-32 and formation of htt aggregates were not found to be prevented in these experimental paradigms.

4.2. INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a potent survival factor for striatal neurons (Ventimiglia et al., 1995) and it induces the differentiation of striatal GABAergic, calbindin- and DARPP-32-positive cells (Mizuno et al., 1994; Nakao et al., 1995). BDNF is synthesized in the cortex and anterogradely transported to the striatum, where it is stored in dense-core vesicles before its release (Sobreviela et al., 1996). BDNF null-mutant mice present a reduction of striatal projecting neurons, but such animals do not survive long enough to evaluate the effects of a chronic depletion of this trophic factor in striatal neurons. (Jones et al., 1994; Ivkovic et al., 1997). Recently, the crossing of transgenic floxed BDNF mice with *Emx1IRES^{cre}* has generated viable mice that lack cortical BDNF. Eighteen-month-old animals display a loss of medium spiny neurons, thus further illustrating the importance of this trophic factor for GABAergic neurons in adults (Baquet et al., 2004). Interestingly, the mice present several analogies with R6/2 transgenic mice, notably the forelimb clasping behavior and a reduction of CNS volume. Evidence that BDNF influences HD pathology was further obtained by cross-mating *bdnf*^{+/-} mice with R6/1 mice; an earlier onset of motor deficits and a lack of DARPP-32 expression in the striatum were observed in the double-transgenic mice (Canals et al., 2004).

In addition, a depletion of striatal BDNF was reported in the brain of HD patients and in transgenic mice (Zuccato et al., 2001). Two mechanisms may contribute to this trophic decrease, since both the cortical BDNF transcription and the vesicular transport of BDNF are impaired by mutant htt (Zuccato et al., 2003; Gauthier et al., 2004). Therefore restoring striatal BDNF could represent a therapeutic approach to prevent medium spiny neuron degeneration. Interestingly, BDNF was proposed as therapy for HD before it was linked to the disease. BDNF was shown to protect GABAergic neurons in drug-induced models of HD (Alberch et al., 2004), as well as in rat striatal cultures expressing a mutant huntingtin fragment (Saudou et al., 1998) (see chapter 2.4.7). In addition to its potential neuroprotective effect, BDNF might induce neurogenesis in the adult rodent striatum (Benraiss et al., 2001; Pencea et al., 2001), which could be especially interesting for a chronic neurodegenerative disease such as HD.

Motivated by these data, we have further tested the protective effect of BDNF in two genetic models of HD which are characterized by mild, respectively severe pathologies. We have

used the YAC128 mice (Slow et al., 2003), which express the full-length huntingtin with 128 CAG repeats. The level of expression of human htt is approximately five times higher than that of the endogenous protein and the polyQ expansion increases in these transgenic mice from 72 to 128 glutamines. As a result, these mice have a more severe phenotype than YAC72 mice (Hodgson et al., 1999). They are behaviorally impaired as recorded by the accelerated rotarod and activity box; at one year of age they show a 20% striatal atrophy and 10% reduction of striatal neuronal sections. To try to reverse this phenotype, we have bilaterally injected Lv-BDNF in the striatum of these transgenic mice. The neuronal pathology was evaluated at 18 months.

The second experimental paradigm was the lentiviral-based rat model expressing the first 853 amino acids with 82 CAG repeats under the control of a tetracycline promoter (Régulier et al. 2003). We have chosen this experimental paradigm to evaluate the potential effect of BDNF in severely lesioned animals. At three months post-injection, the expression of mutant htt fragments induces a severe lesion, which is characterized by neuronal loss, striatal shrinkage and ventricle enlargement.

4.3. MATERIAL AND METHODS

4.3.1. Lentiviral vector production

The cDNAs encoding enhanced GFP, LacZ, human CNTF (Clontech, Palo Alto; USA), human NGF (human pre-proNGF domain [nucleotides 170-532, Genbank X52599] fused to human BDNF [nucleotides 681-1040, Genbank X60201] to obtain a chimera BDNF (Figure 31)) and the first 853 amino acids of human huntingtin containing 19 or 82 CAG repeats, controlled by the PGK or TRE promoter, and PGK-tTA1 were cloned in the SIN-W transfer vector as previously described in chapter 2.3.2.

4.3.2. Cell cultures

E16 rat primary striatal cultures were dissected, cultured and infected with Lv-BDNF or Lv-GFP as previously described in chapter 2.3.1. The concentrations of BDNF were determined by ELISA (R & D, Abingdon, UK) on media samples collected at 2 weeks and stored at -20°C until use. Samples were processed according to the manufacturer's instructions. The detection limit was 20 pg/ml for BDNF.

293T cells were transfected with wild-type BDNF or chimeric preproNGF-BDNF. Two days later media were replaced and collected after two hours, then stored at -20°C until use.

4.3.3. Western blot analysis

Cellular lysates were harvested in lysis buffer (125mM Tris·HCl/0.5% SDS/1% Nonidet P-40) containing protease inhibitors (Roche Pharma, Basel, Switzerland). Protein concentrations of cytoplasmic fractions were determined with the BCA protein assay kit (Pierce Biotechnology Inc, Rockford, USA). Equal amounts of protein (50 µg) or equal amounts of medium (25 µl) were loaded on 10% or 15% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH, Dassel/Relliehausen, Germany). Immunoblotting was performed by using a rabbit polyclonal antibody BDNF; 1:10'000; Santa Cruz, USA or mouse monoclonal antibody DARPP-32; 1:10'000; Chemicon International Inc., Temecula, USA, followed by goat anti-mouse or anti-rabbit horseradish peroxydase (HRP) antibody (1:2'000; DakoCytomation AG, Zug,

Switzerland). Visualization of the proteins was achieved with an ECL and chemiluminescence detection kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) in a Multimage Light Cabinet (Alpha Innotech Corporation, San Leandro, USA).

4.3.4. Animals

The animals were housed in a controlled-temperature room that was maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*. The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for care and use of laboratory animals.

4.3.5. Experiment 1: BDNF assay in YAC128 mice

YAC128 mice and wild-type FVB/N (Iffa-Credo, France) mice (Slow et al., 2003) were used in these studies. Stereotaxic injections were performed as previously described in chapter 3.3.3. Nine-month-old mice were bilaterally injected in the striatum with 2 μ l of lentiviral vectors at p24 of 100'000 ng/ml: YAC128 (N=8) and WT (N=10) with SIN-PGK-BDNF at a p24 of 100 000 ng/ml and control animals YAC128 (N=8) and WT (N=10) were injected with SIN-PGK-GFP at a p24 of 100'000 ng/ml. The animals were sacrificed at the age of 18 months. Additional YAC128 (N=3) and WT (N=8) mice were injected with lenti-BDNF in one hemisphere and lenti-GFP in the other hemisphere and sacrificed after 2 weeks to determine BDNF and GFP expression.

4.3.6. Behavioral tests

a) Spontaneous activity

Spontaneous activity was recorded during 30 minutes with the activity boxes. For details see chapter 3.3.4.

b) Accelerated rotarod

Mice were placed on the rotarod and the speed was progressively increased from 0 to 45 rpm over a period of 120 s. Mice were trained for 3 days with three trials per day. Following this training period, the mice were tested for three consecutive trials in one day, with at least 30min rest between trials and the best score was recorded.

4.3.7. Experiment 2: BDNF assay on the lentiviral rat model

Adult female Wistar rats (Iffa Credo / Charles River, Les Oncins, France) weighing 180-200g were used. The animals were maintained in the “off” state 3 weeks after the first lentivirus injection with the addition of 200 mg/l doxycycline (Dox, Sigma Chemical CO, Saint Louis, USA) in drinking water containing 40g/l sucrose (corresponding approximately to 20 mg Dox/rat/day) and put “on” after the second injection of Lv-BDNF or Lv-GFP.

Concentrated viral stocks were thawed and resuspended by repeated pipetting. Lentiviral-expressing vectors were stereotaxically injected into the striatum of ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthetized animals using a Hamilton syringe with a 34-gauge blunt-tip needle (Hamilton, Reno, USA). A 1:1 ratio in particle contents was used for lentiviral co-injection with 100'000 ng of p24/ml of PGK-tTA1 and 100'000 ng/ml of p24 of TRE-853-82Q. The viral suspensions (4µl) were injected at 0.2µl/min by means of an automatic injector (Stoelting Co., Wood Dale, USA) and the needle was left in place for an additional 5 min. The stereotaxic coordinates were: 0.5 mm rostral to bregma; 2 and 3 mm lateral to midline and 5 mm from the skull surface. The skin was closed using a 6-0 Prolene® suture (Ethicon, Johnson and Johnson, Brussels, Belgium). At three weeks bilateral lentiviral injection was performed with lenti-GFP and lenti-BDNF (N=8); each Lv was injected at 100'000 ng of p24/ml. The stereotaxic coordinates were: 0.5 mm rostral to bregma; 2.5 mm lateral to midline and 5 mm from the skull surface. In addition rats injected only with Lv-GFP, Lv-BDNF or mixed Lv-tTA1 and Lv- TRE-853/82Q were used as control (N=4). Three months later, the rats were sacrificed.

4.3.8. Histological processing

Mice or rats were given a sodium pentobarbital overdose and transcardially perfused with saline and 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 24 h and finally cryoprotected in 25% sucrose /0.1 M phosphate buffer for 48 h. The brains were frozen in dry ice and 25 µm coronal sections were cut on a sliding microtome cryostat (Cryocut 1800, Leica Microsystems AG, Glattbrugg, Switzerland) at

-20°C. Slices throughout the entire striatum were collected and stored in 48 well trays (Costar, Cambridge, USA) as free floating sections in PBS supplemented with 0.12 μ M sodium azide. The trays were stored at 4°C until immunohistochemical processing. Striatal sections from injected rats were processed by immunocytochemistry for DARPP-32 as previously described in chapter 2.3.3. The sections were mounted, dehydrated by passing twice through ethanol and toluene and coverslipped with Merckoglas® (EM Science, Gibbstown, USA).

Image Analysis

The htt lesions were analyzed by directly measuring the area of the lesion in DARPP-32-stained sections (200 μ m between sections) by microscope acquisition at a magnification of 1,5x (software: Analyses, Olympus). Sections throughout the entire striatum were analyzed. Data are expressed as the volume of the lesion in mm³. Ventricle size was analyzed on cresyl violet stained sections (200 μ m between sections) with the same software. Data are expressed as the volume in mm³.

4.3.9. Data analysis

Quantifications are expressed as mean \pm SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test (Statistica 5.1, Statsoft Inc, USA). The significance level was set at $p < 0.05$.

4.4. RESULTS

4.4.1. Lv-BDNF is released and is bioactive in striatal neurons

We have constructed a preproNFG-BDNF vector by fusing the signal peptide of the preproNGF to mature BDNF. This chimeric BDNF should be efficiently processed through the constitutive pathway and the mature BDNF released in the extracellular space (Haubensak et al., 1998; Mowla et al., 1999) (Figure 31).

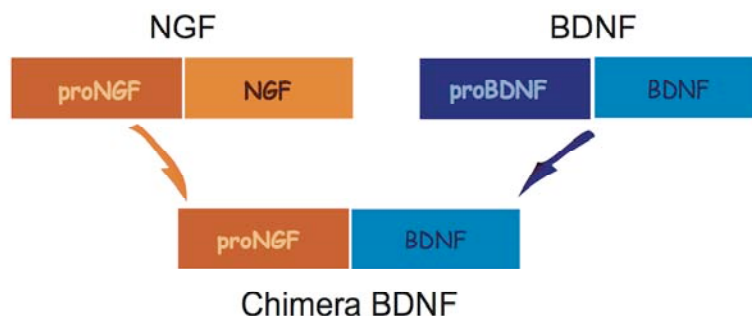


Figure 31: Strategy to increase BDNF secretion

The prepro-domain of BDNF was deleted and BDNF cDNA fused to the prepro domain of NGF to produce the chimeric preproNGF-BDNF protein.

To test if the chimeric preproNGF-BDNF was properly processed and released, we transiently transfected 293T cells with the plasmids coding for wild type or chimeric BDNF and analyzed the samples by Western blot (Figure 32A). The polyclonal antibody recognized both the human recombinant and the chimeric BDNF, and a band with the expected molecular weight of 15 kDa was observed. In contrast, wild type human BDNF was not efficiently secreted. This probably reflects the poor processing of prepro-BDNF and the absence of regulated BDNF secretion in 293T cells. This confirms previous results from our laboratory showing that wild type BDNF is inefficiently produced in different mammalian cells. Based on the titration with the recombinant protein, we have estimated that the amount of BDNF released in the supernatant of 293T cells is between 1-5 ng/ml.

The lentiviral vector coding for the chimeric BDNF was produced and further validated in rat primary striatal cultures. Western blot analysis of media collected 2 weeks post-infection showed that the Lv induced production and processing of mature BDNF (Figure 32B).

Quantitative measurement of BDNF secretion by ELISA showed that 715 +/- 357 pg/ml (N=4) of the protein are present in the media of infected cells. Measurements of non-infected or Lv-GFP infected cultures showed concentrations below the detection limit.

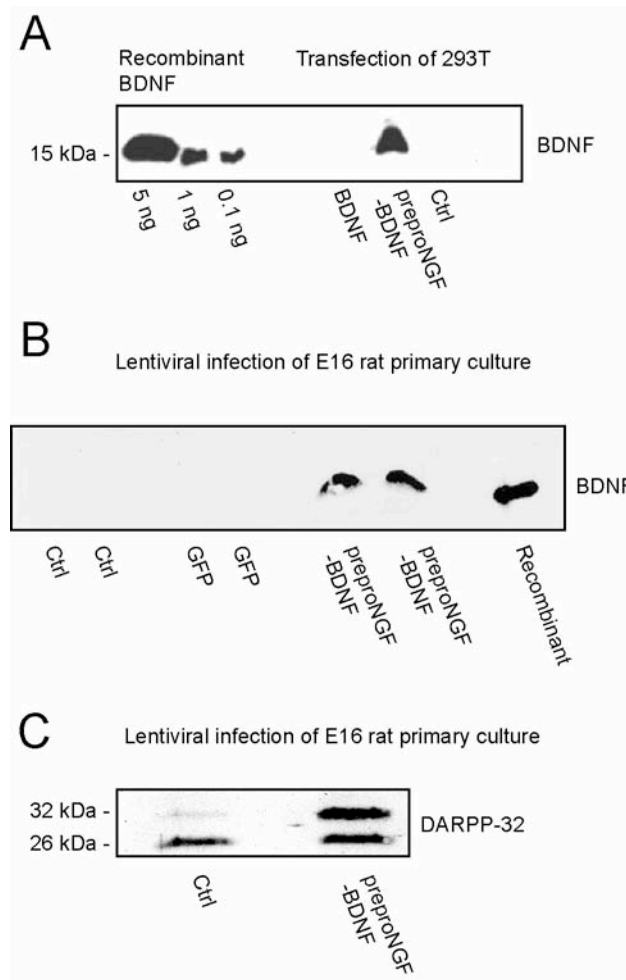


Figure 32: Functionality of the chimeric proNGF-BDNF vector

(A) 293T cells were transfected with BDNF, proNGF-BDNF-plasmids or mock-transfected. The anti-BDNF antibody recognized a band at 15 kDa in the columns loaded with recombinant protein and in the proNGF-BDNF column. (B) Supernatant of rat striatal cultures infected with Lv-preproNGF-BDNF was analyzed by Western blot, showing that BDNF was correctly processed and secreted. (C) Cell fractions of striatal culture infected with Lv-preproNGF-BDNF or control cultures were lysed and blotted against antibody DARPP-32. A faint band corresponding to DARPP-32 was detected in the control cultures, whereas BDNF expression increased the 32kDa signal. An unspecific band at 26 kDa was used to show equal protein loading.

We have also tested the bioactivity of chimeric BDNF in primary striatal cultures. BDNF was shown to promote striatal GABAergic differentiation *in vitro*, notably by enhancing DARPP-32 expression (Ivkovic et al., 1997). At two weeks post-infection, an up-regulation of DARPP-32 expression was observed in Lv-BDNF infected cells, compared to non-treated control cultures (Figure 32C).

4.4.2. Lentiviral-mediated delivery of BDNF in the mouse striatum

Finally, the functionality of the BDNF expression vector was assessed *in vivo* by the injection of Lv-BDNF in mouse striatum. Two weeks later, striatum and hippocampus were dissected, homogenized and the expression of BDNF analyzed by Western Blot. The hippocampus was used as internal control, because it has a high BDNF expression (Conner et al., 1997). In this structure, a 15 kDa band corresponding to the recombinant protein was detected, as well as three higher molecular bands probably representing unprocessed BDNF (Figure 33A). As expected, BDNF expression level was higher in injected striatum compared to control non-injected hemispheres (Figure 33B).

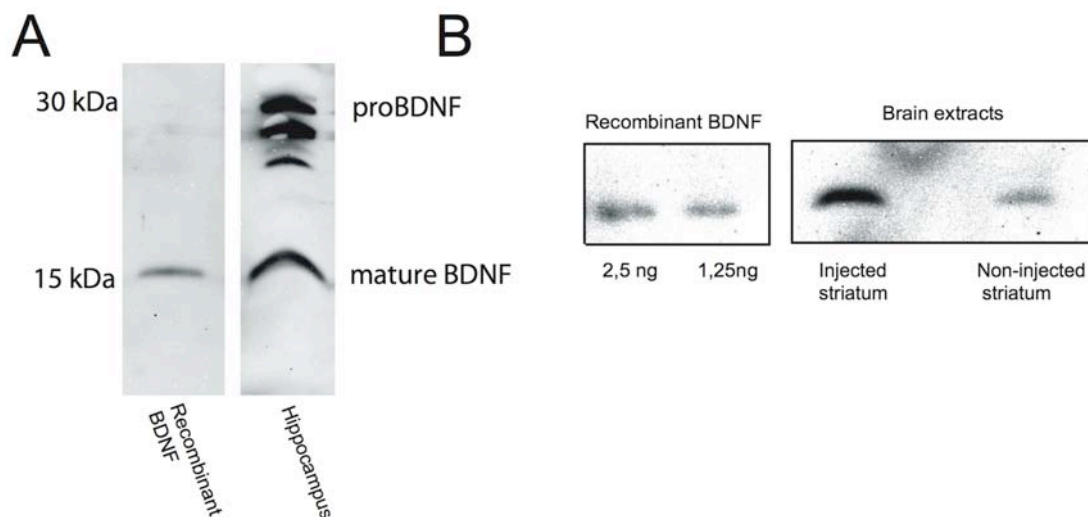


Figure 33: In vivo expression of BDNF

(A) Hippocampus from adult mice was dissected lyzed and endogenous BDNF expression analyzed by Western Blot. A band migrating at the same molecular weight as recombinant BDNF was detected, as well as three higher bands probably representing unprocessed BDNF. (B) Mouse striata injected with lenti-BDNF or control animals were dissected 2 weeks post-surgery and analyzed by Western Blot. A stronger BDNF signal is detected in the injected hemisphere.

4.4.3. Experiment 1: BDNF assay in YAC128 mice

4.4.3.1. Behavior and body weight evolutions

To evaluate the protective effect of BDNF, 9-month-old YAC128 and wild type mice were bilaterally injected with either lenti-BDNF or lenti-GFP. At that stage, the animals display only subtle striatal atrophy with no decrease in neuronal cell body section (Slow et al., 2003). During the following 9 months, the motor deficits were measured with the accelerated rotarod and spontaneous activity tests. Statistically significant differences in the rotarod performance of wild type and YAC128 mice were already observed at 10 months and persisted over

time. Importantly, the delivery of BDNF did not modify the behavioral deficit of HD transgenic mice (Figure 34A). Similar results were obtained with the spontaneous activity test. The Figure 34B showed that wild type animals were more active than YAC128 mice and that BDNF administration did not influence this hypoactivity (Figure 34B)

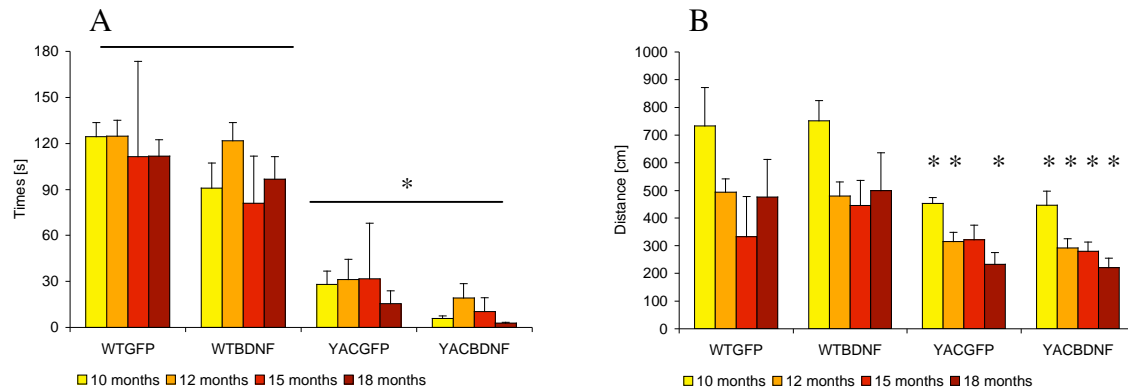


Figure 34: Behavioral tests

(A) Accelerated rotarod tests were performed every 3 months. The latency to fall was recorded and showed a statistically significant decrease in both YAC128 groups compared to wild type groups at each time point ($*p < 0.05$). In both groups the delivery of BDNF did not affect the behavior performance. (B) Spontaneous activity was recorded periodically; wild type mice traveled more than YAC128 mice at each time point ($*p < 0.05$), but the delivery of BDNF did not increase hypoactivity in the transgenic mice.

The decreased latency before falling from the rotarod and the hypoactivity recorded in transgenic mice were correlated with the progressive increase in body mass of HD transgenic mice (Figure 35).

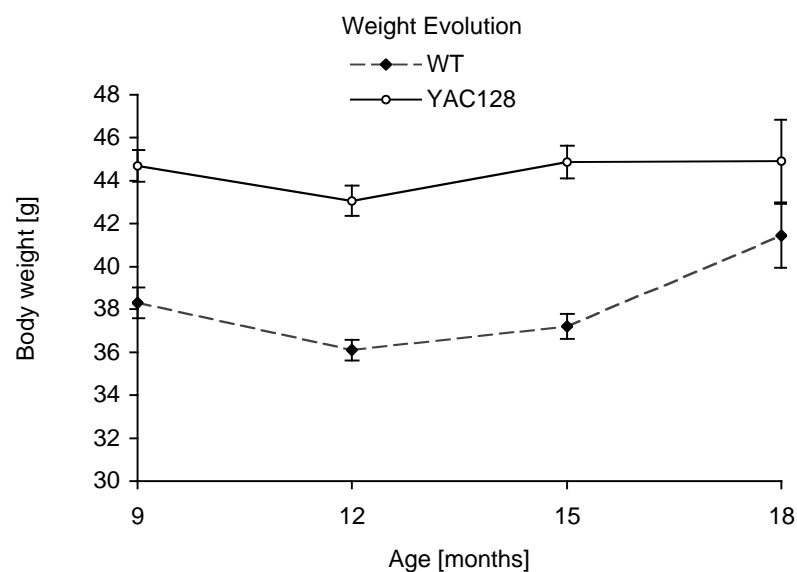


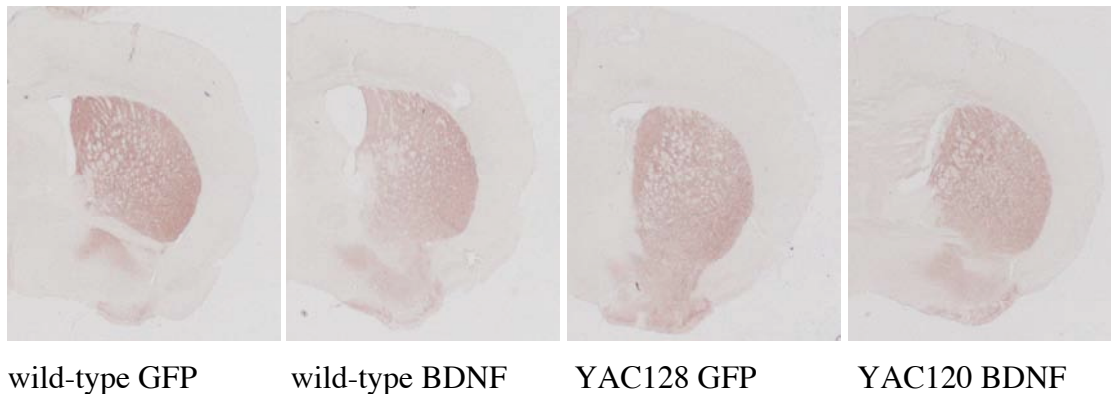
Figure 35: Evolution of the body weight

Body weight of wild type and YAC128 mice was periodically measured. YAC128 weighted more than wild type with a mean difference of 7g (+ 20%). This difference was maintained until 15 months and was reduced only at month 18 ($p < 0.01$ at 9,12,15 months). The groups of BDNF and GFP treated mice were similar and were therefore not represented in this graph.

4.4.3.2. Brain weight and striatal volume

One-year-old YAC128 mice were shown to display a general brain atrophy and in particular a 15% decrease of striatum volume as compared to wild-type mice (Slow et al., 2003). We have therefore used these two parameters to further assess the protective effect of BDNF in 18-month-old YAC128 mice. At this stage DARPP-32 is not downregulated (**Figure 36A**) and rare aggregates are present in the striatum of transgenic mice (**Figure 36B**) as well as in other brain regions (data not shown).

A



B

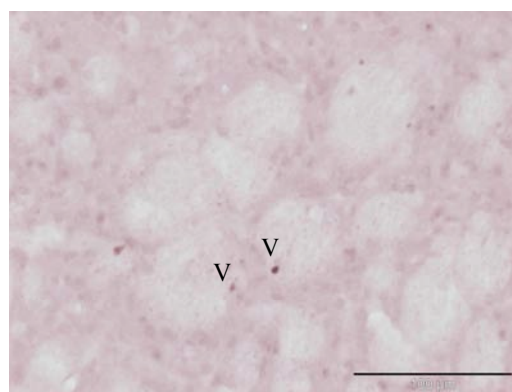


Figure 36: DARPP-32 and ubiquitin staining in YAC128 at 18 months

Wild-type and YAC128 mice brain sections were stained against (A) DARPP-32 and (B) ubiquitin. No difference of DARPP-32 staining was observed between the four experimental groups. Few aggregates were observed in the striatum of YAC128 mice (V).

To our surprise, the brain weights and striatal volumes measured on DARPP-32 stained sections were not significantly different between wild type and YAC128 mice (**Figure 37 A-B**). The protective effect of BDNF could therefore not be evaluated in this experiment.

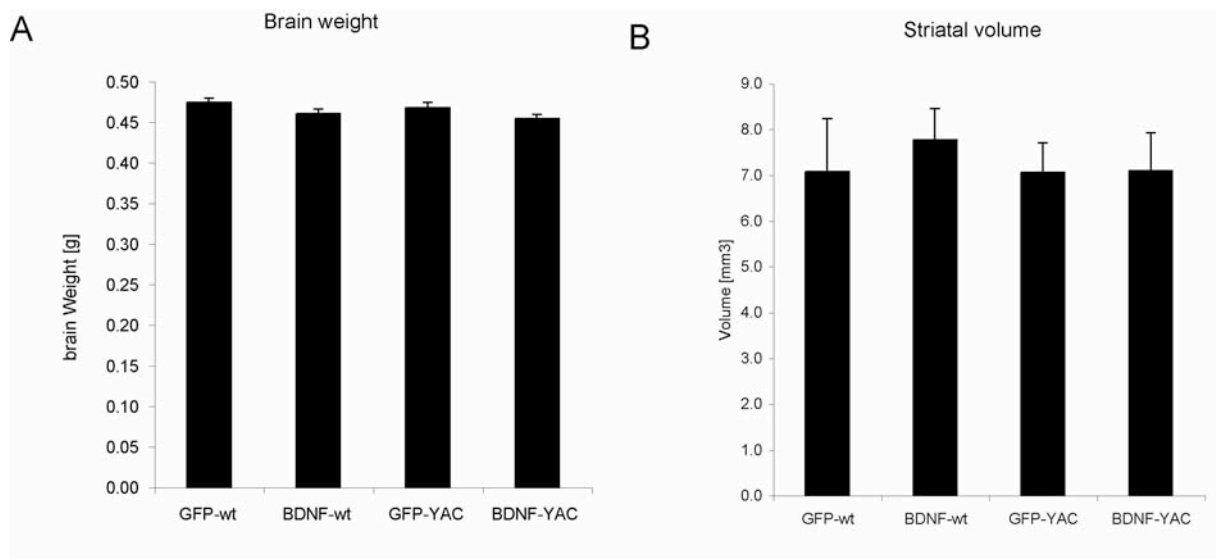


Figure 37: Brain weight and striatal volume at 18 months

(A) Injected YAC128 and wild type mice were sacrificed at 18 months. Histogram shows the brain weights of all four groups. (B) Striatal volume was analyzed on DARPP-32-stained section (Data are expressed as mean \pm SEM)

4.4.4. Experiment 2: BDNF in lentiviral-based rat model of HD

Due to the severity of the pathology in the lentiviral-based model of HD, was chosen as second experimental paradigm to test the protective effect of BDNF. We have previously shown that the delivery of lentiviral vectors encoding mutant htt 853-82Q fragment under the control of a tetracycline-regulated promoter is inducing a striatal pathology, which is characterized by a reduction of DARPP-32, the proteolytic release of N-terminal htt fragments and nuclear aggregation at 3 months (Régulier et al., 2002; Régulier et al., 2003). In these experiments, rats were bilaterally injected with a lentiviral mixture of Lv-PGK-tTA1 and Lv-TRE-853; dox was added in the drinking water to switch off huntingtin expression. Three weeks later, dox was removed and one hemisphere was injected with Lv-BDNF and the other hemisphere with Lv-GFP. In addition, rats expressing only mutant htt, BDNF or GFP were used as controls.

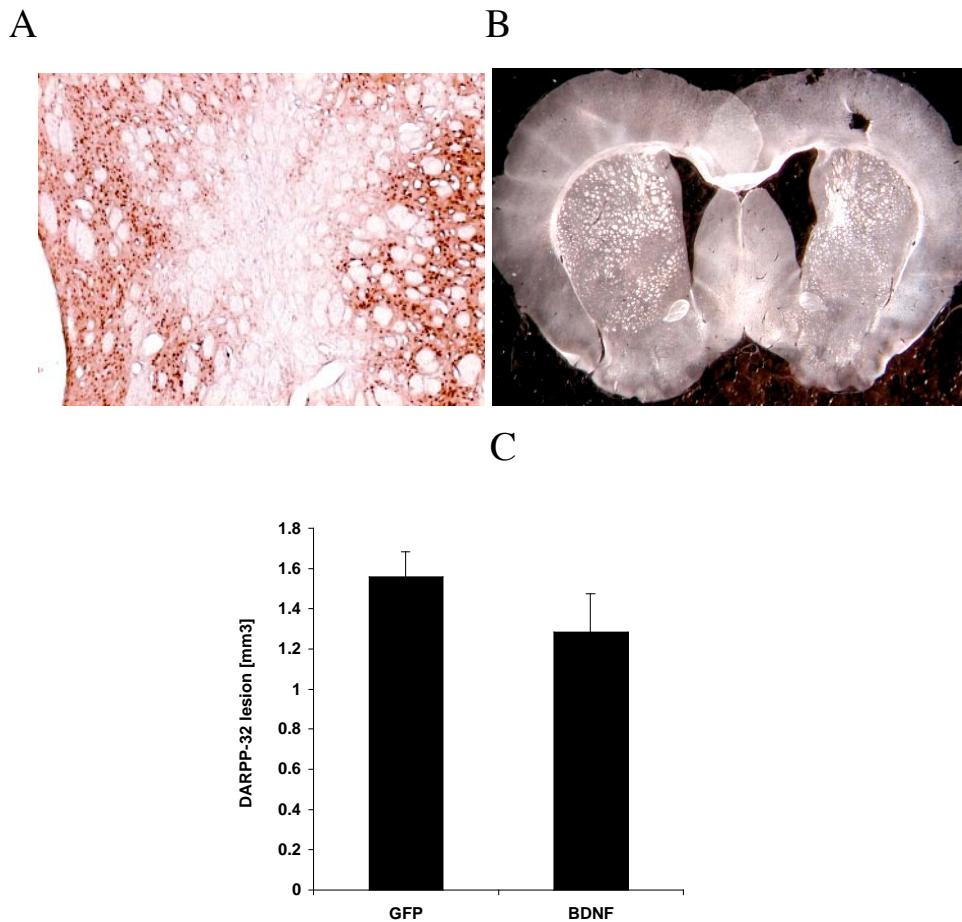


Figure 38: Striatal degeneration in rat striata

Three months post-injection of tet-htt 853-82Q, a severe lesion was observed. (A) The pathology is characterized by a drastic loss of DARPP-32 immunoreactivity, (B) an enlargement of the ventricle due to striatal shrinkage and a coalescence of the internal capsule of the striatum. (C) Quantification of the lesion volume in treated (BDNF) and control (GFP) groups shows that BDNF has no significant protective effect ($p = 0.2$).

As previously reported, mutant htt expression leads to a loss of DARPP-32 immunoreactivity, 3 months post-injection (Figure 38A). In control animals, co-expressing htt853-82Q and GFP reporter gene, a lesion of $1.56 \pm 0.13 \text{ mm}^3$ was observed. This lesion volume was slightly decreased in BDNF-treated animals with a volume of $1.28 \pm 0.19 \text{ mm}^3$, but it did not reach statistical significance. No up-regulation of DARPP-32 expression was observed in BDNF-treated striatum as compared to control hemispheres (data not shown).

In the control group, the HD pathology induced a tissue shrinkage and coalescence of the internal capsule of the striatum (Figure 38B). This striatal shrinkage induced a ventricle enlargement, reminiscent of advanced stages of HD in patients. To take into account tissue contraction, we have quantified the ventricle size and used this as an indirect measurement of striatal shrinkage. Mutant htt induced a two-fold volume dilatation of the ventricle (Figure 39). However, the delivery of BDNF did not reduce this enlargement.

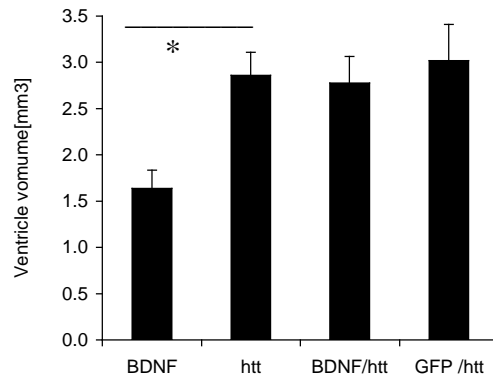


Figure 39: Quantification of the ventricle volume.

Evaluation of ventricle volumes three months post-infection. Mutant htt delivery induced a significant enlargement of the lateral ventricle volume ($p < 0.05$). The co-expression of BDNF or GFP with mutant htt did not modify the ventricle dilatation.

Finally, we analyzed whether the expression of BDNF may alter the formation of htt aggregates. In htt853-82Q injected rats, numerous nuclear and neuritic htt inclusions were present in the striatum (Figure 40). The expression of BDNF did not modify the formation and/or the subcellular localization of aggregates.

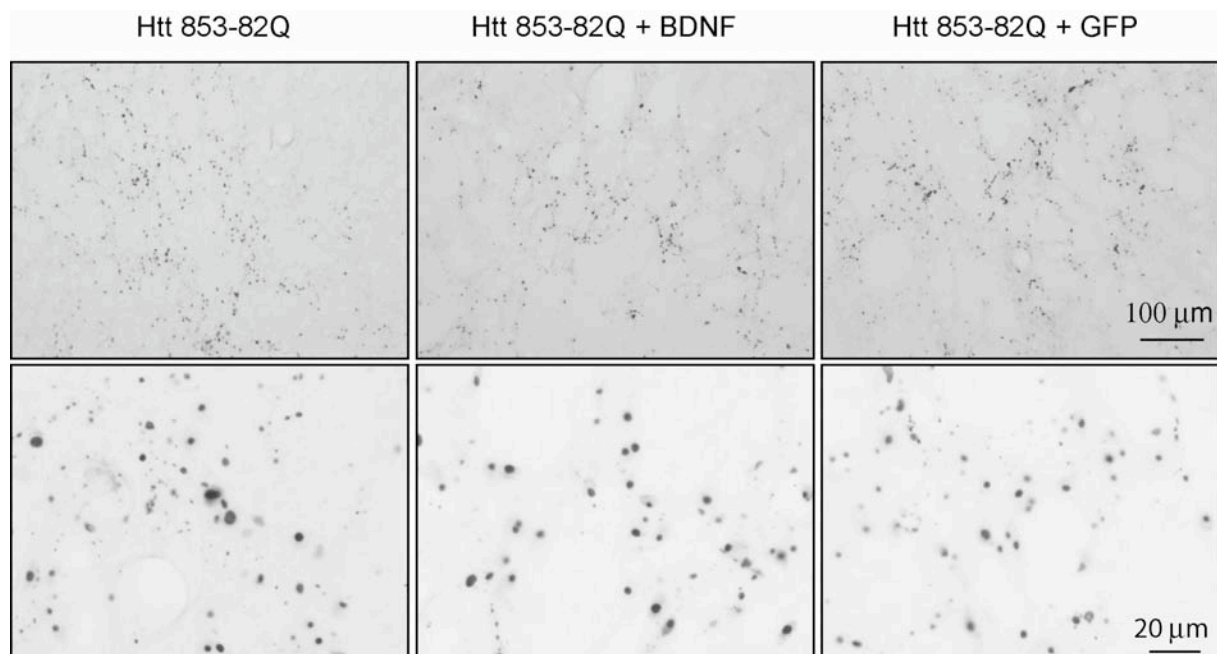


Figure 40: Huntingtin aggregation is not modified by BDNF

Htt staining (mEm48) at three months htt expression showed no qualitative difference of type or number of aggregation or number in the 3 different experimental groups.

4.5. DISCUSSION

Several links have been traced between Huntington's disease and brain-derived neurotrophic. First, mutant htt interferes with BDNF transcription and transport, which leads to a striatal trophic factor depletion that could partially explain the preferential neuronal loss in this brain region. Second, BDNF is a potent survival factor for striatal neurons in various experimental paradigms. Recently, the breeding between R6/1 and BDNF^{+/-} mice demonstrated that a deficit of endogenous BDNF modulates HD pathology. Interestingly, the administration of exogenous BDNF restores neuronal function in these double-mutant mice (Canals et al., 2004). BDNF has been reported to protect striatal neurons expressing a mutant htt fragment in an acute (Saudou et al., 1998) as well as also in a chronic cellular model (see Chapter 2.4.7). *In vivo*, the potential of BDNF has been assessed in the quinolinic acid model (Bemelmans et al., 1999; Kells et al., 2004), but no data are available for the genetic models of HD. The evaluation of its impact in genetic models of HD is, therefore, essential to establish its clinical relevance.

In the present study, a lentiviral vector coding for a chimeric human BDNF was injected in the striatum of adult mice and rats. BDNF is produced as a pre-pro-precursor, which is further processed until it is eventually released through the regulatory secretory pathway (Mowla et al., 1999). NGF, another member of the neurotrophin family, is secreted by the regulated pathway (Mowla et al., 1999), but also by the constitutive pathway (Wu et al., 2004). In a side-by-side comparison, Wu and collaborators showed that in cortical primary cultures infected with adenoviral vectors NGF is released 10 times more efficiently than BDNF (Wu et al., 2004). To increase the secretion of BDNF, we have constructed a fusion protein with the pre-pro peptide of NGF and the mature BDNF. This chimeric protein was efficiently processed and secreted *in vitro* and *in vivo* and its bioactivity was demonstrated in striatal cultures.

In our first attempt to establish the neuroprotective effect of BDNF, we delivered this trophic factor in YAC128 mice. Surprisingly, these mice did not develop major signs of striatal pathology at 18 months. These results are in contradiction with previously reported data describing brain atrophy and neuronal dysfunctions at one year (Slow et al. 2003). One potential hypothesis to explain this discrepancy relates to the housing conditions. It has been shown that enriched environments have a beneficial impact on R6/2 transgenic mice (Hockly et al., 2002; Spires et al., 2004). In addition, subtle environmental changes may have drastic

consequences on the results of behavioral tests (Crabbe et al., 1999). Food dietary could also be a major disease modifier. Indeed, dietary manipulation has been described to influence disease progression in HD transgenic mice (Clifford et al., 2002; Duan et al., 2003). Finally, a recent paper from the Venezuela Research Project shows that environmental factors modulate the age of onset of Huntington's disease (Wexler et al., 2004).

Although we did not observe a striatal pathology in 18-month-old YAC128, a progressive increase in body mass was measured in these animals and this change correlates with the behavioral deficits. It is interesting to note that an increase in body weight has been described in R6/2 (at 8-9 weeks) despite high leptin levels. It was shown to precede a period characterized by weight loss which begins at 12-13 weeks (Fain et al., 2001). HD patients usually experience weight loss, despite an increased caloric intake (Beal, 2000). Therefore hypoactivity observed in the YAC128 is probably due to the increased body weight. Additional studies should be performed to definitely establish the effects of BDNF in HD transgenic mice and determine whether it may alter some of the early and subtle changes occurring in YAC128 animals. In particular, it would be interesting to assess the effect on electrophysiological parameters, which have been identified as indicator of early dysfunctionalities in YAC128 neurons (Zeron et al., 2004).

In the second set of experiments, we used the lentiviral-based model of HD (Régulier et al., 2002). Rats overexpressing htt853-82Q developed a severe pathology at 3 months, which is characterized by neuronal loss, striatal shrinkage and ventricle enlargement reminiscent of advanced stages of HD. However, the delivery of BDNF did not prevent the striatal pathology and inclusion formation. These negative results should, however, be carefully analyzed before drawing any definitive conclusions on the relevance of BDNF for HD.

Long-term exposure to high doses of BDNF could induce a desensitization of the TrkB receptor and a down-regulation of TrkB mRNA *in vivo*. Treatment with high doses of this neurotrophin for extended periods may reduce responsiveness (Frank et al., 1996). In Parkinson's disease, the delivery of high GDNF doses was shown to induce a down-regulation of tyrosine hydroxylase (Rosenblad et al., 2003). In our study, Western blot analysis of Lv-mediated BDNF synthesis showed that BDNF expression was approximately 2-5 times higher than the endogenous protein. Preliminary data on AAV-mediated delivery of BDNF in the striatum of HD transgenic mice indicate that the expression of high levels of BDNF in the striatum of R6/1 mice is associated with mortality (20%) and behavioral abnormalities (Rodriguez et al., 2004). Reducing BDNF expression eliminates these toxic

effects and leads to an up-regulation of DARPP-32, CB1 and ppENK expression in the striatum. In all cases, no improvements of the behavioral deficits were observed.

In YAC128 mice and in the Lv-based rat model, BDNF expression in the striatum was not associated with side effects. It still needs to be established whether this local and sustained BDNF production has modified the expression and function of BDNF receptor subunits. Similarly, additional studies are required to determine the impact of BDNF on striatal markers and neurogenesis.

To deliver BDNF, we have injected lentiviral vector directly into the striatum. The infected neurons in the target region are producing and releasing BDNF, that diffuses into the striatum. Physiologically, cortical afferents deliver BDNF to the striatum. However, as vesicle transport and axonal trafficking are impaired by mutant huntingtin (Gauthier et al., 2004; Trushina et al., 2004), the local delivery of this factor in the striatum should be able to overcome this deficit. However, at this stage we can not exclude that a cortical delivery of BDNF might be advantageous, as it more closely corresponds to the physiological situation. Cortical Lv-injection could be performed in rodents to evaluate whether the site of BDNF production has a major impact on its protective effect in HD models.

In conclusion, this last chapter highlighted the difficulty of testing neuroprotective approaches in genetic animal models of HD when compared to *in vitro* or toxin-induced lesion HD models. A better knowledge of the molecular mechanisms involved in HD and the identification of more specific targets are needed for future therapeutic approaches.

CONCLUSIONS AND PERSPECTIVES

The underlying mechanisms leading to the specific degeneration of striatal neurons in HD are still largely unknown and no therapy is currently available for this fatal disease.

In this thesis, I first developed a cellular model of HD, in order to gain further insight into the molecular pathways leading to cell death, and to identify potential therapeutic agents. The originality of this new *in vitro* model is based on the combined use of primary neuronal cultures and lentiviral vectors. As a result, the majority of neurons express mutant huntingtin and a chronic progression of the pathology is observed. This model will therefore be particularly useful to perform population analyses in primary neurons and to evaluate subtle changes induced by mutant htt, such as electrophysiological abnormalities, gene transcriptional alterations and biochemical changes, as well as factors that would interfere with these primary events. Collaborations have recently been initiated to take advantage of the versatility and reproducibility of this approach and to assess the implication of various intracellular pathways in HD pathology. Biochemical analyses have, for example, highlighted the importance of mitochondrial impairment and disease-modifier candidates have been evaluated. Expanded polyglutamine-mediated transcriptional dysregulation has been identified prior to neuronal death. A large set of gene expression changes have been previously described in R6/2 transgenic mice. The new cellular model has highlighted that cell-autonomous changes in gene expression prior to cell death are induced by mutant htt.

Intrinsically, models are simplifying a complex dynamic process and are therefore affected by several limitations. In our cellular paradigm, the major differences compared to the human pathology are: (i) the expression of a small N-terminal mutant huntingtin fragment, (ii) the presence of two alleles of wild type rat htt, (iii) the time course, which is still short compared to the human disease, and finally, (iv) the artificially simplified setting, which excludes all contributions from other brain regions, such as the cortical glutamatergic input the dopaminergic innervations and neurotrophic delivery which are believed to modulate the disease. Therefore data gathered from a cell culture models have to be validated *in vivo*.

In the second part of the thesis, genetic rodent models of HD were used to validate therapeutic approaches with two neurotrophic factors, CNTF and BDNF. The results obtained with lenti-CNTF are encouraging with regard to both gene therapy approaches and the potential beneficial effects of this molecule in HD patients. These preliminary results have shown the feasibility of the approach and the lack of toxicity. However, additional studies are

required to further test the effectiveness of neurotrophic factors in animal models of HD. None of the therapeutic candidates identified so far in animal models of HD have been successfully tested in HD patients. The most relevant animal models and endpoints for pre-clinical evaluation of new therapy have, therefore, not yet been validated. R6/2 mice, which have been extensively characterized, are still the standard in the field. These mice show neuronal dysfunctions with little evidence of neuronal loss; they have been used to identify disease modifiers. However, these animals manifest several peripheral symptoms that might not be directly related to HD, rendering the assessment of therapeutic strategies more difficult. Lentiviral-based rat models could be an interesting experimental paradigm to mimic late stages of the disease, and be complementary to transgenic models, but the LV-based models, are characterized by a local striatal expression of a mutant htt fragments which is oversimplifying HD pathology. New HD animal models closely reproducing the disease progression and neuronal death are therefore still needed. The emergence of transgenic approaches based on lentiviral vectors could represent a good opportunity to combine the advantages of both techniques and to develop animal models with severe pathologies in different species including primates. This would open new perspectives for longitudinal follow-up of HD pathology by imaging and for more elaborate behavioral analysis.

Delivery of trophic factors into the brain is challenging, in this thesis we took advantage of viral vectors to locally delivery trophic factors into the striatum, but alternative delivery methods including *ex vivo* gene therapy approaches and non-viral delivery systems have been developed. A phase I clinical trial based on encapsulated genetically engineered cells producing CNTF has demonstrated the feasibility and safety of the approach (Bloch et al. 2004). The administration of CNTF in the ventricle of HD patients was not associated with side effects and a mild improvement in electrophysiological parameters was observed. However, no clinical benefit based on UPDRS motor score was obtained, probably due to the implantation of a single capsule in the ventricle, leading to a scarce diffusion of CNTF. The heterogeneity in the survival of encapsulated cells and the periodical capsule replacement every 6 months represent major disadvantages of this approach. Recently, intra-putaminal GDNF delivery with a minipump in Parkinson's disease patients has shown encouraging preliminary results, at least one year post-treatment (Gill et al., 2003). The results of the phase II clinical study, however, did not meet the primary endpoints and four of thirty-four patients developed GDNF-blocking antibodies. In addition, two monkeys that have received unilateral intraputaminal GDNF developed "unusual cerebellar cortical pathology" as reported at the

annual meeting of the American Neurological Association this year. These observations confirm the need for long-term follow-up, as high doses of trophic factor could cause severe damages.

The delivery of trophic factor is by far not the only conceivable therapy. Different strategies have been applied to treat Huntington's disease: rebuilding the brain is the challenge of a pilot study performed on 5 HD patient in Créteil (France). The fetal neuronal transplants in the striatum showed a clinical benefit, one year post-implant on 3/5 patients. Long-term evaluations as well as a phase II clinical trial are currently evaluating this treatment on a larger number of patients (Peschanski et al., 2004).

A consortium of research centers financed by the NIH is using different HD models to screen compounds that have already been approved by the FDA. The hope is that if an agent will be particularly promising, it can be rapidly used in large clinical trials, as these compounds have already been characterized pharmaceutically. At the present time, the absence of overlap between the different models makes this strategy questionable and therefore drugs which are less tested, but biologically more relevant, should probably be reconsidered.

Finally, the results for the HIV-1-derived lentiviral vectors described in this thesis confirmed the interest of this gene-delivery tool for post-mitotic cells, with regard to efficiency, long-term expression and lack of toxicity. Lentiviral vectors are therefore particularly suitable for experimental biology in the field of neuroscience. However, biosafety concerns have to be considered for future clinical applications, as these vectors are derived from a devastating disease, and the risk of recombination with wild-type HIV can not to be excluded. The recent development of new generations of AAV vectors has brought a valid alternative to lentiviral vectors. Moreover, different serotypes might be used to modulate cell tropisms, which opens the possibility for a more specific targeting.

In conclusion, the combination of a primary culture and a lentiviral vector yielded a new relevant model for HD research. The lentiviral delivery of trophic factor has shown the efficiency and the potential of this approach but needs to be validated in other genetic animal models. In particular, major efforts have to develop additional transgenic models are required.

ABBREVIATIONS

3-NP	3-nitropropionic acid
aa	amino acid
AAV	adeno-associated virus
AD	Alzheimer disease
ALS	amyotrophic lateral sclerosis
BDNF	brain-derived neurotrophic factor
CA150	co-activator 150
CAG	cytosine adenine guanine
CBP	c-AMP-response-element-binding protein
ChAT	cholin acetyltransferase
CIP4	cdc42-interacting protein 4
CLC	cardiotrophin-like cytokine
CLF	cytokine-like factor-1
CNS	central nervous system
CNTF	ciliary neurotrophic factor
cPPT	central polypurine tract
CT	C-terminus
CtBP	C-terminal-binding protein
DARPP-32	dopamine and cAMP regulated phosphoprotein
DNA	deoxyribose nucleic acid
DRPLA	dentatorubralpallidoluysian atrophy
EIAV	equine infectious anemia virus
FACS	fluorescence-activated cell sorter
FIP2	for 14.7K interacting protein
FIV	feline immunodeficiency virus
GABA	gamma-amino butyric acid
GFAP	glial fibrillary acid protein
GFP	green fluorescent protein
GPe	external segment of the globus pallidus
GPi	internal segment of the globus pallidus
Grb2	growth factor receptor-binding protein 2
HAP1	htt-associated protein 1
HAP40	htt-associated protein-40
HD	Huntington's disease
HIP1	htt-interacting protein-1
Hip1	htt-interacting protein-1
HIP-14	htt-interacting protein-14
Hippi	Hip-1 protein interactor
HIV	human immuno-deficiency virus
hsp104	heat shock protein 104 kDa
Hsp40	heat shock protein 40 kDa
Hsp70	heat shock protein 70 kDa
htt	huntingtin
HYP-J	htt yeast protein-J
IL-6	interleukin-6
LacZ	lactamase
LIF	leukemia inhibitory factor
LTR	long terminal
Lv	lentiviral vector
MAP-2	microtubule-associated protein 2
MSN	medium sized spiny neurons
NADPH-d	nicotinamide adenine dinucleotide phosphate-diaphorase
NMDA	N-methyl-D-aspartate
N-CoR	nuclear receptor co-repressor
NeuN	neuronal nuclei
NF	neurofilament
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate

NT	N-terminus
P/CAF	p300/CBP associated factor
PACSIN1	protein kinase C and casein substrate in neurones 1
PD	Parkinson's disease
PGK	phosphoglycerate kinase
PKB	protein kinase B
PNS	peripheral nervous system
PRD	proline rich domain
PSD-95	postsynaptic density-95
Q	glutamine
QA	quinolinic acid
RasGAP	Ras GTPase activating protein
REST	RE1-silencing transcription factor
RNA	ribonucleic acid
S421	serin 421
SBMA	spinobulbar muscular atrophy
SC35	splicing component 35 kDa
SCA	spinocerebellar ataxia
SCID	sever combined immunodeficiency
SGK	serum- and glucocorticoid-induced kinase
SH3GL3	SH3-containing Grb2-like protein 4
shRNA	small harpin RNA
SIV	simian immunodeficiency virus
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SOD	superoxide dismutase
Sp1	specificity protein 1
STAT	signal transducers and activators of transcription
STN	subthalamic nucleus
TAFII-130	TATA-binding protein (TBP)-associated factor
TBP	TATA box protein
TH	Thyrosin Hydroxylase
Trk	tyrosine kinase
TUNEL	terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
UCHL-1	ubiquitin carboxyl terminal hydrolase
UPP	ubiquitin-proteasome pathway
VEGF	vascular endothelial growth facto
VSV-G	vesicular somatitis virus G-glycoprotein
WPRE	woodchuck post-transcriptional regulatory element
WT	wild type
YAC	yeast artificial chromosome

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CURRICULUM VITAE

Diana Zala
7748 Campascio

Personal

Born in Poschiavo, Switzerland, December 29th 1972, Swiss citizen

Education

October 1998-present

Research assistant, PhD student

Subject: Use of lentiviral vectors to model and treat Huntington's disease

Initially at University Hospital Lausanne (CHUV); since January 2002 at the Institute of Neuroscience, Federal Institute of Technology Lausanne (EPFL), Switzerland

Advisor: Professor P. Aebischer; Supervisor: N. Déglon

October 1993-April 1998

Master of chemical engineering at EPFL

Project in biotechnology: Characterisation of the growth inhibition of *Saccharomyces cerevisiae* Giv 2009 by 2-phenylethanol

Advisor: Professor U. von Stockar

Summer 1996

Practical training at the Technical school of Prague, Department of Physical Chemistry: experiences with a differential calorimeter and a comparative ebulliometer, correlation of experimental and literature data.

Advisor: Professor V. Ruzicka

June 1993

Maturity type E at Kantonsschule Chur, Switzerland

Publications

Régulier, E., **Zala, D.**, Aebischer P., and N. Déglon. (2004). Lentiviral-mediated gene transfer to model triplet repeat disorders. *Methods Mol Biol* 277: 199-214.

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Submitted manuscript

Zala, D., Benchoua A., Brouillet, E, Zurn, A.D., Aebischer, P., and Déglon, N.
Neuropathological events leading to a progressive and selective cell death in primary striatal neurones expressing mutated huntingtin.

Conference abstracts

Zala, D., Benchoua A., Zurn, A.D., Aebischer, P., and Déglon, N. In vitro model for Huntington's disease: transduction of mutated huntingtin in primary striatal neurones with lentiviral vectors induces neuropathological events leading to a progressive and selective cell death. Forum of European Neuroscience (FENS) 2004, Lisbon, P, 10 – 14 July 2004.

Zala D., Zurn A.D., Aebischer P. and Déglon N. Progressive neuropathology in primary rat striatal cultures expressing mutated huntingtin. Swiss Society for Neuroscience, Lausanne, CH, 17 January 2004.

Zala D., Zurn A.D., Aebischer P. and Déglon N. Progressive neuropathology in primary rat striatal cultures expressing mutated huntingtin. Society for Neuroscience Meeting, New Orleans, USA, 8-12 November 2003.

Zala, D., De Almeida, L.P., Bensadoun, J.C., Leavitt, B.R., Gutekunst, C.A., Aebischer P., Déglon, N., and Hayden, M.R. Lentiviral-mediated delivery of ciliary neurotrophic factor protects striatal neurones in a transgenic model of Huntington's disease. Brain in motion, EPFL, CH, 19 – 22 February 2003.

Zala, D., De Almeida, L.P., Bensadoun, J.C., Leavitt, B.R., Gutekunst, C.A., Aebischer P., Déglon, N., and Hayden, M.R. Lentiviral-mediated delivery of ciliary neurotrophic factor protects striatal neurones in a transgenic model of Huntington's disease. Forum of European Neuroscience (FENS) 2002, Paris, F, 13 – 17 July 2002.

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Gene Therapy approach to Huntington's Disease. 2nd ELBA school of neurosciences: Degeneration and regeneration in the CNS: from biology to disease. Marina di Campo, Isola d'Elba, I, 11-17 September 1999.