**Introduction**

1. **Huntingtin: The Huntington’s disease protein**

   1.1. **Huntingtin: an overview**

   Huntingtin is a large, 348kDa, widely expressed, predominantly cytoplasmic protein that can also be found in the nucleus (Xia, 2003; Kegel, 2002). The interest in huntingtin comes from the fact that an expansion in the variable CAG tract in its encoding gene causes Huntington’s disease (HD), an inherited, fatal, autosomal dominant neurodegenerative disorder characterized by selective loss of the striatal neurons (HDCRG, 1993).

   The dominant character of HD has directed most of the initial interest towards the acquired toxic gain-of-function by mutant huntingtin but, accumulating data point out that loss of wild-type huntingtin function plays also a role in the disease (Cattaneo, 2001). Even if HD pathology is triggered by the presence of the mutation, it has been suggested that some of the disease features may be explained by the loss-of-function from the normal form of the protein. One of these is the selective vulnerability of the striatum and of the deeper layers of the cortex, to undergo degeneration, even if huntingtin is
ubiquitously expressed in the brain and body (Vonsattel, 1998). Moreover, the analysis of the HD gene throughout species evolution revealed a high level of sequence conservation except for the number of CAG triplets carried by the normal gene that differs for each species (Kremer, 1994). This observation suggested that the CAG repeat is not crucial for normal huntingtin function. Consistently, deletion of CAG repeats in the HD gene resulted only in a subtle behavioral and motor phenotype in mice (Clabough, 2006). Taken together, this data led to the hypothesis that the physiological function of normal huntingtin protein might be independent from its polyglutamine tract size but more work needs to be done in order to identify the precise functional domains of this protein. The studies developed in this thesis intend to contribute to a more complete knowledge of huntingtin’s physiological function and to its application for the understanding of Huntington’s disease unclear features.
1.2. Human Huntingtin Gene

The IT15 gene, localized within the chromosome 4p16.3, is the human gene that codes for huntingtin. It’s a 180Kb-long gene, constituted by 67 exons with dimensions that range from 48 to 341bp (HDCRG, 1993).

The HD gene contains several polymorphic sequences near the CAG tract that could act as cis-factors for instability, including a CCG repeat (which is translated into a poly proline tract in huntingtin protein), a codon deletion (delta 2642) and several single-nucleotide polymorphisms (Squitieri, 1994; Rubinsztein, 1993; Rubinsztein, 1995; Coles, 1997; Semaka, 2006). The CCG repeat is immediately adjacent to the CAG tract and tends to vary between 7 and 12 repeats in the general population, with the seven CCG repeat allele found on almost all (~93%) of HD chromosomes. (Andrew, 1994) In HD, the CAG tract is interrupted by a penultimate CAA triplet, which may influence the propensity for the intermediate allele instability (Chong, 1997).

The human huntingtin gene is known for exhibiting numerous GC-rich regions all through its sequence. No typical TATA-box or CCAAT-box elements have been identified but one putative AP2 (Adaptor protein 2) binding site and several potential Sp1 (Transcriptor factor specific 1) were identified within the promoter region (Lin, 1995; Holzmann, 1998). The transcriptional regulation of huntingtin might not be mediated by the classical cis-regulating elements (as is the TATA-box) but by elements within the intronic sequences, in particular the ones in between the first exons, which are unusually long. Additionally, a SRY (Human male sex determining factor sry) binding site has been identified within huntingtin promoter region. Since the SRY is a protein known to be important for both sex determination and testis development the hypothesis that huntingtin expression might have a role in spermatogenesis has been raised (Holzmann, 1998).

Recently, two proteins HDBP1 and HDBP2 (Huntington’s Disease Binding Protein 1 and 2) have been reported to bind the IT15 promoter in a consensus sequence and shown to be able to stimulate huntingtin’s transcription (Tanaka, 2004).

So far, the transcriptional regulation of huntingtin’s gene has not been linked to Huntington’s disease pathology or to its progression.

1.3. Human Huntingtin Messengers

The human huntingtin gene was initially thought to have a single transcript of 10-11kb, but further analysis revealed that this gene produces two distinct transcripts, one of 10kb and another significantly larger of 13.7kb. These two transcripts share the same protein coding sequence, but differ in size and sequence of their 3’ untranslated region. Sequence analysis identified two different polyadenylation signals, which explains the production of two transcripts of different size from the same gene. RNA hybridization has
shown that the larger fragment is the predominant transcript in human brain, while the shorter is mostly present in testis (Lin, 1993). Despite this difference in terms of expression, no defects in the amount or stability of each of the transcripts have been described in Huntington’s disease. In addition, no alternative splicing, which could change the coding sequence, has been identified in the human gene. In mouse, a 1.4kb alternative splicing of the codon sequence has been reported (Lin, 1994). Until now, no different isoforms of the protein have been found.

1.4. Human Huntingtin Protein Structure

Huntingtin’s polyQ tract begins at the eighteenth amino acid and is followed by a proline rich sequence (polyP) composed by 38 amino acids (Figure 4). The polyP stretch is thought to be important in maintaining huntingtin in solution, as if it balances the polyQ extreme insolubility. The first 17aa are highly conserved (between huntingtin orthologues) and the three lysines, K6, K9 and K15, are known to be post-translational modification targets (Steffan, 2004). Recent reports indicate that the first 17aa are indeed important for huntingtin’s shuttling since they interact with TRP (translocated promoter region), a protein of the nuclear pore that actively exports proteins from the nucleus. When huntingtin first 17aa were eliminated, its accumulation in the nucleus was observed (Cornett, 2005). Not only the first 17aa, but also the NES (nuclear export signal) present in the C-terminal portion of the protein, is important for huntingtin cytoplasmic localization. In fact, when the NES sequence was removed or modified, huntingtin passively accumulated in the nuclear compartment. Huntingtin’s amino acid sequence also reveals several potential nuclear localization signals (NLS) in the N-terminal portion (Xia, 2003). Together, these results highlight huntingtin’s preference for the cytoplasmic compartment and its potential role in maintaining and transporting both proteins and mRNA from the nucleus to the cytoplasm.

Up to now, the only putative domains reported through bioinformatic analysis of huntingtin’s sequence are the HEAT repeats. Each of these repeats is approximately 40-aa-long and is a highly conserved sequence. The HEAT repeats occur multiple times within huntingtin’s sequence and their designation is due to the 4 protein’s names within which they were initially found (Huntingtin, Elongation Factor 3, the PR65/A subunit of protein phosphatase 2A, and TOR1 the target of rapamycin) (Andrade, 1995; Neuwald, 2000). Using cross-species comparative analysis, Takano and Gusella predicted that vertebrate huntingtin contains 28–36 HEAT repeats that span the entire protein. These putative HEAT repeats in huntingtin’s sequence can be subdivided in 3 main clusters, but the exact number of functionally active repeats is still controversial (Andrade and Bork, 1995; Andrade, 2000; Li, 1999; Takano, 2002). HEAT repeat containing proteins generally mediate important protein-protein interactions involved in cytoplasmic and nuclear transport, microtubule dynamics, and chromosome segregation (Neuwald, 2000).
Three protease cleavage consensus sites have also been identified in huntingtin’s sequence (Goldberg, 1996; Wellington, 1998; Wellington, 2000, Gafni, 2002, Gafni, 2004). Both wild-type and mutant huntingtin can be cleaved, but the mutant form is more susceptible to proteolysis and has been shown to generate fragments that localize both in the nucleus and the cytoplasm (Davies, 1997; Wellington, 2002, Gafni, 2004; DiFiglia, 1997; Kim, 2001; Lunkes, 2002). Human huntingtin presents active caspase cleavage sites at aa 513 and aa 530 which are specifically recognized by caspase-3, and at aa 586 which are recognized by caspase-6. Besides caspase cleavage, huntingtin can also be cleaved by calpains at aa 469 and 536 (Goffredo, 2002). There are also other cleavage sites with imprecise amino acid position, which are preferentially cleaved in some brain regions (Mende-Muller, 2001) but the importance of huntingtin proteolysis to cell function needs further study. However, when caspase and calpain activity is modified, proteolysis is reduced, mutant huntingtin fragments are no longer produced, toxicity decreases, and ultimately disease progression slows down (Wellington, 2000; Graham, 2006). N-terminal fragments with expanded polyQs readily form inclusions similar to those seen in HD brain. It is still unclear which combination of proteolytic events is required for generation of the toxic fragments (Lunkes, 2002; Goldberg, 1996; Martindale, 1998).

Huntingtin has been shown to be a target for four different post-translational modifications: sumoylation, ubiquitination, phosphorylation and palmitoylation. The first two seem to compete for the post-translational modification of three lysine residues K6, K9 and K15. These three amino-terminal lysines compete for both sumoylation and ubiquitination and the rate of these modifications influence not only huntingtin’s solubility but also it’s shuttling between the cytoplasm and the nucleus (Steffan, 2004).

Phosphorylation of two serine residues in positions 421 and 434 has been documented in physiologic conditions. In the presence of the mutation, in a study developed in the YAC 128 HD mouse model (transgenic mice expressing the entire human huntingtin gene with 128 CAG triplets), phosphorylation levels were dramatically reduced, especially in the striatum. This might partially explain why the striatal neuronal population is particularly vulnerable in Huntington’s disease. Moreover, recent data indicate that huntingtin phosphorylation may protect against the toxicity exerted by the mutant form of this protein (Humbert, 2002; Warby, 2005; Luo, 2005).

Palmitoylation of huntingtin is performed by the palmitoyl transferase Hip14, but the exact residue that is modified is still unclear (Huang, 2004; Yanai, 2006). Palmitoylated proteins are commonly involved in the dynamic assembly of cellular components that control both vesicle trafficking and synaptic vesicle function, and this gives strength to the proposed role for huntingtin as a vesicular trafficking regulator (DiFiglia, 1995; Huang, 2004).

The comparison of huntingtin primary amino acid sequence with other known protein sequences failed in showing similarity. Moreover, very few known sequence motifs can be identified, and no structural domains with clearly defined function have been revealed. But not only this unique amino acid sequence renders huntingtin such a peculiar protein. Huntingtin is also a very large protein and this has lead to the failure of all
crystallization attempts done so far. Perutz and colleagues were the first to propose a structural model for huntingtin in which the polyQ and the polyP region were organized in a β-sheet structure called polar zipper. In the presence of the mutation, the expanded form of the protein may suffer a structural alteration that would in turn favour the interaction with polyQ sequences from other proteins and cause the formation of aggregates (Perutz, 1994). It has also been suggested that huntingtin physiological function is indeed to bind transcription factors containing a polyQ region and that these interactions may become deregulated in the presence of polyQ expansion (Harjes, 2003; Li and Li, 2004; Goehler, 2004). After the first studies from Perutz and colleagues, only recently huntingtin’s structure was further characterized by circular dichroism, dynamic light scattering and electron microscopy. The model proposed by Perutz, in which huntingtin is predominantly composed of HEAT repeats was additionally reinforced by Li and colleagues who have recently shown that this HEAT repeats stack into a rod-like superhelical structure (Li, 2006).

In conclusion, both wild-type and mutant huntingtin forms have an extreme ability to bind other proteins and this might reflects a flexible or multifunctional structure capable of assuming specific conformations and roles depending on its subcellular location and time of maturation in a given cell (Sipione, 2003; Harjes, 2003; Li and Li, 2004, Macdonald, 2003; Marcora, 2003). Alternatively, huntingtin might have appeared as a natively unfolded protein. Most lines of evidences favour the structural flexibility of this unique protein. Moreover, the notion that the elongated polyQ stretch can modify the three-dimensional structure of the entire protein and consequently the interaction with other proteins is supported by independent studies (Perutz, 1994; Harjes, 2003).
1.5. Huntingtin expression and its subcellular localization

Huntingtin’s expression has been detected in many tissues, from the early embryonic stage of gastrulation all through adulthood. Although huntingtin’s widespread expression, only the striatal medium-sized spiny neurons undergo degeneration in the presence of the mutation. The fact that only a specific subset of neurons is affected in Huntington’s disease suggests that huntingtin might have diverse roles depending, at least partially, on the cell type where it is expressed.

The highest levels of huntingtin expression were detected in neurons within the CNS (Trottier, 1995; Ferrante, 1997; Sapp, 1997; Fusco, 1999). Besides the brain and testis, where huntingtin is expressed at moderate levels, also the lungs, heart, kidney and liver express this protein, although in a lower extend (Sharp, 1995; Wood, 1996).

Both wild-type and mutant forms of huntingtin protein show a preference for the cytoplasmic compartment, but mutant huntingtin fragments have also been observed in the nucleus (Hackman, 1999). While most reports agree that huntingtin is predominantly a cytoplasmic protein, conflicting distribution patterns have been demonstrated in terms of its subcellular localization. The search for huntingtin subcellular localization is ultimately a strategy to determine this protein physiological role. Huntingtin localization studies have revealed its association with different organelles, including the nucleus, the endoplasmatic reticulum and the Golgi complex (DiFiglia, 1995; Velier, 1998; Hilditch-Maguire, 2000; Trettel, 2000; Hoffner, 2002). A recent work from Anna Strehlow and colleagues, shows that huntingtin is present as discrete punctate, in the perinuclear region of fibrosarcoma and neuroblastoma cells. This perinuclear distribution overlaps largely with the trans-Golgi and cytoplasmic clathrin-coated vesicles, and as already suggested in previous studies, huntingtin is proposed to have a role in intracellular trafficking of proteins (Strehlow, 2006). Huntingtin has also been found in neurites and at synapses where it associates with vesicular structures and microtubules. These observations suggest a potential role for huntingtin in synapse activity and in cellular transport (Aronin, 1995; Wood, 1996; Gutekunst, 1995; Li, 2003).

If we combine the ability of huntingtin to associate with different cellular structures with its widespread localisation and the lack of information in terms of its biochemical properties it becomes almost automatic to think of huntingtin physiological role as an extremely intricate topic.
2. Huntington’s disease (HD)

2.1. HD history and first hypothesis of its cause

Huntington’s disease is an autosomal-dominant, progressive neurodegenerative disorder which affects 4-7 in every 100 000 individuals, worldwide.

Huntington’s disease designation comes from the name of an English doctor, who vividly characterized this disorder, George Huntington. Several doctors in the 19th century noticed the hereditary nature of the disease but it was George Huntington who introduced the term "chorea", from the Greek word for dance, to describe the involuntary "dance-like" movements shown by his patients (Elliotson, 1832; Hayden, 1981; Harper P in Bates, 2002; Folstein, 1989).

George Huntington published on the Medical and Surgical Report of Philadelphia three paragraphs where he described the hereditary character of the disease, a tendency for the appearance of mental illness as well as a late-adult symptomatology. Although he spent almost all his life studying this disease, and its phenotype, he couldn't find the cause of such illness. Before George Huntington's work, patients were believed to be "drunk" or "possessed" but his clinical descriptions greatly contributed to change the way people affected by the disease were seen at the time (Huntington, 1872). In Salem, in the Boston area, during the Inquisition period, women affected by the "chorea disease" were burned to death, treated as witches ("Salem’s Witches").

In the late 70s, researchers intensified their studies in order to understand why the striatum was selectively affected in HD patients. Several hypothesis were raised at that time, from these the alteration at the level of striatal neurotransmission was the most pursued. In 1976, it was reported that injecting glutamate or an agonist of its receptor in the mouse striatum was able to reproduce almost all neuropathological changes observed in Huntington’s disease. These studies suggested that the striatal neuronal loss could be a result of excitatory phenomena, produced by the glutamatergic afferent neurons (Coyle and Schwarz, 1976; McGeer and MacGeer, 1976). During these years, most of the research was done primarily on brain from HD patients. In one of these studies, an abnormal increased activity of the enzyme the 3-hydroxyanthranilate-oxygenase was observed in the HD affected brain. The production of quinolic acid (QA) depends on this enzyme activity and its excessive accumulation in the brain was found to be toxic for the striatal neurons. This toxic effect was further confirmed by the observation that after injection of QA in mice and rats brains, striatal neurons would undergo selective death in both animal models. Indeed, the QA seems to give rise to an excitatory action, behaving as an agonist of the glutamate receptor NMDA (N-methyl-D-aspartate). Only in 1981, thanks to a study conducted by the Wexler family, a big step towards the cause of this disease was given. Nancy Wexler went to Lake Maracaibo, a region in Venezuela with one of the greatest focus of HD patients, and collected blood and genealogical background from families and families of affected people. With these samples and genealogical
information, Nancy Wexler and collaborators identified a genetic marker for Huntington’s disease. Gusella, Wexler and Connelly’s further genetic analysis of this marker led to the identification of the CAG polymorphism in the human chromosome 4 (Gusella, 1983). A decade after, and with a great endeavor from 58 scientists, the gene responsible for Huntington’s disease was discovered (HDCRG, 1993).

<table>
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<tr>
<th>Year</th>
<th>Event</th>
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<tbody>
<tr>
<td>1374</td>
<td>Epidemic dancing mania described</td>
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<tr>
<td>1500</td>
<td>Paracelsus suggests CNS origin for chorea</td>
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<td>1686</td>
<td>Thomas Sydenham describes post-infectious chorea</td>
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<td>1832</td>
<td>John Elliotson identifies inherited form of chorea</td>
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<td>1872</td>
<td>George Huntington characterizes Huntington’s disease</td>
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<td>1953</td>
<td>DNA structure elucidated</td>
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<td>1955</td>
<td>Huntington’s disease described in Lake Maracaibo (Venezuela)</td>
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<td>1967</td>
<td>Word Federation of Neurology meeting on Huntington’s disease</td>
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<tr>
<td>1976</td>
<td>First animal model (kainic acid) of Huntington’s disease described</td>
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<td>1981-83</td>
<td>Gene marker for Huntington’s disease discovered</td>
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<tr>
<td>1993</td>
<td>HD gene identified, Huntington study group formed for clinical trials</td>
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<tr>
<td>1996</td>
<td>Transgenic mouse developed</td>
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<td>2000</td>
<td>Drugs screened for effectiveness in transgenic animal models</td>
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(Adapted from “Huntington’s disease”, Francis O Walker, Lancet 2007)

Table 1: History of Huntington’s disease

With the HD gene discovered, a huge step towards the understanding of this disorder was given, but further studies need to be made in order to elucidate the mechanisms at the base of this disorder.

Following the initial hypothesis that proposed the HD striatal vulnerability as a consequence of excitatory phenomena, other lines of evidences pointed out that this selective degeneration was the result of an alteration in the energetic metabolism. In humans, the ingestion of the 3-nitropropionic-acid, an acid that is known to inhibits the activity of the succinate-dehydrogenase enzyme (a key enzyme for the mitochondrial complex II), resulted in the selective death of striatal neurons. These observations were further confirmed in both striatal cell cultures and rats injected with 3-NPA (Beal, 1993; Broillet, 1993). From these observations, an alteration in the mitochondrial metabolism stood up as another important piece of data for the understanding of striatal selective vulnerability in HD. Moreover, other abnormalities were reported at the cellular metabolic level: huntingtin polyQ tract seems to be able to interact with the glyceraldehyde-phosphate dehydrogenase (GADPH) an essential enzyme for the glycosis process, by reducing the glucose used in the basal ganglia (Burke, 1996). The oxidative stress hypothesis was suggested after the observation of an inadequate functioning of the mitochondria and a consequent energetic deficit in the cell coupled with an increased
production of free radicals and oxidative agents (Coyle and Puttfarken, 1993; Dugan, 1995). Some of these hypotheses were further tested and confirmed in the transgenic animal models that were created right after the discovery and the cloning of the human HD gene.

### 2.2. Clinical Genetics

The CAG triplet repeat appears on the first exon of the huntingtin encoding gene, and in normal alleles varies from 11 to 26 repeats. In HD, exon 1 CAG repeats can span from 27 to 35 in unaffected individuals with intermediate HD penetrance, from 36 to 39 in affected individuals with reduced HD penetrance or more than 40 in affected individuals with full penetrance (Semaka, 2006). While normal alleles rarely show germ line instability, HD alleles have shown to undergo repeat expansion upon transmission to the next generation (Kremer, 1995; Macdonald, 1993). Germ line repeats instability results in CAG repeat heterogeneity. CAG heterogeneity refers to an observed difference in repeat lengths within or between tissues of an individual (Telenius, 1995; Clearly, 2005; Chong, 1997). Instability is greater in spermatogenesis than oogenesis, in that large expansions of CAG repeat on replication happen almost exclusively in males (Kremer, 1995; Ranen, 1995; Trottier, 1994). These findings account for the occurrence of anticipation, in which the age of onset of Huntington’s disease becomes earlier in successive generations, and the likelihood of paternal inheritance in children with juvenile onset symptoms. Similarly, new-onset cases of Huntington’s disease with a negative family history typically arise because of expansion of an allele in the borderline or normal range (27-35 CAG repeats), most usually on the paternal side (Harper, 2002). Notably, in HD, repeat size heterogeneity is not confined to the germ line but variable levels of repeat heterogeneity are found in brains of affected individuals and in animal models of the disease (Telenius, 1994; Aronin, 1995; Kennedy, 2000; Semaka, 2006).

### 2.3. Diagnosis

The disease can be easily diagnosed using genetic testing. However, whether people who have a family history of the disease but no symptoms should be tested is controversial.

Huntington’s disease may be difficult to recognize in the early stages because symptoms are subtle. The disease may be suspected based on symptoms and a family history.

Furthermore, computed tomography (CT) or magnetic resonance imaging (MRI) can detect atrophy of the basal ganglia, which is characteristic of the disease.
Routine MRI and CT in moderate-to-severe HD show a loss of striatal volume and increased size of the frontal horns of the lateral ventricles, but in early stages of the disease these scans are inadequate for diagnosis. Both MRI and PET functional studies revealed that even before symptoms arise, changes in affected brains take place (Kuring, 2000; Lawrence, 1998; Paulsen, 2004).

2.4. Epidemiology

Huntington’s disease shows a stable prevalence in most populations of white people of about 4-7 affected individuals per 100 000. Exceptions can be seen in areas where the population can be traced back to a few founders, such as Lake Maracaibo in Venezuela (Young, 1986). In Japan, China and Finland the prevalence of the disorder has been reported to be much lower. African populations show a similarly reduced prevalence, although in areas where intermarriage with white people takes place the frequency is higher (Kremer, 2002; Harper, 2002; Wright, 1981). In USA, Huntington’s disease strikes 1 in every 10 000 individuals, with a total of 30 000 patients. At least 150 000 others carry a 50 percent risk of developing the disease, and thousands more of their relatives live with the possibility that they might develop HD (Li and Li, 2006). Huntington’s disease appears in a similar frequency both in man and woman.

Studies have revealed that no consistent increase or decrease in the number of children of affected individuals has been shown (Harper, 2002). Besides, the HD gene does not seem to confer any promising health benefits, other than a potentially lower incidence of cancer (Sorensen, 1999).

2.5. Symptomatology

Huntington’s disease was initially known as Huntington’s chorea due to the distinct involuntary movements performed by the affected people, which somehow remembered a dance.

Individuals with Huntington’s disease can become symptomatic at any time of their lives, but more frequently, and as a late-onset disease, this happens around their 30s-40s; before then they are healthy and have no detectable clinical abnormalities (Myers, 2004). The age of HD onset is inversely correlated with the number of CAG repeats (Brinkman, 1997; Duyao, 1993). Generally, individuals with larger CAG repeats have an early age of onset, and this relationship is further supported by the finding that individuals with higher number of CAG repeats present juvenile-type of HD. The average lifespan after HD onset is 10-20 years but, the younger the age of onset the more rapid is the disease progression (Telenius, 1993; Nance, 2001).
Symptoms usually evolve slowly and vary from person to person, even within the same family. Some individuals may be affected first cognitively (depression, forgetfulness, impaired judgment), while others may be primarily affected in their motor skills (chorea or dystonia, unsteady gait). Huntington’s disease in juveniles (onset before the age of 20 years old) and in some adults can take place with rigidity without signs of chorea. Indeed, although being useful for diagnosis, chorea is a poor marker of disease severity. Patients with early-onset might not develop chorea, or it might arise only transiently during their illness. Most individuals have chorea that initially progresses but then, with later onset of dystonia and rigidity, it becomes less prominent (Young, 1986; Mahant, 2003). Another finding in Huntington’s disease that contributes to patient over activity is motor impersistence- the inability to maintain a voluntary muscle contraction at a constant level (Walker, 2007). Motor impersistence is independent of chorea and is linearly progressive, making it a possible surrogate marker of disease severity (Reilmann, 2001). Cognitive dysfunction in Huntington’s disease often spares long-term memory but impairs executive functions, such as organizing, planning, checking, or adapting alternatives, and delays the acquisition of new motor skills (Folstein, 1989; Craufurd, 2002). Psychiatric and behavioral symptoms come out but do not show stepwise progression with disease severity. Depression is typical and suicide is estimated to be five to ten times higher respect to the general population. Besides, manic and psychotic symptoms can be developed (Folstein, 1989; Craufurd, 2002; Baliko, 2004; Di Maio, 1993; Robins, 2000; Wellington, 1997). As motor and cognitive deficits become severe, patients eventually die, usually from complications of falls, inanition, dysphagia or aspiration.

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<tr>
<th>Behavioral/ Emotional</th>
<th>Motor</th>
<th>Cognitive</th>
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<tr>
<td>Irritability</td>
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<td>Depression</td>
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<td>- Chorea</td>
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<td>- Dystonia</td>
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<td>Anxiety</td>
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Table 2: General symptoms in Huntington’s disease
2.6. Neuropathology

Neuropathological changes in HD are strikingly selective, with prominent cell loss and atrophy in the caudate and putamen (Figure 1). GABAergic medium sized spiny striatal neurons are the most vulnerable, resulting in atrophy of the caudate nucleus, putamen, and globus pallidus. The striatal neurons that contain enkephalin and that project to the external globus pallidum are more involved than neurons that contain substance P and project to the internal globus pallidum (Gutekunst, 2002; Rubinsztein, 2003; Vonsattel, 1998). Medium sized spiny neurons containing somastatin, neuropeptide Y, and NADPH diaphorase in addition to cholinergic interneurons and parvalbumin containing GABAergic neurons are relatively spared (Martin, 1999). These findings go along with the hypothesis that chorea dominates early phases of the disease due to a preferential involvement of the indirect pathway of basal ganglia-thalamocortical circuitry (Paulsen, 2005).

Other brain areas are also greatly affected in HD, including the substantia nigra, the cortical layers 3, 5 and 6, the CA1 region of the hippocampus, the angular gyrus in the lateral lobe, Purkinje cells in the cerebellum, lateral tuberal nuclei of the hypothalamus, and the centromedialparafascicular complex of the hypothalamus (Spargo, 1993; MacDonald, 2002; Macdonald, 1997; Jeste, 1984; Kremer, 1992; Kremer, 1991; Heinsen, 1999, Fusco, 1999). Recently, work from Rosas and colleagues revealed that some HD patients exhibit a diffused degeneration of all brain areas, starting from early disease stages (Rosas, 2003).

Huntington’s disease progression can be classified in 5 grades. Grade 0 includes those individuals who show no neurodegeneration but whose family history of HD renders them potential HD patients. In early symptomatic stages of HD, the brain could be free of degeneration (Gomez-Tortosa, 2001; Mizuno, 2000; Myers, 1991). However, evidence of neuronal dysfunction is abundant even in asymptomatic individuals, where the levels of several proteins associated with synaptic function, cytoskeletal integrity and axonal transport are reduced (DiProspero, 2004; Modregger, 2002). The following grades of the pathology are characterized by a progressive reduction of the brain mass. In grade 4, approximately 90% of the striatal neurons have been lost, and both gliosis and strong atrophy have been reported (Reiner, 1988).

One of the pathological hallmarks of Huntington’s disease is the appearance of nuclear and cytoplasmic inclusions. Although inclusions appear only in pathology, apparently in affected individuals long before the first symptoms onset, mounting evidence suggest that these inclusions are not predictors of cellular dysfunction or disease. Instead, it has been shown that it is the presence of intermediate stages of polyglutamine aggregates that triggers mutant huntingtin- dependent cellular dysfunction (Gomez-Tortosa, 2001; Davies, 1997). The role of these nuclear inclusions is still controversial. Inclusions formation can be correlated with disease progression but so far no association with neuronal degeneration has been undoubtedly documented (Gutenkunst, 1999; Slow, 2005). Moreover, several lines of evidences suggest that huntingtin inclusions are
protective against huntingtin-mediated toxicity in cultured cells (Saudou, 1998; Arrasate, 2004). In conclusion, so far, there is no correlation between aggregates formation in cellular and mice HD models and the appearance of inclusions in human HD brain, and onset of dysfunction or neurological symptoms (Menalled, 2003; Arrasate, 2004; Zuccato, 2005; Van Raamsdonk, 2005).

Figure 1: Neuropathology in Huntington’s disease. Control (left) and a Huntington’s disease (right) brain sections.
3. Huntington’s disease: two main lines of research

3.1. Gain- and Loss-of-function in Huntington’s disease

Huntington’s disease first studies were based on both genetic analysis and disease manifestation in patients. The fact that this disorder emerges only in the presence of the mutation had initially directed most research interest towards the understanding of mutant huntingtin mechanism of action. The first evidences supporting the gain-of-function hypothesis came from the fact that the deletion of one huntingtin allele does not result in HD, meaning that at least one mutant allele is needed for disease manifestation. Moreover, heterozygous individuals for the mutation develop the full range of HD phenotypes. These evidences clearly sustain the dominant feature of the disorder (Housman, 1995). Furthermore, some studies preceding the discovery of HD gene reported that homozygous patients were clinically indistinguishable from the heterozygous individuals for the mutation. This conclusion may be, at least partially, due to the fact that some of the earlier studies were done before the HD gene discovery and so homozygotes were identified by linkage analysis (Wexler, 1987; Myers, 1989; Durr, 1999). In other CAG triplet diseases, homozygoty enhances the phenotypic severity but in HD this correlation is not that strong (Kremer, 1993; Brinkman, 1997; Telenius, 1993). It is also important to emphasize the overlap of psychiatric, cognitive, and motor manifestations, typical of the first HD stages, and consequently, the equivalence between homozygous and heterozygous HD subjects is far from being completely established. From these clinical genetic studies, the contribution of normal huntingtin loss-of-function to HD cannot be excluded.

Important revelations came from the huntingtin knockout mice studies which, for the first time, showed an essential role of normal huntingtin. In this model, the absence of huntingtin resulted in cell degeneration and early embryonic lethality (Duyao, 1995; Nasir, 1995; Zeitlin, 1995). In addition, conditional knockout mice work revealed degeneration also in adult neuronal cells (Dragatsis, 2000). These observations have given further strength to the hypothesis that loss of huntingtin function may also play a part in HD pathology. One of the most striking features of HD is the striatal selective vulnerability. Since huntingtin is expressed ubiquitously, the simple presence of mutant huntingtin fails in answering why a subpopulation of neurons selectively dies throughout the disease progression. How the mutant form of the protein might cause the loss of striatal, and later on of cortical neurons, is not clear. One of the raised hypotheses suggests that mutant huntingtin could interact with striatal specific proteins but, so far, no such proteins have been identified. Another hypothesis is based on the fact that mutant huntingtin causes aggregates formation in cells, however this phenotype is not striatal specific and additionally, many authors view the aggregates as an epyphenomenon and not as a cause of the degeneration. The loss-of-function hypothesis, represents an alternative for the understanding of some of the disease features that mutant huntingtin acquired toxic ativity cannot explain. While the mutation is certainly responsible for the disease initiation, the
loss-of-function hypothesis suggests that the selective vulnerability feature might be linked to the loss of striatal-specific beneficial functions endowed in the normal huntingtin protein context and independent of the CAG expansion.

The view of Huntington´s disease as both a gain and loss-of-function disorder is becoming more accepted: from one side, the toxic gained activity of mutant huntingtin, from the other, the loss of the beneficial activity of normal huntingtin. Loss of huntingtin normal function and the stress-responses activated by the expanded polyglutamine stretch of mutant huntingtin, may act together, contributing to the progression of the disease. Indeed, a study done in a transgenic HD mice model revealed that the overexpression of mutant huntingtin results in both a decrease of the endogenous, normal huntingtin and an increase of its fragmentation (Zhang, 2003). Therefore, it might be that normal and mutant huntingtin cross talk to each other resulting in a dominant negative effect of the mutant over the normal form of the protein (Cattaneo, 2001).

3.2. Mutant huntingtin: Gain-of-function

The first experimental evidence of the gain-of-function theory was given by a work of Housman in which the deletion of one huntingtin allele did not result in HD pathology (Housman, 1995). Other evidences support this theory wherein mutant huntingtin gains a toxic function, for example, heterozygous HD knockout mice are known to live normally, without developing an HD phenotype (Duyao, 1995; Nasir, 1995; Zeitlin, 1995). In HD patients, it is not well demonstrated if homozygous and heterozygous individuals exhibit differences in terms of symptomatology. This fact led to the early thought that the presence of one allele of normal huntingtin had no influence on the pathology and so mutant huntingtin mechanisms of action are the key to understand HD (Wexler, 1987; Durr, 1999).

The identification of the HD gene allowed the generation of several mice models in which mutant huntingtin is expressed in the presence of endogenous normal huntingtin and still, these mice develop neurological symptoms, die early, even when endogenous normal huntingtin is expressed at normal levels (Davies, 1997; Schilling, 1999). Moreover, mutant huntingtin can rescue the embryonic lethality of huntingtin null mice (Hodgson, 1996), which also suggests that HD mutation can lead to neuronal toxicity, even in the absence of normal huntingtin (Li and Li, 2006).

3.2.1. Huntington’s disease Mouse Models

Most of the studies describing mutant huntingtin acquired toxic effects were done on a number of rodent models created to provide in vivo evidences for HD pathology.

i) Transgenic HD mice

Several transgenic mice have been generated using either the human huntingtin promoter or neuronal promoters. In 1996, Davies and colleagues produced the R6/2
transgenic mouse that would soon become the most used HD mouse model. The R6/2 mice express a mutant version of the human huntingtin exon, which is under the control of the human HD gene promoter and is translated into an expanded N-terminal portion of human huntingtin with 115-150 CAG repeats. This model reproduces some of the HD phenotypes, confirming that the presence of an expanded CAG is sufficient to give rise to the pathology. At the cellular level, nuclear inclusions are observed and although the striatum is not selectively vulnerable, a general neurodegeneration is present (Davies, 1997). A microarray study with different brain regions from symptomatic R6/2 mice revealed a significant alteration in the transcriptional levels of several genes, but no correspondence with a particular brain region was observed (Luthi-Carter, 2002). An important feature of these mice is that they closely mirror the reduction in cortical brain-derived neurotrophic factor (BDNF) mRNA levels seen in HD patients (this will be further discussed in the Results and Discussion sections).

Another available transgenic mouse model that uses the human HD gene promoter is the YAC (Yeast Artificial Chromosome) transgenic mouse model. In this case, the human HD gene promoter drives the expression of full-length mutant huntingtin. Several mouse models were created with this technology, such as the HD mouse model expressing normal huntingtin known as YAC 18 and the ones expressing mutant huntingtin with different numbers of polyglutamine residues commonly called YAC 46, YAC 72 (Hodgson, 1999; Graham, 2006). These transgenic mice, even at early ages, display electrophysiological changes, indicating cytoplasmic dysfunction prior to observed nuclear inclusions or neurodegeneration. Later on, around their 12th month of age nuclear inclusions appear and selective striatal neurodegeneration is observed. In this HD model, nuclear inclusions seem not to be the cause of neurodegeneration. Furthermore, the emergence and progression of the pathology is proportional to the levels of mutant huntingtin. Another similar model, the YAC 128 (with 128 CAG repeats) represents a relevant HD model since these mice develop also motor dysfunction, and this symptom is tightly related with the striatal neuronal loss (Slow, 2003).

The N171-82Q transgenic mice represent another widely used HD model. These mice express the first 171 amino acids with 82 glutamines under the control of the neuronal prion promoter (Schilling, 1999). Both their neurological and behavioral phenotypes resemble those from HD affected individuals.

ii) Knock-in HD mice

Besides the transgenic HD mice also the knock-in mice models have been generated. In this case, the mice model was created by the insertion of an expanded repeat into the endogenous mouse HD gene (Lin, 2001; Shelbourne, 1999; Wheeler, 2000; Menalled, 2002).

Most HD mouse models fail in exhibiting the overt neurodegeneration seen in HD patients. It is possible that the short life span of the mouse does not allow the
development of evident neurodegeneration, although some earlier pathological events do occur. Indeed, more recently a transgenic rat model of HD (tgHD rats) was developed, and this model exhibits enlarged ventricles, striatal atrophy and pycnotic pyramidal cells in frontal cortical layer V (Kántor, 2006). So far the tgHD rat is the only animal HD model with selective striatal loss and alterations in behavior.

3.2.2. Nuclear and Cytoplasmic effects of Mutant Huntingtin

It has become clear that the polyQ expansion can cause mutant huntingtin to misfold and to aggregate. Misfolded huntingtin tends to abnormally interact with other proteins involved in several different processes, including transcription and cell signaling. Additionally, mutant huntingtin is known to accumulate in the nucleus and in neuronal processes. For both its misfolding and aggregates formation, mutant huntingtin proteolysis is required. Accordingly, many cleavage sites have been identified in the N-terminal region of huntingtin. Given its favored nuclear localization and its increasing number of interactors it is likely that mutant huntingtin is involved in a number of pathogenic pathways:

a) Proteolysis and aggregates formation

b) Abnormal interaction with other proteins

c) Transcriptional dysregulation

d) Mitochondrial dysfunction

e) Excitotoxicity
a) Proteolysis and aggregates formation

A number of protease cleavage sites, including those for caspase-3, caspase-6, calpain and unknown aspartic protease, have been found within the first 548 amino acids of huntingtin (Graham, 2006; Kim, 2001; Gafni, 2002; Wellington, 2002; Lunkes, 2002).

A particular event that takes place in the HD cellular context is the proteolytic cleavage of mutant huntingtin into smaller N-terminal fragments that subsequently can enter in the nuclear compartment. Although some immunostaining and nuclear fractioning studies have shown that normal huntingtin can also be localized in the nucleus, it is clear that the majority remains in the cytoplasm (Hoogeveen, 1993; Kegel, 2002). In agreement, antibodies raised against the N-terminal region of the protein recognize only nuclear huntingtin fragments (Difiglia, 1997; Gutekunst, 1999).

Both normal and mutant huntingtin are substrates for caspase-3 activity, but there is a difference in the rate of cleavage of each substrate. In wild-type mice, huntingtin fragments have been observed suggesting that the proteolytic cleavage is a physiologic event in the cell, but the mutant form of the protein acquires a conformation that renders it the preferred enzymatic substrate, respect to normal huntingtin. The cleavage of mutant huntingtin results in the generation of a number of N-terminal huntingtin fragments that can enter the nuclear compartment. Studies of N-terminal htt fragments have failed in demonstrating that these fragments contain nuclear localization sequences, thus it may be that these fragments passively enter the nucleus, and the expanded polyglutamine tract prevents their export back to the cytoplasm (Cornett, 2005). The presence of mutant huntingtin fragments in the nucleus and the various cleavage sites in the N-terminal region of huntingtin support the hypothesis that htt proteolysis leads to generation of toxic htt fragments (Kim, 2001; Gafni, 2002; Wellington, 2002; Lunkes, 2002; Sun, 2001). Consistently, smaller N-terminal htt fragments appear to be more toxic than large-sized fragments in both cultures cells (Hackman, 1998) and transgenic animals (Davies, 1997; Schilling, 1999; Yu, 2003). It has been reported that these fragments preferentially accumulate in the nucleus of cortical neurons, giving strength to the cortico-striatal dysfunction in Huntington’s disease (Wellington, 2000).

Kim and colleagues have revealed that partial proteolysis by calpain of the human caspase 3-cleaved N-terminal htt fragment can occur in vitro resulting in the production of smaller N-terminal products. Indeed, products of similar size appeared when mouse brain protein extracts were treated with calpain. These results support the idea that sequential proteolysis by caspase 3 and calpain may regulate huntingtin function at membranes and produce N-terminal mutant fragments that aggregate and cause cellular dysfunction in HD (Kim, 2001).

It has also been reported that endogenous wild-type huntingtin is promptly cleaved by calpains in primary neurons, producing fragments of 60 and 75kDa. Moreover, the exposure of primary neurons to glutamate or 3-nitropropionic acid increased intracellular calcium concentration, leading to loss of intact full-length wild-type huntingtin (Goffredo, 2002).
The htt N-terminal fragments produced upon caspase and calpain cleavage can form nuclear and cytoplasmic aggregates that exhibit a fibrillar morphology and are known to be SDS resistant. These aggregates have been reported in several cellular models, such as the PC12 cells, the N2a neuroblastome or the HEK293 fibroblasts, where mutant huntingtin was transfected, and their appearance seems to be dependent on the type of cell and on the levels of exogenous protein expression (Davies, 1997; Martingdale, 1998). Pronounced neuronal intranuclear inclusions have also been observed in transgenic mice models, expressing the exon 1 of the human HD gene carrying 115 to 156 CAG repeat expansions, and were further characterized as containing the proteins huntingtin and ubiquitin (Davies, 1997). Htt N-terminal formed aggregates also in the YAC 46 and YAC 72 mice models (Hodgson, 1999) and were reported be also present in post-mortem tissue brain from HD patients. (DiFiglia, 1997)

To study the biological importance of these aggregates, Saudou and colleagues used a cellular model in which nuclear localization of mutant huntingtin was blocked and consequently, its ability to form intranuclear inclusions and to induce neurodegeneration was suppressed. The exposure of mutant huntingtin-transfected striatal neurons to conditions that suppress the formation of inclusions resulted in an increase of mutant huntingtin-induced death. These results suggested for the first time that the formation of intranuclear inclusions might reflect a cellular mechanism to protect against huntingtin-induced cell death (Saudou, 1998). The role of these nuclear inclusions remains controversial, since their formation is correlated with disease progression, but is not linked with neuronal degeneration. Furthermore, several studies have shown that htt inclusions are protective against htt toxicity in cultured cells (Saudou, 1998; Arrasate, 2004). Despite the controversy on the exact role of htt inclusions, they represent a pathological hallmark for the accumulation of toxic mutant htt and reflect protein misfolding caused by polyQ expansion.

b) Abnormal interaction with other proteins

Early studies have reported that mutant htt increases caspase activity (Ona, 1999; Li, 2000) and affects various signaling pathways (Cepeda, 2001; Zeron, 2002). These findings indicate that mutant htt acts in the cytoplasm, affecting different cellular functions. Both yeast two-hybrid and in vitro binding assays have revealed a number of htt partners (See Table 3). Most of these studies aimed at identifying striatal-specific htt interacting proteins, but so far none of the htt partners fulfills this requisite.

Many proteins have been reported to interact with htt and to be involved in different cellular functions, such as vesicular trafficking, synaptic transmission, signaling transduction or transcription regulation. Of these, htt-associated protein 1 (HAP1) and htt-interacting protein 1 (HIP1) have been extensively studied. Both proteins are involved in intracellular trafficking. While HAP1 binds more strongly to mutant htt than to normal htt, HIP1 shows the inverse affinity (Li, 1995; Kalchman, 1997). HAP1 has been shown to co-localize with microtubules and synaptic vesicles in axonal terminals, and its hypothalamic
function seems to be crucial for feeding behavior and metabolism (Sheng, 2006). HIP1, besides binding htt, has also been described to interact with clathrin and alpha-adaptin subunit AP-2. The function of HIP1 seems to be important for the assembly of the cytoskeleton and for endocytosis (Kalchman, 1997).

c) Transcriptional dysregulation

The aberrant nuclear accumulation of mutant htt is likely to cause gene transcriptional dysregulation. Indeed several nuclear transcription factors are found to bind htt and these interactions may occur at various binding sites. In order to have a general view of the transcriptional alterations in the presence of the mutation, several microarray studies were performed in both cellular and animal HD models (Sipione, 2002; Luthi-Carter, 2002; Chan, 2002). Microarrays studies have shown that the transcription of several genes is altered in the presence of mutant huntingtin. The most accepted reason for this observation is that mutant huntingtin interacts with several transcription factors, through its N-terminal tract. Many of these transcription factors contain a polyQ-rich region, and likely it is through a polyQ-polyQ interaction that these factors and huntingtin interact. Since mutant htt fragments have a preference for localizing in the nucleus, this is possibly a reason for the abnormal interactions with the transcription factors present in this cellular compartment. The colocalization of some transcription factors in nuclear polyQ inclusions also led to the idea that recruitment of transcription factors into the inclusions reduces the level of these transcription factors.

Mutant htt was shown to be able to interact with both the polyQ (Nucifora, 2001) and the acetyltransferase domains (Steffan, 2001) of cAMP response element-binding protein (CREB)-binding protein (CBP). In the presence of mutant htt, CBP becomes abnormally ubiquitinated resulting in its higher degradation and in reduced transcriptional expression of CREB-controlled genes (Jiang, 2003). Deletion of CREB in the brain causes selective neurodegeneration in the hippocampus and striatum (Mantamadiotis, 2002). Other transcription factors have been reported to interact with mutant htt, such as TAFII130, a factor that together with TBP (TATA Binding Protein) is fundamental for RNA polymerase II activity (Shimoata, 2002; Dunah, 2002), the N-CoR (Nuclear Corepressor Receptor), the m-Sin3a (Nuclear Corepressor Sin3a) and the transcription factor Sp1 (Specific protein 1) (Dunah, 2002; Li, 2001). Many neuronal genes that lack a TATA box require Sp1 for their transcription.

Altered expression of genes involved in lipid metabolism, cellular signaling, vesicular trafficking, RNA processing, neurotransmission have been reported in the presence of mutant huntingtin (Sipione, 2002). Further studies revealed that in both transgenic HD mice models and fibroblast from HD patients there is a reduced cholesterol biosynthesis, and this might be due to the reduction of the nuclear levels of SREBP (Sterol Regulator Element Binding Protein), an important transcription factor of several key genes for cholesterol biosynthesis (Valenza, 2005).
<table>
<thead>
<tr>
<th>Designation</th>
<th>Function</th>
<th>Htt region of interaction</th>
<th>PolyQ influence on binding affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP1</td>
<td>Trafficking, endocytosis</td>
<td>1-230</td>
<td>Increase</td>
</tr>
<tr>
<td>HIP1</td>
<td>Endocytosis, pro-apoptotic</td>
<td>1-540</td>
<td>Decrease</td>
</tr>
<tr>
<td>HIP14</td>
<td>Trafficking, endocytosis</td>
<td>1-550</td>
<td>Decrease</td>
</tr>
<tr>
<td>PACSIN 1</td>
<td>Endocytosis, pro-apoptotic</td>
<td>Polyproline</td>
<td>Increase</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Synaptic transport</td>
<td>Unknown</td>
<td>Decrease</td>
</tr>
<tr>
<td>CA150</td>
<td>Transcriptional activator</td>
<td>Unknown</td>
<td>No effect</td>
</tr>
<tr>
<td>HIP1</td>
<td>Endocytosis, pro-apoptotic</td>
<td>1-540</td>
<td>Decrease</td>
</tr>
<tr>
<td>HIP14</td>
<td>Trafficking, endocytosis</td>
<td>1-550</td>
<td>Decrease</td>
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<td>PACSIN 1</td>
<td>Endocytosis, pro-apoptotic</td>
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<tr>
<td>PSD-95</td>
<td>Synaptic transport</td>
<td>Unknown</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

Table 3: Summary of proteins reported to interact with huntingtin.
d) Mitochondrial dysfunction

In eukariotic cells, mitochondria provide energy from aerobic metabolism. This organelle plays an important regulatory role in apoptosis, produces and detoxifies free radicals, and serves as a cellular calcium buffer.

Early studies demonstrated that a systemic administration of the mitochondrial toxin 3-nitropropionic acid (3NP), in rodents and non-human primates, replicated most of the clinical and pathophysiological hallmarks of HD, including spontaneous choreiform and dystonic movements, frontal-type cognitive deficits, and progressive heterogeneous striatal degeneration. Moreover, 3NP produced the preferential degeneration of the medium-sized spiny GABAergic neurons with a relative sparing of interneurons and afferents, as was observed in HD striatum (Brouillet, 1999).

Several lines of research have shown that mitochondrial activity is indeed important for the neuropathological events observed within the striatal neurons (Orth, 2001; Beal, 1993). Mitochondria from HD cells have been shown to have a lower membrane potential and to depolarize with lower calcium loads, when compared to mitochondria from control cells. These effects have been observed in diverse models, such as the fibroblast from HD patients and brain mitochondria from transgenic mice expressing full-length mutant huntingtin. Consistently, N-terminal fragments from mutant huntingtin were found in neuronal mitochondrial membranes. Furthermore, incubating normal mitochondria with a fusion protein containing an abnormally long polyglutamine repeat produced a mitochondrial calcium defect similar to the one seen in HD human patients and transgenic animals (Panov, 2002). By taking advantage of a striatal immortalized cell line expressing mutant htt, Rigamonti and colleagues have shown that under toxic stress conditions, cells expressing mutant htt exhibit an increased release of the pro-apoptotic factor, cytochrome c, and an activation of caspase 3 and 9 (Rigamonti, 2001).

Together, these studies propose that mitochondrial calcium abnormalities occur early in HD pathogenesis and that these defects may be a direct effect of mutant huntingtin on the organelle. Still, the mechanisms underlying these abnormalities remain unclear.

e) Excitotoxicity

Excitotoxicity is the pathological process by which neurons are damaged and killed by the overstimulation of receptors for the excitatory neurotransmitter glutamate, such as the N-methyl-D-aspartate (NMDA) receptor and non-NMDA, AMPA receptor. NMDA, kainic acid and pathologically high levels of glutamate bind these receptors and cause excitotoxicity by allowing high levels of calcium ions to enter the cell.

Excitotoxicity has been a long-standing theory to account for the pathogenesis of HD. Glutamate activates ionotropic glutamate receptors, specially the NMDA and non-NMDA (AMPA/ kainite) receptors. In HD, the overactivation of glutamate receptors by high
levels of extracellular glutamate induces excitotoxicity (Beal, 1994; Coyle, 1976; DiFiglia, 1990).

Administration of NMDA receptor agonists to the striatum of animals causes a selective loss of medium spiny neurons and produces neurological symptoms similar to those seen in HD patients (Hantraye, 1990), whereas NMDA receptor antagonist effectively reduced the excitotoxicity in a HD animal model (Greene, 1993). It has also been observed the reduction of glutamate receptors in HD brain, in particular of the mGluR2 receptor (Cha, 1999). Finally, neurons from a HD transgenic mouse model were shown to have an increased NMDA receptor activity (Zeron, 2002; Levine, 1999).
3.3 Wild-type huntingtin: function and its loss in HD

Several lines of evidences account for the need to investigate wild-type huntingtin function, in order to fully understand Huntington’s disease.

Huntington’s disease is one of the eight inherited neurodegenerative diseases in which the expanded CAG repeat has been found. This class of pathologies includes the spinocerebellar ataxias (SCA) 1, 2, 3 (also known as Machado Joseph disease), 6, 7 and 17, the spinobulbar muscular atrophy (SBMA or Kenedy’s disease) and the dentatorubral-pallidoluysian atrophy (DRPLA). It is now clear that the expansion of the CAG repeat in diverse genes causes distinct neurodegenerative pathology in each of the previously mentioned disorders. For each disease, a different brain area selectively undergoes degeneration in the presence of the CAG expansion. This observation implies that the presence of the triplet expansion is not the cause of the loss of a specific neuronal population. This selective neuronal loss became a hallmark of this class of pathologies and its explanation might only be found by investigating the loss-of-function from the wild-type protein involved in a given neurodegenerative disease. In the HD case, our group has proposed that, wild-type htt exerts a beneficial effect to striatal neurons and that the expanded CAG disrupts this ability (Cattaneo, 2001).

Mutant htt acquired toxicity is not striatum-specific: aggregates have been found in all brain areas and also in some periphery tissues, the proteolytic cleavage is not exclusively confined to the striatum and so far, no striatum-specific protein interactors/pathways have been identified as being altered in the presence of mutant htt.

Early clinical studies with HD patients have always favored the gain-of-function hypothesis, by reporting no differences between homozygous and heterozygous patients (Wexler, 1987; Myers, 1989; Durr, 1999). Much has been done in order to develop more accurate clinical testing and phenotype measurements, and recent clinical data reveal that the disease duration is different between homozygous and heterozygous HD patients (Squitieri, 2003). In agreement, homozygous transgenic mice expressing mutant htt have a shorter lifespan when compared with heterozygous mice (Reddy, 1998).

Another important consideration came out from the analysis of the HD gene. The sequence of this gene reveals a high level of conservation throughout species evolution, but strikingly the number of CAG triplets carried by the normal gene is different for each species (Kremer, 1994). This observation raised the idea that the CAG repeat is not crucial for normal huntingtin function. Furthermore, a recent study revealed that the deletion of CAG repeats in the HD gene results only in a subtle behavioral and motor phenotype in mice (Clabough, 2006).

Interestingly, studies with a conditional knockout mouse, where wild-type htt expression was inactivated in the forebrain and testis, resulted in a progressive degenerative neuronal phenotype and sterility (Dragatsis, 2000). These outcomes imply that huntingtin is required for neuronal function and survival in the brain. Collectively, this data supports the hypothesis that loss-of-function mechanism may contribute to HD
pathogenesis and that the study of wild-type htt function will possibly lead to a more complete perception of Huntington's disease.

### 3.3.1 Normal huntingtin function in early embryogenesis and in neuronal development

Huntingtin is essential for mammalian early embryonic events since huntingtin knockout mice (Hdh-/-) die before embryonic day 8.5, before gastrulation and formation of the nervous system (Duyao, 1995; Nasir, 1995; Zeitlin, 1995). These studies revealed the importance of huntingtin for the conclusion of gastrulation, somites formation and organogenesis. In one of these studies, the heterozygous mice (Hdh+/-) displayed increased motor activity and cognitive deficits along with significant neuronal loss in the subthalamic nucleus (Nasir, 1995); while in the other two studies, the heterozygous mice did not present any phenotype (Duyao, 1995; Zeitlin, 1995). The deficits observed in the Hdh+/- mice (Nasir, 1995) were proposed to be a consequence of the 20kDa N-terminal fragment of htt that was still being expressed in that model. The small htt fragment may produce a dominant negative effect over the full-length form of htt, altering its functionality or simply reducing htt function(s) (O'Kusky, 1999). Together, this data show that the HD gene is essential for post-implantation development. Furthermore, the significantly higher number of apoptotic cells in the ectoderm of the huntingtin knockout embryos raised the hypothesis of htt being involved in anti-apoptotic processes throughout embryonic development.

In 1997, important findings came out from a work done in an htt knock in mouse model. White and colleagues extended the polyglutamine tract of the mouse htt by targeted introduction of an expanded human HD CAG repeat, creating mutant HdhneoQ50 and HdhQ50 alleles that expressed reduced and wild-type levels of altered huntingtin, respectively. The homozygous mice, with reduced htt levels displayed characteristic aberrant brain development and perinatal lethality, indicating a critical function of htt in neurogenesis. However, mice with normal levels of mutant huntingtin did not display these abnormalities, indicating that the expanded CAG repeat does not eliminate or detectably impair huntingtin's neurogenic function in vivo. For neurogenesis it is crucial that htt is present in the correct amount, independently of being wild-type or mutant. In fact, in post-gastrulation stages, huntingtin becomes essential for neurogenesis since a dose of htt below 50% allows gastrulation but is not enough for the proper formation of the epiblast, the structure that will form the neural tube. Mice expressing less than 50% normal dose of htt exhibited abnormalities in morphogenetic gradients that are known to be critical for the determination of the anterior-posterior, ventro-dorsal and medio-lateral axis throughout brain development (White, 1997). These observations implicate htt in the early organization of the embryo and in the formation of the central nervous system.

An important piece of data came from a work done with mice chimaeras created by injecting mouse embryonic stem cells knockout for huntingtin (ES Hdh -/-) into normal
blastocysts. Some brain regions from these chimaeras were properly populated by neurons Hdh +/- (although the functionality of these neurons was not evaluated), but few donor cells were found in cerebral cortex, striatum and basal ganglia. These observations suggest that htt may play a region-specific role in neuronal survival during development, and that neuroblasts in these brain regions need to synthesize huntingtin in order to progress throughout development and differentiation processes (Reinier, 2003).

In overview, the available data indicate that htt is required at different developmental stages, probably by exerting diverse roles in a time and tissue-specific manner. Huntingtin is essential for early embryonic development, more specifically for gastrulation conclusion. Afterwards, becomes important for neurogenesis and for the formation of the CNS. Finally, huntingtin physiological function in the adult life might be linked with neuronal survival by acting in a region-specific manner. Taking all these observations into account, htt seems to have an extreme ability for exerting diverse functions during mammalian development and throughout adult life. These considerations headed the hypothesis that htt may be a protein that accumulated different activities, that have somehow developed along species phylogenetic evolution (Cattaneo, 2005). The starting point of this hypothesis is that, if htt function has evolved, then we can identify its original and acquired roles by looking at ancestral species. By following htt roles in ancestral species, and moving back in time, we will arrive to those species with poorly organized or absent nervous system and in these, it will be expectable to identify an early, possibly, non-neural role of htt. Accordingly, looking at the other extremity of species evolution, we find the higher vertebrates (such as mammals) where huntingtin has been implicated in neuronal-specific activities that can be revealed in post-mitotic neurons that most probably have evolved later.

3.3.2. Normal huntingtin function in adulthood

Several lines of evidences have shown that normal htt is able to protect brain cells from apoptotic stimuli (Rigamonti, 2000; Hackman, 2000; Dragatsis, 2001) and has a neuroprotective effect in vivo (Zhang, 2003). One of the strongest evidences came from a work done on an htt conditional knockout mouse, where htt expression was eliminated from the forebrain region. As a result of htt forebrain depletion, a progressive neurodegeneration and neurological dysfunctions were observed.

Elimination of endogenous wild-type htt expression from the widely used YAC128 HD mouse model, resulted in the exacerbation of the pathology, but there was only a mild effect on striatal phenotypes. This observation suggests that a simple increase of wild-type huntingtin dose may not be sufficient to improve all disease symptoms (Van Raamsdonk, 2005).

Huntingtin has been implicated in the transcriptional regulation of the Brain-Derived Neurotrophic Factor (BDNF), a Neuron Restrictive Silencer Element (NRSE)-containing gene. The NRSE element is inactivated by normal htt, but this activity is no
longer exerted in the presence of the mutant form of the protein (Zuccato, 2003). Moreover, it has also been proposed that besides its role in BDNF transcription, htt might act at the level of BDNF vesicular transport, from the cortex to the striatum (Gauthier, 2004). In addition, huntingtin has also been involved in fast axonal trafficking in mammalian neurons (Trushina, 2004).

Only by understanding huntingtin’s physiological function, its functional domains and respective downstream effectors, it will be possible to take advantage of htt’s potential beneficial function(s) and apply them in the Huntington’s disease context.

Even if huntingtin essential role during embryonic development has been recognized more than one decade ago (Nasir, 1995; Duyao, 1995; Zeitlin, 1995), its function in brain cells and throughout adult life is still an enduring research. Defining how important it is loss of huntingtin in HD requires a full understanding of htt physiological function in the adulthood.

At this point, a more detailed revision of the studies concerning huntingtin roles, in the adult brain, will be presented in the following order:

a) Anti-apoptotic effect of normal huntingtin;

b) Normal huntingtin controls fast axonal trafficking and mitochondrial motility;

c) Normal huntingtin is implicated in synaptic activity;

d) Normal huntingtin regulates BDNF transcription;

e) Normal hematopoiesis requires normal huntingtin.
a) Anti-apoptotic effect of normal huntingtin

The first evidence that huntingtin could act as an anti-apoptotic protein came from the observation of an abnormally high number of apoptotic cells in the HD knockout embryos (Nasir, 1995; Duyao, 1995; Zeitlin, 1995). The heterozygous mice produced in one of the early HD knockout works (Nasir, 1995) were further analyzed and morphometric and ultrastructural studies revealed apoptotic cell death in the basal ganglia and adult brain, along with behavior abnormalities (O’Kusky, 1999).

Meanwhile, in vitro studies, in a conditionally-immortalized striatum-derived cells overexpressing the human wild-type huntingtin, provided the first evidence of wild-type huntingtin neuroprotective role. This in vitro system was exposed to several apoptotic stimuli, such as serum deprivation, mitochondrial toxins, transfection of genes involved in death pathways, and consistently, cells overexpressing wild-type huntingtin were protected from apoptotic cell death (Rigamonti, 2000).

Other in vivo evidences of this anti-apoptotic effect of huntingtin came from the htt conditional knockout in the adult brain (and testis). The neuronal inactivation of htt gene led to extensive apoptotic cell death in the hippocampus, cortex and striatum, accompanied by neurologic abnormalities and death by the age of one year (Dragatsis, 2000).

How htt exerts its anti-apoptotic effect is a question that has been addressed in several studies. One of the first hypotheses was that wild-type htt protective effect involved a mechanism of action somewhere upstream of caspase-3 activation. In fact, wild-type htt was shown to inhibit the cleavage of pro-caspase-9 into active caspase-9, and consequently inhibiting the apoptotic cascade (Rigamonti, 2001). Hackman and colleagues proposed an alternative/complementary explanation for this wild-type huntingtin beneficial effect. Their study suggested that the anti-apoptotic action of normal htt was mediated by its interaction with HIP1 (Huntingtin Interacting Protein 1), a protein known to interact with huntingtin and to possess pro-apoptotic activity. The overexpression of HIP1 in vitro resulted in rapid caspase-3 dependent cell death but when HIP1 and htt were coexpressed, HIP1 mediated cell death was significantly reduced. These results point out that the binding of htt to HIP1 protects cells from apoptosis (Hackam, 2000). Consistently, in the presence of mutant htt, there is less binding of HIP1, which instead has been shown to associate with another protein Hippi (HIP1 protein interactor). In turn, Hippi is known to activate pro-caspase-8 and of causing apoptotic cell death (Gervais, 2002).

More recently, htt was reported to be a substrate of Akt (a serine/threonine kinase) and that phosphorylation of htt by Akt is critical for htt to exert its neuroprotective effect. Huntingtin has also been implicated in the phosphoinositide 3-kinase (PI3K)-Akt pathway by stimulating the expression of pro-survival genes (Humbert, 2002; Rangone, 2004).

In an in vivo study, overexpression of wild-type htt was shown to protect against ischaemic injury (Zhang, 2003). Finally, normal htt seems also to protect against the
toxicity produced by the increased activation of the NMDA receptors, observed in HD (Van Raamsdonk, 2005).

b) Normal huntingtin controls fast axonal trafficking and mitochondrial motility

Studies with RNA interference for huntingtin in Drosophila melanogaster have shown that in flies expressing reduced levels of the protein in the eye caused defects in the fast axonal trafficking of vesicles and degeneration. A similar effect was observed after the expression of an exogenous fragment of mutant huntingtin (Szebenyi, 2003; Gunawardena, 2003). In agreement, expression of full-length mutant htt impaired vesicular and mitochondrial trafficking in mammalian neurons in vitro and in whole animals in vivo (Trushina, 2004). An important evidence of a role for normal htt in trafficking came from a study done on embryonic striatal neurons taken from mice genetically engineered to express only one copy of normal htt or less than 50% of normal htt levels upon CRE-mediated recombination. The trafficking defect was worse in neurons from mice that were complete knock out for htt than in those expressing less than 50% of htt, suggesting a dose-dependent effect of loss of normal htt. The trafficking defects in both neurons without, or expressing less that 50% of normal htt, were more extensive than those exhibited by neurons isolated from mice expressing mutant htt (Trushina, 2004).

c) Normal huntingtin is implicated in synaptic activity

Normal htt has been shown to interact with a number of cytoskeletal and synaptic vesicle proteins known to be essential for exo- and endocytosis at the synaptic terminals (Smith, 2005). One of these proteins is the Post-synaptic density 95 (PSD-95), a member of the membrane- associated guanylate kinase (MAGUK) family of proteins, that has been reported to bind the N-methyl-D-aspartate (NMDA) and the kainate receptors (Sheng & Skim, 2002). Normal htt is able to interact with the SH3 domains present in the PSD-95. In the presence of the mutation, less PSD-95 binds to mutant htt and this, results in a higher release of PSD-95 in HD that affects the activity of NMDA receptors and ultimately might lead to excitotoxicity. If normal htt is overexpressed, neuronal toxicity induced by NMDA receptors and by mutant htt presence seems to be attenuated (Sun, 2001). Moreover, htt binds to proteins that have been involved in the normal receptor recycling at the nerve terminals. One of these proteins is the PACSIN1/ Syndapin, a neurospecific phosphoprotein that has a key role in synaptic vesicle and receptor recycling. Huntingtin has been reported to bind syndapin through its Polyproline- rich region and this interaction is dependent on the length of the polyglutamine- rich region in htt. In the presence of mutant htt (and its expanded polyglutamine stretch), the interaction with syndapin is enhanced. This abnormal binding between syndapin and mutant htt results in the removal of syndapin from synapses and neuronal processes, which eventually conducts to an altered synaptic transmission (Modregger, 2002).
d) Normal huntingtin regulates BDNF transcription

Huntingtin has a pro-survival role that has been observed in diverse types of brain cells as well as in cells isolated from periphery tissues. Additionally, htt has been involved in the control of fast axonal transport and in synaptic transmission. None of these roles is striatal specific and so they fail in explaining why striatal neurons selectively die during HD progression.

A possible explanation for this selective degeneration is based on the results of a study conducted by Ivkovic and Erlich, in which the Brain-Derived Neurotrophic Factor (BDNF) was shown to be required for the maturation of a large subset of medium spiny neurons (the ones that die in HD) both in vivo and in vitro (Ivkovic and Erlich, 1999). Furthermore, BDNF is a pro-survival factor, essential for striatal neurons, since they strictly depend on the cortical-produced BDNF that is delivered to the striatum via the cortico-striatal afferents (Jovanovic, 2000). By taking this data in consideration, Zuccato and colleagues proposed that BDNF levels were altered in HD and that this could be, at least partially, the reason why striatum is the most affected brain region in the pathology (Zuccato, 2001).

Before describing how huntingtin was found to be involved in regulation of BDNF gene transcription, it is crucial to have an overview of BDNF gene structure (Reviewed in Zuccato, 2007). As first shown by Timmusk and colleagues, the rodent BDNF gene has four 5’ exons (I-IV) associated with distinct promoters and one 3’ exon (V) that encodes BDNF protein (Metsis, 1993; Timmusk, 1993). Alternative use of these promoters and differential splicing generate four BDNF mRNAs with different 5’ untranslated regions fused upstream of the same coding exon. It is also known that each transcription unit uses two different polyadenylation signals at the 3’ end of exon V, thus generating eight distinct BDNF transcripts (Timmusk, 1993). Recently, work from two independent groups has reorganized BDNF gene structure. Studies from Liu and Timmusk groups identified respectively three, and four, novel BDNF exons (Liu, 2006; Aid, 2007).

Huntingtin was shown to be able to regulate BDNF gene transcription by acting at the level of BDNF promoter II, more specifically on the Neuron Restrictive Specific Element (NRSE) present in this promoter. The NRSE element is a conserved sequence of 21-23bp that can bind the transcriptional repressor REST/NRSF (RE-1 silencing transcription factor/neuron-restrictive silencer factor). When REST binds to the NRSE element in the BDNF promoter II, the transcription of this gene is inhibited. Normal htt has been shown to bind REST, trapping this transcription factor in the cytoplasm and avoiding its binding to the NRSE sequence. This ability is no longer exerted by mutant htt and in HD lower levels of BDNF mRNA have been reported (Zuccato, 2003). Together these studies sustain that normal huntingtin controls BDNF gene transcription in cerebral cortex, which is then delivered to its striatal targets. In the disease state, supply of cortical BDNF to the striatum is strongly reduced and this possibly leads to the HD striatal vulnerability. Further analysis has shown that a reduction in cortical BDNF messenger level correlates with the progression of the disease in the R6/2 mouse model of HD. Reduction of BDNF messenger
levels, in cortex and blood, from R6/2 mice over time of the disease progression, will be more extensively discussed since this data represent one of the central topics of my PhD studies.

Not only BDNF mRNA levels have been reported to be impaired in HD, also the transport of the BDNF protein along microtubules seems to be inhibited in the presence of mutant htt. In vitro studies revealed that full-length normal htt stimulates BDNF vesicular trafficking in neuronal cells, while mutant htt represses this process. Moreover, BDNF transport is attenuated when the levels of normal htt are decreased with the RNAi technique. The ability of normal htt to enhance BDNF vesicular transport involves the Huntingtin-Associated Protein-1 (HAP1) and the p150Glued subunit of dynactin which is a multisubunit protein complex containing several other subunits that are organized into an elaborate structure. It is currently believed that the bulk of the dynactin structure participates in interactions with a wide range of cellular structures, many of which are cargoes of the dynein motor. Normal htt interact with the p150Glued subunit via HAP1 and by doing so it is believed to stimulate BDNF transport (Gauthier, 2004).

e) Normal hematopoiesis requires normal huntingtin

Expression of huntingtin is detected in spleen and thymus. To determine the function of huntingtin and to provide insight into potential pathologic mechanisms in HD, the role of huntingtin in hematopoietic development has also been addressed. In vitro hematopoiesis was assessed in mouse embryonic stem (ES) cells expressing both, one or none of the htt alleles. All ES cells formed primary embryoid bodies (EBs) with similar efficiency, the numbers of hematopoietic progenitors detected at various stages of the in vitro differentiation were reduced in the ES cell lines expressing only one or none of the htt alleles. Surprisingly, expression analyses of the hematopoietic markers within the EBs revealed that primitive and definitive hematopoiesis occurs in the absence of huntingtin. However, further analysis using a suspension culture in the presence of hematopoietic cytokines demonstrated a highly significant gene dosage-dependent decrease in proliferation and/or survival in the ES cells with only one or none of the copies of the htt gene. Enrichment for the CD34(+) cells within the EB confirmed that the impairment is intrinsic to the hematopoietic cells. These observations suggest that huntingtin expression is indeed required for the generation and expansion of hematopoietic cells (Metzler, 2000).
3.3.3. Wild-type huntingtin counteracts mutant huntingtin

Collectively, the data described above account for the beneficial roles of normal huntingtin in the mature brain. In order to investigate how the loss of normal htt may contribute to Huntington’s disease pathogenesis, several lines of research are being followed. It is also worth to note the ability of normal huntingtin to mitigate the damage caused by the mutant form of the protein may represent an alternative way to look at potential therapeutical methodologies.

**Wild-type huntingtin reduces mutant huntingtin toxicity**

The first demonstration that normal htt can reduce the toxic effects exerted by mutant htt came from studies conducted by Leavitt and colleagues. In these studies, mice with a reduced allelic dose of normal htt and expressing mutant htt, exhibited higher apoptotic cell death in the testis. This effect was then rescued by the overexpression of normal htt in the same mice. These data provided the first direct in vivo evidence of a role for normal htt in decreasing the cellular toxicity of mutant htt (Leavitt, 2001). Further in vitro studies, confirmed that normal htt protects both neuronal and non-neuronal cells from death. Normal huntingtin was shown to be protective in different cell types by acting against the toxicity caused by a mutant huntingtin fragment as well as against a full-length transgene (Ho, 2001).

**Reduced wild-type huntingtin in HD models increases damage**

To investigate the contribution of wild-type htt function to the pathogenesis of HD, Van Raamsdonk and colleagues generated the YAC128+/- mice (expressing one allele of mutant huntingtin with 128 CAG repeats, over the two endogenous wild-type huntingtin endogenous alleles) and the YAC128-/- mice (expressing only mutant huntingtin, and no endogenous wild-type huntingtin). The loss of wild-type huntingtin in the YAC128-/- mice led to a modest worsening in striatal atrophy and neuronal loss, and to a small but significant decrease of neuronal cross-sectional area. Additionally, testis from YAC128+/- mice showed atrophy and degeneration, which was markedly worsened in the absence of normal htt. Loss of normal htt had a clear impact on motor dysfunction, hyperkinesia, testicular degeneration and impaired lifespan in YAC128 mice. The mild effect of normal htt absence on striatal phenotypes in YAC128 mice suggests that the characteristic striatal neuropathology in HD is caused primarily by the toxicity of mutant htt and that replacement of normal htt may not be sufficient to counteract all HD phenotypes (Van Raamsdon, 2005). The fact that reduction of wild-type huntingtin worsened the behavior of HD mice in the absence of striatal pathology, suggests that a simple increase in the wild-type huntingtin allelic dose might not be sufficient to counteract all HD phenotypes. Most probably, a combination of strategies should be designed at restoring the activity of the downstream targets of wild-type huntingtin. However, the level of wild-type huntingtin protein in this study was always >50%, whereas this is not always the case in HD patients. Consistently with this idea, studies conducted in a mouse model stably expressing <50%
of wild-type huntingtin protein showed an early phenotype in striatum. Morphological analyses of these mice indicated that when wild-type huntingtin protein level is below 50%, striatal defects appeared (White, 1997).

### 3.3.4. Neurotherapeutics in HD

Currently there is no cure for HD, and there are no therapies that slow the progression of the disease or delay its onset. Patients are commonly treated with antidepressant drugs and neuroleptics without significant benefits.

However, basic science research dedicated in understanding HD pathogenesis has generated a number of different and promising areas of neurotherapeutics that are below summarized.

**Drugs reducing excitotoxicity**

The excitotoxic hypothesis is a well-studied mechanism in HD pathology and has led to the identification of possible neuroprotective compounds. Injection of quinolinic acid into the striatum of rodents recapitulates many of the features of HD and has therefore been used as a model to identify and test potential neuroprotective agents. N-acetyl-aspartyl-glutamate, a compound that acts as an antagonist at NMDA receptors and has an agonist activity at the mGluR3 metabotropic glutamate receptor, has been found to reduce lesion volume in quinolinic-acid-treated rat (Orlando LR, 1997). (S)-4-Carboxy-3-hydroxyphenylglycine, which acts at the metalotropic glutamate receptors, has also been shown to protect against excitotoxicity in rats that have received intrastriatal injection of quinolinic acid (Orlando LR, 1995). Riluzole, an inhibitor of glutamate release that has been used for Amyotrophic Lateral Sclerosis (ALS), was found to be protective against quinolinic lesions (Mary V, 1995). An open-label human trial found that riluzole reduced chorea and showed decreased levels of cerebral lactate, as assessed by magnetic resonance spectroscopy (Rosas HD, 1999). Another open-label trial of riluzole also demonstrated transient motor improvement in human HD subjects (Seppi K, 2001). A large multi-center placebo-controlled human clinical trial of riluzole is currently underway in Europe. A recent multi-center trial called (CARE-HD) (Coenzyme Q10 and Remacemide in Huntington’s Disease) tested the effects of coenzyme Q10, an antioxidant and cofactor involved in mitochondrial electron transfer, and remacemide, a noncompetitive NMDA-receptor antagonist (Huntington Study Group, 2001). In two different lines of transgenic mice, combination of these two compounds produced beneficial effects (Ferrante RJ, 2002; Schilling G, 2001). However, in the CARE-HD trial, there was no evidence of any benefit of remacemide, either alone or in combination with coenzyme Q10. No significant change in total functional capacity (TFC) was observed, although patients treated with coenzyme Q10 showed a trend toward slowing in TFC decline (13%) over 30 months. In addition, beneficial trends on certain cognitive tests and tests of behavior were found in the coenzyme Q10-treated group.
Drugs acting on mitochondrial dysfunctions

Molecules able to increase energy by boosting ATP stores have been studied for their potential neuroprotective effects. Both creatine and cyclocreatine have been demonstrated to be neuroprotective in animal HD models. Body weight was significantly greater in creatine-treated transgenic mice and motor ability, as measured by performance of the Rotarod test, was significantly improved in those animals. These data support a role for metabolic dysfunction as a pathogenic component of HD and suggest a potential role for ATP repletion via creatine supplementation as a therapeutic strategy (Ferrante RJ, 2000). Other studies have investigated the therapeutic potential of antioxidants. Both vitamin E and idebenone have been tested in clinical trials and found to have no significant impact on functional decline observed in HD (Peyser CE, 1995; Ranen NG, 1996).

Drugs counteracting apoptotic cell death and aggregates formation

Although there is scant evidence for apoptosis in human HD, the demonstration that inhibiting caspase-1 could extend lifespan in transgenic HD mice gave credence to the idea that apoptosis was a legitimate target for therapeutics (Ona VO, 1999) Minocycline, a second-generation tetracycline, has been shown to inhibit caspase-1 and was recently tested in transgenic HD mice. A delay of motor decline (as measured by Rotarod performance) and extend survival time by 14% were reported. By contrast, in another study minocycline administration was not beneficial in mice (Chen M, 2000).

Cystamine, a caspase inhibitor, has also been studied in transgenic HD mice. Cystamine was originally tested as a therapeutic candidate given its ability to inhibit transglutaminases. Transglutaminases have been implicated in the mechanism of aggregate formation, by possibly cross-linking molecules of mutant huntingtin. Because mutant huntingtin aggregation appeared to correlate with disease phenotype, a transglutaminase inhibitor such as cystamine was tested for its potential ability to reduce aggregate formation and, in turn, extend survival. Both oral and intraperitoneal administration of cystamine extended survival in transgenic HD mice and reduced the number and size of aggregates. Recently, cystamine has been found to inhibit caspase-3 activity in vitro, suggesting that it may work through a variety of mechanisms, including caspase inhibition to prolong neuronal survival in HD (Dedeoglu A, 2002; Karpuj MV, 2002; Lesort M, 2003).
Drugs restoring transcriptional deficits

The role of transcriptional dysregulation in HD has been an exciting area of research over the last few years. Studies indicate that transcriptional repression is the main result of transcriptional dysregulation and that it could be explained by the recruitment and sequestration of transcription factors by mutant huntingtin. Because many of these nuclear factors are involved, directly or indirectly, in histone acetylation, research focused on histone deacetylase inhibitors (HDACs). Recent studies in cell culture, yeast, and Drosophila models of polyglutamine disease have shown that HDACs can reduce polyglutamine toxicity (Steffan JS, 2001; Hughes RE, 2001). The effects of suberoylanilide hydroxamic acid (SAHA) were recently accessed in a transgenic mouse model of HD (McCannell A, 2001; Hockley E, 2003). Mice treated with SAHA demonstrated improved motor impairment and less striatal neuronal loss although there was no significant effect on weight or polyglutamine aggregation. The testing of other HDAC inhibitors in mouse models of HD is underway.

Cell transplantation

One obvious approach to reduce the damage caused by dead striatal neurons is to transplant a new source of neurons. Animal studies demonstrated that the plasticity of the adult brain allows the anatomical and functional integration of grafted foetal neural tissue, and that the maturation of these neurons can sustain recovery of motor and cognitive functions (Rosser AE, 2000). Several groups have already started with the first clinical trials. In 2000, the results of a pilot study (Créteil, France) were published and established the basic parameters of foetal cell transplantation in HD (Bachoud-Levi AC, 2000). Results from this study demonstrated long-term clinical benefits in three out of five patients. Improved speed of movements and of some cognitive functions was observed. In another trial, an HD patient who had received a foetal cell transplant died of presumably unrelated causes 18 months later (Hauser RA, 2002). Autopsy analysis demonstrated that the implanted cells had survived, expressed appropriate neurochemical markers, and received innervation by host dopaminergic cells, demonstrating that transplanted tissue could persist for 18 months (Freeman TB, 2000)

While there is no definitive demonstration of clinical improvement with transplantation, a multi-center European trial is ongoing (Rosser AE, 2002). However the rationale of foetal striatal transplantation is questionable, its application in animal models has produced some benefits.

Growth factors administration

Another approach under evaluation is based on the administration of growth factors in the HD brain given their beneficial role in neurons. The importance of BDNF for striatal neuron survival has been previously discussed and on this basis different studies considered the direct administration of BDNF in the brain, as well as the transplantation of cells engineered to express it. These studies will be treated with more detail in the general discussion section.
3.3.5. An alternative way to investigate normal huntingtin function

3.3.5.1 The Phylogenetic approach

Several roles have been anticipated for huntingtin but still no functional domains have been identified in this extremely “active” protein.

One way to address this challenge is to follow a Biologic approach, by either deleting the gene that codes for huntingtin or by inducing its over-expression in both in vitro and in vivo models. This is an approach already being pursued by several laboratories, ours included.

Alternatively, a protein’s function can be investigated through Structural studies. In the huntingtin’s case this revealed to be a hard route. So far, it hasn’t been possible to crystallize huntingtin due to its biochemical properties and to the fact that it is a particularly big protein (348kDa). In the absence of a three-dimensional structure, researchers started looking for functional domains through Bioinformatic analysis of huntingtin amino acid sequence. Analysis of htt primary amino acid sequence reveals very little about which might be its physiological function(s), since only a few known sequence motifs and no structural domains with known function have been identified. In higher vertebrates, the polyQ stretch is followed by a proline-rich sequence (polyP), and downstream to these regions a series of HEAT repeats can be found (Andrade, 1995). The presence of several HEAT repeats favors the hypothesis that htt might be a scaffold protein, capable of mediating different cellular processes. Indeed, htt has been implicated in cell survival, signal transduction, endocytosis, cytoskeletal structure, transcription and axonal transport (Rigamonti, 2000; Trushina, 2004; Zuccato, 2003; Gunawardena, 2003; Gusella, 1998). The increasing number of htt putative functions is also a consequence of the large number of proteins that have been reported to interact with htt in different experimental models (Harjes, 2003). Comparison analysis of huntingtin with other proteins of known function and/ or domains has only revealed a lack of homology between them, giving strength to the notion of huntingtin as a peculiar protein.

The alternative approach that we propose, and which represents about half of my PhD studies, is a comparative approach of huntingtin orthologues isolated from different species in a broad range of taxonomic classes- Phylogenetic approach. These species were selected as they represent key points throughout species evolution. By comparing huntingtin’s gene/ protein sequences from these animals separated by large spans of evolutionary time, we hope to identify structural domains and functional elements of huntingtin, and eventually understand how huntingtin activities and domains evolved during species phylogenesis.

In the absence of information on huntingtin’s three-dimensional structure and domains, comparing huntingtin isolated from progressively more ancient species and testing their biological activities in complementation assays, developed in a cellular model
where huntingtin was previously knocked-out, may be an imperative way to define huntingtin’s conserved, and/or latter-acquired functional domains. In summary, isolating huntingtin orthologues from ancient species and testing their biological activities in complementation assays may therefore represent an extremely useful and informative procedure to identify functionally active domains and give a time and biological frame of their appearance during species evolution.

The three main steps of this approach include:

- **Bioinformatic comparative analysis of available huntingtin sequences.** Recover of RNA from selected key species, isolation of **Huntingtin Orthologues** and, production and validation of expression constructs containing portions, or full-length, huntingtin orthologues.

- **Development of Biological Assays** in a mammalian cell model lacking huntingtin: *Hdh* null mouse embryonic stem (mES *hdh*-/-) cells.

- **Complementation Assays:** application of the developed biological assays in mES *hdh*-/- cells stably expressing the diverse constructs containing portions, or full-length, huntingtin from different species.

My contribution to the long-term studies intended to define huntingtin’s physiological function, by linking the classic biologic approach with the novel Phylogenetic approach.

I was initially involved in the production of expression constructs containing the N-terminus part of huntingtin from different species and in testing their respective expression in our cellular model, but my main contribution to this project consisted in the development of cell-based assays in the extremely useful cellular model, the *Hdh* null mouse embryonic stem (mES *hdh*-/-) cells. These assays aimed at identifying specific molecular and biological phenotypes in cells lacking huntingtin, when compared to wild-type cells (mES *hdh* +/+) both in proliferation and under neural differentiation conditions. Moreover, and as a first step towards huntingtin complementation, I have applied the same biological assays in a mouse ES *hdh*-/- cell line stably expressing the first 548 amino acids of human huntingtin.
3.3.5.2 Huntingtin orthologues

With the ultimate goal of revealing the molecular basis of Huntington’s disease, numerous groups have been analysing huntingtin gene, isolated from different species, in order to create animal models that render possible the study of this gene/protein function.

a) Description of huntingtin gene orthologues

- Vertebrates: mammals
  The first huntingtin orthologue to be isolated was the one from mouse. In this specie, the HD gene was mapped in the chromosome 5, 67 exons were identified and a 86% of nucleotide sequence similarity with the human HD gene was reported. Comparison between human, mouse and rat HD genes shows the same number of exons (67) as well as the conservation of the majority of the exon boundaries. The length of the exons is highly conserved, while in the regulatory regions is less conserved, indeed the gene is 180Kb in human and 150Kb in both mouse and rat (Barnes, 1994). Although this gene sequence was quite conserved, each species exhibits a different number of CAG repeats. In mouse, 7 CAG triplets are present, while in rat 8 CAG triplets interrupted by a CAA triplet (coding for glutamine, as CAG) in the third position has been observed (CAG)2CAA(CAG)5 (Lin, 1994). The number and the position, within the CAG, of the CAA triplet variants had been suggested to reduce the slippage that creates the mutant allele.

  Comparing coding sequence between mammals, and even more generally between vertebrates, the number of CAG triplets is the main difference. In the majority of the vertebrates, the CAG triplets are present in a number below ten and interrupted by CAA triplets. The gorilla was shown to have (CAG)₆ CAA CAG, and the dog (CAG)₄ CAA (CAG)₅ (Pêcheux, 1996). The only known exception is the pig that exhibits a CAG polymorphic stretch of (CAG)₁₇₋₁₈ CAA (CAG)₂ and a sequence similarity with the human gene of 96%. Together, these studies suggest that the ability to expand larger CAG triplet repeats remains typical of humans. In addition, the murine coding sequence contains also a CCG (coding for proline) polymorphic tract that is only found in mammals, and that in pig is not polymorphic (Matsuyama, 2000).

- Vertebrates: fishes
  The HD gene orthologue in zebrafish (a specie at the base of the vertebrates) shows the same number of exons present in mammals (67). Interestingly, the regulatory regions are reduced and in this case, the gene has 80kb and is 79% identical at the nucleotide level to the human gene. The zebrafish coding sequence has only (CAG)₃ followed by one CAA triplet (only one proline in the zebrafish htt protein).

  As in the human HD gene, also in zebrafish, the promoter region lacks a TATA box, CCAAT box, moreover the Sp1 binding sites present in the human gene sequence are no longer found in zebrafish (Karlovich, 1998). This data indicates that even if HD gene structure was quite conserved in terms of number and length of its exons, the regulatory
sequences, introns and untranslated regions are totally different in terms of nucleotide composition. In fugu (another fish at the base of the vertebrates), the HD gene is still organized in 67 exons but its only 23kb-long that is 7.4-fold smaller than the human gene (170 kb). All 67 exons and the exon-intron boundaries are conserved and the coding region is 69% identical at the nucleotide level. The exons are of a comparable size to the human exons but the intronic regions are considerably smaller, ranging from 47 to 1476 bp in Fugu compared with 131 to 12286 bp in human. The fugu coding sequence contains 2 CAG triplets followed by 2 CAA triplets: (CAG)$_2$ (CAA)$_2$. In a work from Sathasivam and colleagues, the fugu promoter was found to be functional in mouse cells. The gene was transcribed both in vitro and in vivo, revealing a possible conservation of the sequences involved in transcriptional regulation. Transgenic mice for the fugu HD gene were then produced and an analysis across the entire 10kb transcript revealed the presence of many aberrant splice forms that were found to be incompatible with the production of the fugu huntingtin protein. Moreover, the transcript was correctly polyadenilated but was incorrectly processed in mouse cells both in vitro and in vivo resulting in translational problems. These results shed doubt on the usefulness of Fugu genes for transgenesis studies (Sathasivam, 1997).

- Non-vertebrates: fruit fly

From all the completely known htt gene orthologues, the most divergent is the one from the fruit fly (Drosophila melanogaster). The gene has a totally different exonic organization and the number of the exons decreases to 29 with a length that varies from 82 to 1151 nucleotides. Furthermore, the intron-exon boundaries are no longer conserved, even if the total length of the gene is identical to the one of the human gene (170kb) (Li, 1999).

Evidences account for the thought that huntingtin gene is highly plastic and in drosophila this is even more evident. Creating a link between vertebrate’s htt gene orthologues and htt gene in drosophila is still an enduring research.

b) Description of huntingtin messengers from different species

- Vertebrates: mammals

Murine and rat htt messengers show 80% similarity, in terms of the 5’untranslated region (5’UTR), to the human one, and about 60% similarity at the 3’UTR region (Barnes, 1994; Holzmann, 1998). In human, mouse and pig at least two different length of 3’UTR have been identified and this result from the presence of different polyadenilation sites. Both messengers are widely expressed but the longer one is particularly present in the brain (Matsuyama, 2000)

- Vertebrates: fish

In all stages of zebrafish development htt seems to be expressed, in particular in the adult head (Karlovich, 1998). As in mammals, also fish htt exhibits different
polyadenilation sites, two in zebrafish and three in fugu. In this last case, all three resulting messengers have around 10kb and the shortest one is highly present in the fugu nervous system (Sathasivam, 1997).

- **Non-vertebrates: fruit fly**

  In drosophila, htt seems to be also expressed at all stages of the embryonic development and also throughout adult life (as in humans). So far, only one polyadenilation site has been reported and the resulting messenger is 12kb long (Li, 1999).

c) Comparison of huntingtin protein from different species

If the polyglutamine (polyQ) and polyproline (polyP) tracts are excluded, the huntingtin protein is 80% identical from human to fishes. In the case of drosophila, huntingtin is only 49% identical to the human htt protein, and in particular the N-terminal portion of the protein is significantly diverse since it shows an aminoacidic insertion. The human htt protein has 3144 amino acids, while the drosophila protein has 3583 amino acids. The available data raises the possibility of some similar roles for drosophila and vertebrate’s htt. It might also be that htt from drosophila may have evolved in a different direction, developing different activities, particularly concerning the N-terminus part of the protein. Interestingly, htt from drosophila exhibits no polyQ or polyP tracts.

The polyQ seems to be a feature of the vertebrates and its expansion arises only in the most recent species (mammals), in phylogenetic terms. In fact, with an exception observed in pig, htt shows a polymorphic polyQ tract only in human. Even if polyQ is present is almost all the studied organisms, the same is not true for the polyP stretch. An interesting finding is that the longer is the polyQ tract, the longer is the polyP tract. Biochemical properties of prolines seem to help htt to remain in solution by “balancing” the effect caused by the presence of an expanded polyQ region (Bates, 2002).

The comparison of htt protein from different vertebrates species has revealed some other similarities: functionally active calpain cleavage sites, caspase cleavage sites (Gafni, 2002 and 2004; Goldberg, 1996; Wellington, 1998 and 2000 and 2002; Li, 1999). The HEAT repeats have been reported to be present both in diverse vertebrate species and also in drosophila. More research needs to be done in order to clearly demonstrate the number of functionally active HEAT repeats (Takano and Gusella, 2002).
3.3.5.3. Phylogenetic tree and key organisms

Organisms have evolved through the ages from ancestral forms into more derived forms. New lineages generally retain many of their ancestral features, which are then gradually modified and supplemented with novel traits that help them to better adjust to the environment they live in.

Many human genes have originated within the Metazoa at various times during evolution and have been conserved. Those genes that encode proteins and RNAs involved in multicellular functions are most likely to have arisen within the Metazoa — for example, genes involved in morphology, physiology, behavior, and multicellular development, processes that are biological innovations of the Metazoa. A number of these have been implicated in disease; indeed, one viewpoint is that the genes and pathways underlying these innovations that lead to more complex anatomy and physiology are also the ones that are affected by disease in humans.

In terms of protein evolution, evolutionary distances of 500 million years are in some cases too short to produce much useful information about the evolution of sequences of highly conserved domains. The most important changes are usually revealed by comparisons among the genomes of a set of organisms that are genetically distant.

In order to follow huntingtin along species evolution, a number of organisms were selected as they fulfilled two main demands: a) to be genetically distant and b) to present anatomical and/ or physiological characteristics that can be linked to our final aim of revealing huntingtin physiological function. The selected organisms represent key points of species evolution since they carry some type of novelty. In our particular case, the selection was based on species that exhibit innovations related to nervous system organization and neuronal specialization.

Figure 2 is a schematic illustration of a phylogenetical tree showing some of the animals that represent divergence points of species evolution and that represent the tools for our alternative approach to study huntingtin function. Each divergence point (or node) corresponds to a unique set of major changes in biology- development, physiology, morphology, and behavior- and most probably in genome. Animals were divided in Deuterostomes (blastopore never becomes the mouth, but often the anus) and Protostomes (blastopore turns into the mouth). The arthropods (eg: Drosophila), which are included in the Protostomes, represent a very diverse group but share a segmented body and jointed appendixes. On the Deuterostome side, the echinoderms (eg: Sea urchin) are often considered the deuterostome ancestors, showing pentameric body organization with a diffuse nervous system, only locally condensed. As basal chordates, the urochordates (eg: Ciona) possess the main chordate traits, notochord, dorsal hollow nerve cord, gill slits, endostyle and post-anal tail. As a step towards the vertebrates, there are the cephalochordates (eg: Amphioux) that show the same chordate traits as the urochordates, introducing cefalization but still lacking the anatomical and physiological specialization observed in vertebrates. The jawless fish (eg: Lamprey) are considered the basal vertebrates, representing the earliest point at which a “true vertebrate” can be
identified. This is a divergence point where several anatomical and physiological innovations were introduced. Some of the innovations of this group are the presence of peripheral and enteric nervous system, cranial and spinal ganglia, mid and forebrain and also a brain region homologous to the striatum. The ray-finned fish (eg: Zebrafish, Fugu) represent the species that diverged before the conquest of land by the amphibians and latter by mammals (eg. Mouse, Human). (Comparative Genome Evolution Working Group, 2004)

Figure 2: Schematic illustration of a phylogenetic tree with key points of species evolution. Circled species represent the tools for our alternative approach to study huntingtin function.
3.3.5.4. Development of biological assays

The second step of our phylogenetic approach consisted in the development of cell-based assays that could be used as "read-outs" of normal huntingtin function.

The cellular system selected for these studies was the mouse ES hdh +/- (expressing both htt alleles), ES hdh +/- (expressing only one huntingtin allele) and ES hdh -/- ( htt null) cell lines (Duyao, 1995; Nasir, 1995; Zeitlin, 1995).

By taking advantage of the viability of both Hdh null mouse embryonic stem (ES) cells and neurons that are obtained from these cells differentiated in a monolayer type of culture (Ying, 2003), biological assays were developed both under self-renewal (proliferation) conditions and throughout the neural commitment and differentiation process.

Pluripotent mouse embryonic stem (ES) cells can be expanded in culture indefinitely while retaining the capacity to produce every type of fetal and adult cell (Smith, 2001). ES cell differentiation in vitro is thought to recapitulate in vivo development programs (Keller, 1995).

The biological assays were primarily developed in ES hdh +/-, ES hdh +/- and ES hdh -/- ( htt null) cells propagated in self-renewal conditions. Undifferentiated ES cells were kept under stress-induced conditions and their cell survival, cell death (caspase 3 activation, LDH release) and ability to produce BDNF mRNA was assayed.

Since our final aim is to identify huntingtin physiological function in adult brain, the same cells were differentiated to neurons in a monolayer differentiation (Figure 3) culture and normal huntingtin activities were assayed.

Figure 3: Representation of the consecutive lineage restrictions that embryonic stem (ES) cells undergo throughout a monolayer neural differentiation protocol. Both NEP (neuroepithelium) and NS (Neural stem cells) are transient cellular populations present at intermediate stages of ES neural differentiation.
Under monoculture condition, in the absence of leukaemia inhibitory factor (LIF), ES cells lose their pluripotent status and predominantly commit to a neural fate. ES cells undergo consecutive lineage restrictions and a variety of neural precursor populations arise before neurons start to appear. The subsequent step towards neural lineage is the transition into neural progenitors, also known as neural stem cells. This transient population becomes apparent from 5 days after LIF withdrawal when rosettes start to appear. The neural rosette is the developmental signature of neuroprogenitors in cultures of differentiating embryonic stem cells. Rosettes are radial arrangements of columnar cells that express many of the proteins expressed in neuroepithelial cells in the neural tube. Besides exhibiting a similar morphology, neuroprogenitors within neural rosettes differentiate into the main classes of progeny of neuroepithelial cells in vivo: neurons, oligodendrocytes and astrocytes. Subsequently, neurons appear and increasingly populate the culture. It is also important to retain that neuronal differentiation is most efficient if cells are replated at the neural precursor stage, around day 7 from initial plating (See Material and Methods, and Results Sections).

The monolayer differentiation represents an extremely useful tool to dissect huntingtin activities throughout neural determination, since the entire process by which pluripotent ES cells acquire neural specification can be followed and recorded at the level of individual colonies. Moreover, analysis of knockout ES cell lines is particularly useful in cases where a targeted mutation results in embryonic lethality, as is the huntingtin case. Briefly, ES cells undergoing neural commitment and differentiation were tested in terms of: i) survival vs apoptotic-dependent cell death, ii) tendency to maintain self-renewal properties, iii) ability to produce neural progenitors, iv) spatial organization of neural progenitors, v) neurogenic potential, vi) astrocytes quota, vii) BDNF mRNA production and viii) propensity to escape neuronal differentiation. Since Hdh null mouse embryonic stem cells can efficiently produce neurons (Metzler, 1999) it seems that the absence of huntingtin has no impact on these cells neurogenic potential, at least under the tested culture conditions, but instead it seems to affect cell survival and the transient populations of neural progenitors.

3.3.5.5. A step towards huntingtin complementation: The first 548 amino acids of human huntingtin

After checking if the constructs, containing a portion or the full-length of huntingtin isolated from each of the selected animal, were indeed being expressed, and recognized by the available antibodies, in our cellular model (ES hdh -/-), the subsequent step was to produce cell lines that stably express each of the available constructs.

We have started by evaluating the ES hdh -/- N548 human cell line, which stably expresses the first 548 amino acids of (wild-type) human huntingtin. The biological assays mentioned above were applied to these cells, both in undifferentiated and under neural differentiation conditions.
The first 548 amino acids of human huntingtin have been proposed as a functional domain of the protein.

Several proteases, including caspases and calpains, cleave htt within the N-terminal region (Wellington, 1998; Lunkes, 2002; Gafni, 2004) and in vitro studies have demonstrated that expanded N-terminal htt fragments have enhanced cytotoxicity (Hackam, 1998).

It has also been shown that the expression of a truncated form of huntingtin (up to amino acid 548) results in the formation of perinuclear aggregates. Furthermore, this fragment has been consistently identified in transfected cells undergoing stress (Martindale, 1998). When mES hdh +/- and mES hdh -/- cells were transfected with a truncated form of huntingtin (the first 1955 nucleotides, corresponding to the first 548 amino acids) with 15 glutamines, no (in ES hdh +/- cells) or very few (in ES hdh -/- cells) aggregates were observed. In contrast, the frequency of aggregates was increased in both ES hdh +/- and ES hdh -/- cells transfected with the truncated form of huntingtin expressing 128 glutamines (1955-128Q). Once more, the aggregates were found to localize in the perinuclear region of the ES cells (Hackam, 1998).

In a 293T cell model, expression of the first 548 amino acids of huntingtin with an expanded polyglutamine tract (128Q) resulted in higher toxicity than that with shorter tracts (15Q). However, even cells expressing truncated huntingtin containing 15 polyglutamines (corresponding to a normal polyQ tract) have an increased susceptibility to cell death (albeit less than seen with mutant huntingtin), which suggested a role for wild-type huntingtin in the regulation of cell viability (Hackam, 1998; Martindale, 1998). Interestingly, these observations lead to the conclusion that in 293T cells, the expression of the truncated form of huntingtin even with a normal sized polyglutamine tract confers increased susceptibility to cell death from apoptotic stimuli. Similar findings of toxic effects for other wild-type truncated polyglutamine-containing proteins have been reported (Ellerby, 1999).

Work from our group, in immortalized striatal cell lines stably expressing either: i) full-length wt, ii) full-length poly-Q expanded, iii) truncated wt (N548 with 15Q) or, iv) truncated mutant (N548 with 128Q) htt constructs has shown that while mutant htt induces apoptotic cell death, wt htt acts as an anti-apoptotic protein in neural cells. In these immortalized striatal cell lines, wt htt functions as a pro-survival molecule, both the full-length and the truncated form (N548 with 15Q) of the protein. This data highlights a possible neuronal protective activity exerted by the first 548 amino acids of human htt. At the same time, striatal-derived cells overexpressing mutant htt undergo cell death when challenged with various stress stimuli (serum withdrawal, 3-Nitropropionic acid). A worth of note finding was that the truncated versions induce a wider spectrum of cytotoxic events than the full-length proteins (Rigamonti, 2000).

Data produced in different cellular systems suggested that the first 548 amino acids of wt htt can have either a pro-apoptotic (293T cells) or an anti-apoptotic (immortalized striatal cells) activity. One hypothesis for these differences is that the first 548 amino acids of wt htt act indeed as a functional domain but in a cell- and neural
differentiation stage-dependent manner (See Results and Discussion Sections). Taken together, these findings propose a role for the first 548 amino acids of huntingtin in the balance between cell death and survival and eventually in the formation and/or functioning of the mammalian nervous system.
4. General Aim

The purpose of this thesis was to investigate wild-type huntingtin physiologic function, and how loss of normal huntingtin function may be involved in Huntington’s disease pathogenesis.

4.1. Specific Aims

In order to study normal function of huntingtin and explore the possibility that loss-of-function effects may contribute to HD pathology, two different lines of research were followed:

Study of huntingtin function in BDNF gene transcription

Aim I: Analysis of BDNF progressive loss in a Huntington’s disease mouse model.

Aim II: Investigation of BDNF mRNA levels in blood from R6/2 mice throughout disease progression and upon treatment with CEP-1347.

Identification of huntingtin functional domains through a phylogenetic approach:
The first 548 amino acids of human huntingtin.

Aim III: Validation of constructs expressing the N-terminus from huntingtin orthologues in huntingtin-knockout mouse embryonic stem (ES hdh-/-) cells.

Aim IV: Development of read-out assays to identify loss-of-function defects in ES hdh-/- cells.

Aim V: Test ability of the first 548 amino acids of human huntingtin in complementing the defects found in mouse ES hdh-/- cells.
5.1. BDNF gene transcription is reduced in R6/2 mice cortex during HD progression (paper I)

Previous work showed that BDNF mRNA level is strongly reduced in several in vitro and in vivo models of HD. Increasing number of studies account for a consistent reduction of BDNF mRNA levels in HD brain (Zuccato, 2007). Furthermore, wild-type huntingtin was shown to be able to stimulate the production of BDNF by acting at the level of the Neuron-Restrictive Silencer Factor (NRSE), a transcriptional regulatory sequence lying on BDNF promoter II (Fig.4). By doing so, wild-type huntingtin controls BDNF gene transcription in cerebral cortex, which is then delivered to its striatal targets (Zuccato, 2003). Here, we investigated the levels of BDNF mRNA transcribed from BDNF exons II, III and IV in vivo during the progression of HD phenotype. For this, we have used cortical samples from R6/2 transgenic mice that represent the most used transgenic model of HD and express exon-1 of human huntingtin, with 150 glutamines, under control of the human IT15 promoter. These mice are known to develop a progressive neurological phenotype with some features of HD (Mangiarini L, 1996). Symptoms begin at 6 weeks as subtle
neurological dysfunctions which become more prominent at eight-nine weeks and which become progressively worst. The neurological status of the mice declines over time until death, which occurs at 13-14 weeks of age.

BDNF exons II, III and IV mRNA levels were analyzed at pre-symptomatic (1 and 4 weeks of age), early symptomatic (6 and 8 weeks of age) and in symptomatic stages (12 weeks of age) (Fig.5). We did not analyze BDNF exon I mRNA because it is expressed only after kainic acid induction (Metsis M, 1993). At each of the time points indicated, we isolated the cerebral cortex from 2-3 controls and 2-4 HD mice. Semi-quantitative radioactive RT-PCR was performed using primers that specifically recognized each of the three BDNF exon-specific mRNAs.

No differences in the mRNA levels produced from BDNF exon II were observed in cortex from HD and control mice until 4 weeks of age, while a moderate reduction of BDNF exon II mRNA level was found in HD mice at 6 weeks of age. A significant 24% reduction was evident only in 8 weeks old HD mice, compared to controls. In 12 weeks old symptomatic HD mice, BDNF exon II mRNA level showed a 58.7% reduction compared to controls. Analyses of BDNF exon III mRNA level at the same time points showed a different profile of expression. While no difference was observed between HD and control mice at 1, 4, 6 and 8 weeks, a 47.1% reduction was found at 12 weeks in HD mice compared to controls. Finally, when we looked at BDNF exon IV mRNA level, no difference in expression was found between HD and control mice at 1 and 4 weeks of age. However, a statistically significant 29.1% reduction was observed at 6 weeks in HD mice compared to controls. A progressively reduced mRNA level transcribed from this exon was also detected at later time points. In particular, a 35.9% and 60.7% reduction was detected at 8 and 12 weeks of age, respectively, in HD mice with respect to controls.

We concluded that mRNA levels produced from BDNF exons II, III and IV are differently modulated over time in R6/2 transgenic mice and that reduction in BDNF gene transcription occurs in the cortex of R6/2 transgenic mice starting from a pre-symptomatic stage. In particular, BDNF exon IV mRNA level was the first to be affected (at 6 weeks) while a significant 24% reduction was evident for exon II mRNA only at the 8 weeks. On the basis of this study we conclude that the beginning of the symptoms in R6/2 mice is accompanied by a significant reduction in total cortical BDNF gene transcription. The different kinetics of down-regulation of the three BDNF mRNAs analyzed might be explained by the different ability of wild-type and mutant huntingtin to influence, over time, the transcriptional complexes that regulate their expression.
5.2. Depletion of BDNF mRNA in blood from R6/2 mice follows disease progression and is restored by CEP-1347 treatment (paper II)

Here we measured total BDNF mRNA levels by Real-Time PCR in blood from R6/2 HD mice. Our analysis revealed that total BDNF messengers level in peripheral blood from R6/2 mice is much lower than the levels found in brain, which was expectable since BDNF is a neurotrophic factor. Total BDNF mRNA level was analyzed at pre-symptomatic (2 and 4 weeks of age), early symptomatic (6 and 8 weeks of age) and in symptomatic stages (12 weeks of age). For each time point, blood from 6 WT and 6 R6/2 mice was analyzed (Fig.6B). Results from Real-Time PCR for total BDNF messenger levels in blood from R6/2 mice at different stages of the pathology showed that BDNF mRNA level decreases over disease progression. This data suggests that mutation in huntingtin interferes with BDNF messengers level in blood from R6/2 mice and that the observed reduction parallels the well-documented decline of BDNF mRNA level in HD brain. To investigate if we could monitor the predictable reduction of BDNF mRNA level over disease progression (Kinetic study), we recovered blood from 3 WT and 3 R6/2 mice. Each mouse was followed over time and blood was collected at 4, 8 and 12 weeks. BDNF mRNA level in blood from each of these animals was assayed and results were plotted over time in the same mouse (Fig.6B, 7A). Results clearly show that BDNF transcript follows the exact same trend as observed in our initial findings. By following 3 WT mice over time, we confirmed that the maximum BDNF mRNA level is observed at 8 weeks and that at 12 weeks this level slightly decreases. Results from all 3 R6/2 mice gave further strength to the progressive reduction of BDNF mRNA in blood.

To further address the mechanism of BDNF transcriptional dysregulation in HD periphery, particularly in blood, we conducted qualitative PCR for each of the BDNF messengers and checked for their expression in blood from WT mice (Fig.7A). Our results point out that only BDNF messenger III is expressed in mice blood. It is possible that other messengers accounting for total BDNF mRNA are expressed in mice blood, but we could only detect messenger III even when using maximum amount of material. For BDNF messenger III we further confirmed that its expression is compromised in blood from R6/2 mice, when compare to age-matched control licttermates (Fig.7B).

In this study we had also the opportunity to examine total BDNF mRNA levels in mice blood, by Real-Time PCR, from a CEP-1347 acute (4 hours) and chronic (4 weeks) treatment study recently performed in R6/2 mice. For each tested condition, blood from 6 mice was analyzed (Fig.8). Consistently with our initial findings, we observed a significant down-regulation of BDNF in R6/2 untreated (vehicle only) mice when compared to untreated WT mice. Analysis of acute CEP-1347 treated WT animals revealed a non-significant increase of total BDNF messenger, when compared to untreated WT mice. The same treatment caused a significant 3.5-fold increase of total BDNF messenger levels in R6/2 mice treated with CEP-1347, when compared to untreated R6/2 mice. This result point out that CEP-1347 acute treatment had no effect on WT mice but it enabled BDNF mRNA level to be restored in R6/2 mice. Real-Time PCR was performed for evaluating the
levels of BDNF mRNA in blood from chronic treated animals. Results confirmed the ability of this compound to increase levels of the BDNF transcript.
6.1. Validation of expression constructs containing the N-terminus from huntingtin orthologues.

In this section, results from the Phylogenetic approach will be presented. As a step towards the understanding of wild-type huntingtin function, huntingtin orthologues were isolated and a list of expression constructs became available. The ability of these constructs to be expressed in our selected cellular system, the mouse embryonic stem (ES) huntingtin-depleted cells, was tested by transient transfection followed by immunostaining analysis of huntingtin expression.

**Huntingtin expressing constructs.** Expression constructs were designed at both the N- and C-terminus of huntingtin, but in the present thesis only the first ones will be discussed (Fig.9). Figure 10A is a schematic phylogenetic tree where the animal species selected for our evolutionary approach are circled in red. From these species, we have collected RNA and in some cases isolated the huntingtin orthologues. The available huntingtin expression constructs are listed in figure 10B.

**Cellular system used for testing the expression of huntingtin constructs.** For validating the expression of huntingtin constructs we used the huntingtin-depleted mouse embryonic stem (ES hdh-/-) cells. Figure 11A shows representative contrast photos of the mouse ES cells expressing both (ES hdh+/+), only one (ES hdh+/-) and none (ES hdh-/-) of the huntingtin alleles. Huntingtin expression was tested by Western blot (Fig.11B) and by immunostaining (Fig.12) with the anti-huntingtin monoclonal antibody- mAb2166. Two immortalized striatal cell lines overexpressing full-length (ST Clone 9.58) and a truncated form (ST Clone 7.36) of huntingtin were used as positive control for huntingtin immunostaining. ES hdh-/- cells were considered as negative control.

**Validating huntingtin constructs in ES hdh-/- cells.** Before testing the newly produced expression constructs we have transfected by nucleofection the pCI-N548 construct, which contains the first 548 amino acids of human huntingtin. Since cell lines stably expressing the pCI-N548 construct have been produced, we used it as a positive control of transfection method. Co-nucleofection of pCL-N548 and a GFP-containing construct into mouse ES hdh-/- cells was performed. After 36 hours from nucleofecting both constructs, cells were fixed and double immunostained with anti-huntingtin and anti-GFP antibodies (Fig.13A). Approximately 50% of the cells were positive for both constructs, meaning that nucleofection could be used as transfection method with the selected cell system. We moved on testing the pcDNA3.1 N548 mouse construct, containing the first 548 amino acids of mouse huntingtin and the same methodology was followed. In this case, approximately 40% of the cells were positive for both pcDNA3.1 N548 mouse and the pAcGFP construct (Fig.13B). The next step was to test a construct containing the N-terminal portion from an ancient specie- the sea urchin. The pcDNA3.1 N519 sea urchin construct was co-nucleofected with the pAcGFP construct and double
immunostaining was performed. Approximately 40% of the cells stained positively for GFP but no huntingtin-positive (red) cells were found (Fig.14A). To exclude that this result was not due to a problem at the transcription of this construct, RT-PCR was done on nucleofected cells and results confirmed the sea urchin construct was being transcribed. These results indicate that the construct has been successfully transfected and transcribed but most probably i) the anti-huntingtin antibody did not recognize sea urchin huntingtin or ii) maybe the construct was not being correctly translated. Since the commercially available antibodies for huntingtin have only been tested against this protein from mammalian origin and we planned on testing constructs containing huntingtin from non-mammalian species, HA-huntingtin fusion constructs were produced. By following this strategy, the expression of constructs containing the N-terminal of huntingtin from ancient species could be done by staining transfected cells with an antibody against the selected tag.

The pcDNA3.1 N519 sea urchin construct was fused to a HA-tag and its expression was tested. Immunostaining with anti-HA revealed that few cells presented a positive signal (Fig.14B). These cells had a stressed-like morphology and in most of the cases exhibited apoptotic nuclei. Moreover, in some of the HA-positive cells a sort of aggregate could be seen in the perinuclear region of the cell, as a brighter red dot, in HA-staining image, or as a dark spot in the respective phase contrast image (Fig.14B).

Further effort is being done for optimizing the expression of the constructs containing the N-terminus fragment of sea urchin huntingtin. Different expression vectors, with diverse promoters, are being tested and the same approach will be followed for all the other constructs designed for expressing huntingtin from the selected ancient species.
6.2. Development of read-out assays to identify loss of huntingtin function defects in ES hdh-/- cells.

In this section, results from the Phylogenetic approach will be presented. Cell-based assays were developed to identify differences between cells expressing both (hdh+/+), only one (hdh+/-) and no (hdh-/+) of huntingtin alleles. Read-outs of huntingtin activities in these cells were designed both under self-renewal and monolayer neural differentiation conditions.

**Undifferentiated huntingtin-depleted mouse ES cells are more vulnerable to serum-deprivation than wild-type mouse ES cells.** Under proliferation conditions, MTT reduction to formazan was used as an index of cell viability. The growth rate of huntingtin depleted mouse embryonic stem cells was compared to the one of wild-type mouse embryonic stem cells over time under basal proliferation conditions. The resulting growth curve of undifferentiated ES cells expressing both (ES hdh+/+), only one (ES hdh+/-) and none (ES hdh-/-) huntingtin alleles reveals that the absence of huntingtin does not impair the growth rate within the first 48 hours of culture. Only after 48-96 hours, in proliferation medium, huntingtin-depleted ES cells start to show a lower capacity to reduce MTT, meaning that less viable ES hdh-/- cells were present at those time points, when compared to both ES hdh+/+ and ES hdh+/- cell lines (Fig. 15B). The same assay was repeated with cells kept in proliferation media but deprived of serum for up to 96 hours. It is important to note that under this condition of serum deprivation, none of the cell lines undergo differentiation.

Under this stress-induced condition, the growth curve of all three cell lines was affected, but the number of viable huntingtin depleted cells was significantly lower than that of wild-type cells (Fig.15C).

To determine if the absence of huntingtin affects cell death in undifferentiated ES cells, LDH release into the medium was measured after 12, 24, 36 and 48 hours of culture. Only after 48 hours, cell death was observed as all three cell lines started to release LDH into the medium. For each time point, cell lines were compared and a significant increase in cell death extend was observed both in ES hdh+/+ and ES hdh-/- cells when compared to wild-type ES cells (Fig.16B). Serum deprivation resulted in an earlier release of LDH in ES hdh-/- cells. After 24 hours in serum-deprived medium, ES hdh-/- cells showed higher cell death when compared to both wt and ES hdh+/+ cells. After 36 hours of serum deprivation, both ES hdh+/+ and ES hdh-/- cells show significantly greater LDH release when compared to wt cells, and no significant difference was observed between cell lines following 48 hours of serum deprivation, as the majority of the cells, in all three cell lines, was dying (Fig.16C). Consistent with the compromised ability to reduce MTT, and to the higher release of LDH into the medium, ES hdh-/- cells show a greater increase of caspase-3 activity, following 6 hours of serum deprivation, when compared to wild-type cells (Fig.17). These findings clearly indicate that huntingtin-depleted mouse embryonic stem cells (ES hdh-/-) are particularly vulnerable to serum deprivation.
A mitochondrial complex II inhibitor does not affect viability of Huntingtin-depleted mouse ES cells. To determine if in the absence of huntingtin and under proliferation conditions, ES cells were selectively vulnerable to mitochondrial complex II inhibition, ES wt, hdh +/- and hdh -/- cell lines were treated with 3-NP, an irreversible mitochondrial complex II inhibitor. Treatment with three different doses (1, 5 and 10mM) of 3-NP for 24 hours (Fig.18B) resulted in a modest decrease in cell viability, but no difference between cell lines was observed. Treating cells with same doses of 3-NP but for 48 hours resulted in a significant decrease in cell viability but still all three cell lines behaved similarly (Fig.18C). These results suggest that the well-documented mitochondrial impairment described in several HD models, might be exclusively due to a gain of function from mutant huntingtin and independent from loss of function of wild-type huntingtin, at least in this cell type.

Undifferentiated huntingtin-depleted mouse ES cells produce less BDNF mRNA than wt ES cells. Real-time PCR analysis of BDNF level in undifferentiated ES cells revealed that although the amount of transcript present in these cells is very low, there was a significant decrease of BDNF mRNA level in both ES hdh+/- and hdh-/- cells with respect to ES hdh++/ cells (Fig.19).

Throughout ES cells monolayer neural differentiation, total number of cells is lower in huntingtin-depleted monolayer cultures. Huntingtin is expressed during neurogenesis in developing mouse embryos (Bhide, 1996). In order to define if huntingtin depletion could be involved in aberrant cell proliferation or had an effect on developmental programmed cell death we have analyzed cell survival, caspase-3 activation and cell division throughout neural differentiation of mouse ES hdh+/- and hdh-/- cells, and compared them to ES hdh++/ cells. To investigate the impact of huntingtin depletion in the ability of ES cells to differentiate into neural progenitors, and subsequently into neurons, we applied a monolayer neural differentiation protocol to ES hdh++/, hdh+/- and hdh-/- cell lines. Over 21 days, cells are kept under neural induction conditions (Fig.20A). For some experiments, after the first 7 days of neural induction, a replating step was performed. Neuronal differentiation has been shown to be most efficient if cells are replated at the neural precursor stage (around day 7). Replating step includes counting cells and this revealed that total cell number in hdh-/- monolayer cultures were repeatedly lower when compared to wt and hdh +/+ monolayer cultures (Fig.20B). Total cell number was evaluated by dissociation and counting of monolayer cultures at different days of monolayer neural differentiation (Fig.21A). Parallel cell counts were performed with Trypan blue and with Cell counter. Both counting methods showed a reduced total number in hdh+/- and to a greater extend in hdh -/- compared to wild-type monolayer cultures (Fig.21B, Graphics 1 and 2). When the replating step was performed the difference, in terms of total number of cells between wt, and hdh +/- and hdh -/ monolayer cultures became even more striking (Fig.21B, Graphics 3 and 4).
**Hdh -/- monolayer cultures show higher caspase 3 activation and lower quota of mitotic cells.** To test if the lower number of cells in huntingtin-depleted monolayer cultures was associated with higher apoptotic cell death, western blot for active caspase 3 was performed at different time points of neural differentiation (Fig.22A). Quantification of the results revealed that in the absence of huntingtin (hdh -/- monolayer culture) there is higher caspase-3 activation both in early (Day 2) and late (Day 21) stages of neural *in vitro* differentiation, when compared to wt monolayer cultures. At the end of the differentiation and particularly when the replating step is performed, huntingtin dose is inversely proportional to caspase-3 activation (Fig.22B). These results clearly show that expression of huntingtin has an anti-apoptotic effect during monolayer neural differentiation. Another possible explanation accounting for the reduced number of cells in the absence of huntingtin was that fewer cells were undergoing mitosis during the initial stage of neural precursors formation. To check this hypothesis, cultures were fixed at the forth day of monolayer differentiation and stained for Phospho histone H3, a marker of mitotic cells. We found that indeed the quota of mitotic-positive cells was lower in hdh -/- compared to wt monolayer cultures (Fig.23). These results suggest that lack of huntingtin affects programmed cell death and may be also involved in altered cell proliferation.

**Following conversion of huntingtin-depleted ES cells into neurons in adherent monoculture.** Throughout monolayer neural differentiation, ES cells suffer sequential transitions into neuroepithelial progenitors (NEP), then into radial glia and finally into neurons and glia. In order to investigate how huntingtin is involved in ES neural differentiation, how it may affect proliferation of neural precursors cells and cell viability through different developmental stages, we selected a series of key marker genes that are associated with each transient cellular population and compared differences between hdh +/+ , hdh +/- and hdh/- monolayer cultures. Markers analyzed will be referred in the following order: Oct4 (pluripotent cells), Nanog (pluripotent cells), Nestin (radial glia), BLBP (mature neuroprogenitors), β-III tubulin (early neurons), MAP2 (mature neurons), NeuN (mature neurons), GFAP (astrocytes) and Cytokeratin (non-neural differentiated cells) (Fig.24).

**Huntingtin-depleted monolayer cultures tend to retain undifferentiated cells throughout neural differentiation.** Under self-renewal (proliferation) culture conditions, in the presence of LIF and serum (Day 0), ES hdh +/+ , +/- and -/- cells show no overt difference in growth rate or undifferentiated morphology, and all three cell lines express pluripotency markers such as Oct4 and Nanog (Fig.25B Day 0). Following LIF and serum withdrawal, and under neural differentiation conditions, cells loose the expression of these two key markers of the pluripotent state but throughout the 21 days of ES monolayer differentiation, a minor population of cells resists neural differentiation and persists as undifferentiated ES cells. Interestingly, we found that in the absence of huntingtin, more cells kept their pluripotency, as more cells stained for Oct4 after 7 and 14 days of neural differentiation conditions (Fig.25B- Day 7 and 14NR). Moreover, when the replating step
was performed at day 7, almost no Oct4 positive cells were observed in hdh+/+ and +/- monolayer cultures after 14 days, but in hdh-/- monolayer cultures groups of Oct4-positive cells were still present (Fig.25B- Day 14R). RT-PCR analysis confirmed that hdh-/- monolayer cultures tend to retain undifferentiated cells, as higher levels of Oct4 and Nanog transcripts were found after 7 and 14 days of neural induction (Fig.25C). Collectively these data indicate that huntingtin may promote initial stages of ES cell differentiation and that in its absence more cells keep their undifferentiated state.

**Smaller and fewer neural rosettes are generated in the absence of huntingtin.** The neural rosette is the developmental signature of neuroprogenitors in cultures of differentiating embryonic stem cells. Rosettes structures are composed of radially oriented bipolar Nestin+ cells (NEP). While undifferentiated ES cells do not express nestin, neural rosettes express this marker gene and we captured its expression after 7 and 14 days of neural differentiation conditions. Rosettes were much larger in wt than in hdh+/+ and hdh-/- monolayer cultures. Moreover the structure of the rosette was remarkably more organized in wt than in hdh+/+ and hdh-/- monolayer cultures. In huntingtin-depleted cultures, cells forming the rosettes were not always radially aligned (Fig.26B). Moreover, when cells were dissociated and replated, the quota of nestin-positive cells after 14 days (Day 14R) was lower than in non-replated monolayer cultures (Day14NR) (Fig.26B). Indeed, when cells are replated at the neural precursor stage, neuronal differentiation is driven more efficiently. Cultures were fixed at day 7, stained for nestin, and a quantification of small, big and total number of rosettes was done in 30 fields from 3 independent monolayer experiments (Fig.26C). Total number of rosettes was similar in hdh +/+ and +/- monolayer cultures but significantly lower in hdh -/- monolayer cultures. Although the total number was similar between hdh +/+ and hdh +/- cultures, the size of the rosettes was diverse. While big rosettes were 3 times more frequent in hdh +/- than in both hdh +/+ and hdh -/- monolayer cultures, small rosettes were particularly present in hdh +/- cultures. This observation led us to hypothesize that in the presence of only one allele of huntingtin, differentiation of monolayer cultures was being delayed. To further investigate this hypothesis, monolayer cultures were fixed at day 5 and day 7 and immunostained for nestin and phospho-histone H3 (Fig.26D). In agreement, morphology and staining exhibited by hdh +/- at day 7 of neural differentiation was similar to the one observed at day 5 in hdh +/- monolayer cultures indicating that half-dose of huntingtin may be correlated with a delay in neural differentiation.

**ES cells can differentiate into mature neurons and astrocytes in the absence of huntingtin.** As previously demonstrated (Metzler M, 1999), both neurons and astrocytes can be generated from huntingtin-depleted ES cells. Also following monolayer neural differentiation protocol, neurons were detected in control (hdh +/+), hdh +/- and hdh-/- monolayer cultures by immunostaining with β-III tubulin at 7 and 14 days after initial plating (Fig.27B). At day 7, neurons were observed in all monolayer cultures analyzed, but their presence was higher in control monolayer culture. No obvious differences were found
after 14 days ("No Replate") of neural differentiation between the different genotypes. Even when the replating step was carried out, no differences in neurogenic potential were observed (day 14 "Replate"). These results were further confirmed by western blot analysis of β-III tubulin (Fig.27C). Consistently with immunostaining results, at day 7, control monolayer cultures have a higher content of β-III tubulin protein (early neurons) respect to huntingtin-deficient cultures, but this is no longer true at later stages (day 10) of differentiation of ES cells. Not only early (pre-synaptic) neurons, but also more mature (post-synaptic) neurons can be produced in the absence of huntingtin. After 17 days from initial plating, monolayer cultures were fixed and stained for MAP2 and NeuN, two markers of mature neurons (Fig.28B). No significative differences were observed in morphology and number of mature neurons between control and hdh+/− and hdh -/- monolayer cultures. Density of neurons developed in hdh -/- monolayer cultures was similar to control cultures. Immunostaining with anti-GFAP revealed that astrocytes were absent at the seventh day in all monolayer cultures, but after 14 days of differentiation similar quota of astrocytes was observed in control, hdh +/− and -/- monolayer cultures (Fig.29B). Replating step reduced the appearance of astrocytes in all monolayer cultures. Taken together, these data indicate that in our ES monolayer neural differentiation model, the absence of huntingtin does not seem to down-regulate neurogenesis.

**Huntingtin suppresses non-neural differentiation.** Throughout the 21 days of ES monolayer differentiation, a population of cells escapes neural differentiation as they no longer expresses ES cell markers and lack neural markers. These cells appear around day 6 and exhibit a large, flat, non-neural differentiated morphologies, as can be seen in phase contrast photos of figure 30B. Many of these cells express cytokeratin 8, a differentiation marker that is not expressed in ES cells or neural lineages (Lowell S., 2006). Immunostaining for cytokeratin was done on monolayer cultures fixed at day 7 and 14, both in non-replated, and replated, cultures (Fig.30B). Replating step is known to be crucial for the elimination of these large and flat cells from neural monolayer cultures, indeed these cells are usually considered “contaminants” of cultures undergoing in vitro neural differentiation. Results from immunostaining show that non-neural cells are present already at day 7 in all monolayer cultures. After 14 days and when cells are not replated, non-neural cytokeratin-positive cells become highly present, especially in hdh +/- and -/- monolayer cultures. The presence of these “contaminant” cells is significantly reduced when cells are replated, but still in hdh-/- monolayer cultures their presence can be observed, in marked contrast to the barely detectable cytokeratin immunoreactivity levels in control and hdh+/− monolayer cultures. Western blot analysis of protein lysates prepared at the same time points confirmed the staining results (Fig.30C). Once more, replating step almost eliminated non-neural differentiated cells from hdh +/- and +/- cultures, but in huntingtin-depleted monolayer cultures non-neural differentiation still occurred. These observations suggest that huntingtin suppresses in some way non-neural differentiation and that in the absence of huntingtin cells tend to escape neural differentiation.
Lower BDNF mRNA level in huntingtin-deficient neural monolayer cultures.
Quantitative RT-PCR for BDNF throughout neural differentiation revealed that in the absence of huntingtin the level of this transcript is lower respect to wt cultures (Fig.31). At day 14, along with the appearance of the first mature neurons, a striking increase of BDNF transcript is observed in control monolayer cultures. Although BDNF mRNA in hdh +/- and -/- cultures increases between day 10 and 14, the levels are significantly lower than the ones in control cultures. At day 17 and 21 a decrease of BDNF transcript is registered, and this might be due to the senescence of the cultures at these latter stages of monolayer differentiation.
6.3. Complementation assays in mouse ES hdh -/- cells expressing the first 548 amino acids of human huntingtin.

A first step towards complementation of hdh/- cells was done by stably expressing the first 548 amino acids of human huntingtin (ES hdh/- N548 hu) and by testing which defects of huntingtin - knockout ES cells were rescued by this huntingtin potential (N-terminal) domain (Fig.32A and B).

Checking huntingtin expression in ES hdh -/- N548 human huntingtin cells. To check for huntingtin expression in the ES hdh/- N548 human huntingtin cell line, immunostaining and western blot analysis was performed with the widely used mAb2166 antibody (Fig.33A and B). As positive controls of the immunostaining, we used two immortalized striatal cell lines stably overexpressing huntingtin full length (Fig.33A, image i) and stably overexpressing the N548 construct (Fig.33A, image ii). As negative control of huntingtin expression, ES hdh/- cells were used (Fig.33A, image iii). Western blot analysis confirmed huntingtin expression in all 4 ES hdh -/- N548 human huntingtin clones (Fig.33B).

Undifferentiated ES hdh -/- N548 human huntingtin cells behave similarly to huntingtin-depleted ES cells. Under self-renewal growth conditions, ES hdh -/- N548 human huntingtin cells although slightly smaller, show a morphology that is similar to the one exhibited by their parental cells (ES hdh/- cells) (Fig.32B). Caspase-3 activity was measured in cells kept in basal and serum-deprived conditions for 6 hours. Serum deprivation led to an increase of caspase-3 activation in all three cell lines, but the highest activation was recorded in the ES hdh/- N548 hu cells (Fig.34A). This result was further confirmed by western blot analysis for cleaved caspase-3, an index of caspase activation (Fig.35B). Together, these results indicate that under proliferation conditions, undifferentiated ES cells expressing the first 548 amino acids of human huntingtin behave similarly to huntingtin-depleted ES cells. The ability of this portion of human huntingtin to rescue cell death, induced by serum-deprivation, in undifferentiated ES cells is limited.

N548 portion of human huntingtin affects cell viability throughout neural differentiation of ES cells. To investigate if the N-terminus of human huntingtin could rescue the defects identified in neural differentiation of ES hdh/- cells we have applied the same 21 days-monolayer differentiation protocol to ES hdh/- N548 human cells. Total cell number was assessed by dissociation and counting of monolayer cultures at different days of neural differentiation. Both Trypan blue (graphics 1 and 3) and Cell coulter count (graphics 2 and 4) showed that total cell numbers in hdh/- N548 human monolayer cultures fell between hdh+/ and -/- monolayer total cell numbers. Moreover, replating step did not influence this result (Graphics 3 and 4) (Fig.35). We then evaluated if caspase-3 activation was affected throughout neural differentiation by the presence of the N-terminal fragment of human huntingtin (Fig.36). A neuroprotective role for N-terminus
fragment of human huntingtin has been suggested in adult brain cells (Rigamonti et al, 2001), but this same portion has been shown to be proapoptotic in 293T cells (Hackman et al, 1998). Western blot for cleaved caspase-3 revealed that apoptosis was significantly higher in hdh/-/- N548 human monolayer cultures throughout the initial transitions of ES cells into neuroepithelium (days 0-2), respect to wt monolayer cultures. Only after 21 days of neural differentiation, when mature neurons represent the prevalent cellular population, -/- N548 human monolayer culture shows a lower caspase-3 activation, respect to huntingtin-depleted monolayer cultures. These results confirm that the first 548 amino acids of human huntingtin have a role in cell viability but this activity is most possibly cell- and developmental stage-dependent. Together this data gives further strength to the initial hypothesis that the N-terminal portion of human huntingtin has a protective role only in adult brain cells.

**Hdh-/— N548 human monolayer cultures also maintain undifferentiated cells but replating step eliminates them.** As observed in hdh-/-, also hdh-/- N548 hu monolayer cultures showed a tendency to keep groups of Oct4-positive cells, after 7 and 14 (in non-replated cultures) days of differentiation (Fig.37). When replating was carried out on the seventh day of differentiation, pluripotent (ES) cells were no longer observed at later days of neural differentiation (day 14 in replated cultures). These results indicate that the N-terminal portion of human huntingtin is not as efficient as full-length huntingtin (hdh+/+) in promoting initial stages of ES cell differentiation, as some cells keep their undifferentiated state throughout neural differentiation. Interestingly, opposite to what happens in hdh/- monolayer cultures, where even when replating step was performed, Oct4-positive cells tended to reappear, replating of hdh/- N548 hu monolayer was able to eliminate most of these ES (Oct4-positive) cells.

**Neural rosettes appear earlier in wt and Hdh-/— N548 human monolayer cultures.** First rosettes appeared after 4 to 5 days of neural differentiation in hdh+/+ (wt) and hdh-/— N548 human monolayer cultures while in hdh+/— and -/- the first rosettes could only be observed after 6 to 7 days. Moreover, the structure of the first hdh-/— N548 hu rosettes was remarkably similar to the wt one (Fig.38B). Hdh-/— N548 human cultures were fixed at days 7 and 14 (from both non- and replated monolayer cultures) and nestin staining confirmed the presence of radial glia organized in rosettes structures (Fig.38C). Consistent with the earlier appearance of rosettes we found significantly higher levels of nestin transcript in hdh+/+ and N548 hu monolayer cultures at day 5 of differentiation by Real-Time PCR (Fig.38D). For Real-time PCR analysis, monolayer cultures were not replated and this may account for the late (day 14) presence of nestin transcript.

**ES hdh-/— N548 human cells can differentiate into mature neurons and astrocytes.** Under monolayer neural differentiation conditions, ES hdh-/— N548 human cells can efficiently differentiate into neurons and into astrocytes (data not shown). Since huntingtin complete depletion did not impair the generation of neurons and astrocytes, the
ability of ES hdh/- N548 human cells to efficiently differentiate into neurons and astrocytes was expectable.

**The first 548 amino acids of human huntingtin suppress non-neural differentiation.** Monolayer cultures obtained from neural differentiation of mouse ES hdh+/+, +/-, -/- and hdh/- N548 hu cells were fixed and immunostained for cytokeratin at days 7 and 14, both in non-replated, and replated cultures. Interestingly, no cytokeratin-positive cells were found at day 7 in the hdh/- N548 hu cultures and very few could be observed after 14 days of neural differentiation in a non-replated monolayer culture. Cytokeratin staining at day 14 of replated hdh/- N548 hu cultures revealed a total elimination of these non-neural differentiated cells (Fig.39B). This result was additionally verified by western blot analysis of cytokeratin expression in monolayer cultures at days 7 and 14 (non- and replated). Not only the signal for cytokeratin in hdh/- N548 hu protein lysates was completely absent at day 7 as it was weak and almost non-existent at day 14 non- and replated, respectively (Fig.39C). Taken together, these results highlight an ability of huntingtin to suppress non-neural differentiation an ability that is lost in the absence of huntingtin, and that the N-terminal portion of human huntingtin can outstandingly rescue.

**Expression of human huntingtin N-terminal may rescue hdh/- low BDNF levels only at late stages of neural differentiation.** Real-time PCR for BDNF throughout neural differentiation of hdh/- N548 hu monolayer cultures revealed that expression of this huntingtin portion is not sufficient for restoring BDNF mRNA level. Almost up to the end of monolayer differentiation, hdh/- N548 hu cultures show BDNF levels similar, or even lower than the ones of huntingtin-depleted cultures. At the end of neural differentiation (day 21), a modest increase of BDNF mRNA level is observed in hdh/- N548 hu, respect to hdh/- cultures, reaching the hdh+/+ BDNF level. (Fig.40).
Huntingtin is a 3144 amino acids protein characterized by the presence of a polyglutamine tract on its amino-terminal portion. The abnormal expansion of this polyglutamine tract is known to be the cause of Huntington’s disease, a progressive neurodegenerative disease. The recognition of HD as a dominant disorder has attracted most of the research attention towards mutant form of huntingtin. Even if the toxicity elicited by the polyglutamine expansion in mutant huntingtin is the disease-triggering event, it does not completely clarify all HD features. In fact, the polyQ-associated toxicity fails in giving an explanation for the selective striatal vulnerability observed in HD. In this context, loss of wild-type huntingtin function studies appeared as an alternative approach to solve some of the unclear features of the disease (Cattaneo E, 2001). Furthermore, in order to fully understand the consequences of wild-type depletion, it is critical to identify its physiological function. The study of wild-type huntingtin normal function will hopefully lead to a better knowledge of this protein functional domains and consequently to the understanding of which roles are lost in the presence of the mutation. Restoring wild-type huntingtin beneficial activities may eventually contribute to HD cure. Strategies aimed at inhibiting depletion of wild-type huntingtin (caspase inhibitors or gene therapy) or restoring huntingtin function (BDNF) merit evaluation as therapy aimed at interfering with HD progression.
Studies from Rigamonti and colleagues have provided the first direct evidence that wild-type huntingtin is important for the survival of CNS-derived cells, as it protects neuronal cells from apoptotic death. In vitro studies revealed the pro-survival effect of wild-type huntingtin in cells expressing a N-terminal fragment of human wild-type huntingtin containing 548 amino acids or the full-length wild-type protein. In contrast, cells expressing the huntingtin fragment comprising a N-terminal portion of 63 amino acids were no longer protected, as they showed the same survival profile as parental cells. This data suggests that the protective effect of wild-type huntingtin requires a portion of the protein between amino acids 63 and 548 and more importantly that huntingtin might be organized in multiple domains with different activities. Interestingly, no protection was observed when wild-type huntingtin was expressed in peripheral cells. (Rigamonti D., 2000; Rigamonti D., 2001). Consistently with these results, expression of the truncated (N548) form of human wild-type huntingtin in 293T (non-neural) cells led to an increased susceptibility to cell death from apoptotic stimuli (Hackman A., 1998). Taken together, these results indicate that the anti-apoptotic function of huntingtin is most probably brain-specific.

Further studies demonstrated that wild-type huntingtin prevented caspase-9 processing thus reducing caspase-3 activation (Rigamonti D., 2001). It was also proposed that the anti-apoptotic neuroprotective effect occurred through the sequestration of Hip-1 (Huntingtin-interacting protein 1), a pro-apoptotic molecule containing a novel death-effector domain (Hackman A., 2001).

A work performed in a huntingtin conditional knock-out mouse in which both copies of the huntingtin gene were switched off in adult mice. When huntingtin gene was inactivated, mice stopped producing huntingtin protein and this conducted to the development of severe brain damage. Studies on this conditional HD mouse model demonstrated that depletion of wild-type huntingtin in postnatal stages lead to neuronal degeneration, motor phenotypes and early mortality, features that were similarly reported in transgenic animal models overexpressing mutant form of huntingtin (Dragatsis I., 2000). Additional evidences established that the expression of wild-type huntingtin in vivo was able to protect against the pro-apoptotic effects induced by mutant huntingtin (Leavitt, 2001).

The discovery that wild-type huntingtin has a neuroprotective role, which is somehow impaired in the presence of the mutation, opened the possibility that loss of wild-type huntingtin function may contribute to HD (Cattaneo E., 2001).

All the described findings show that wild-type huntingtin exerts a beneficial effect in adult brain but are unsuccessful in explaining the selective striatal vulnerability observed in HD. An important step towards the comprehension of how wild-type huntingtin neuroprotective activity in adult brain may be linked to HD striatal selective vulnerability came out from the work of Zuccato and colleagues. Wild-type huntingtin was shown to be able to stimulate the transcription of BDNF (Zuccato C, 2001), a crucial growth factor for the development and survival of neurons in the striatum. Importantly, BDNF production in striatal neurons is minimal and for this reason, striatum is particularly dependent on the cortical produced BDNF, which is then delivered via the cortico-striatal afferents (Altar CA,
Analysis of BDNF protein levels in CNS cells overexpressing full-length wild-type (wt) or mutant (mu) huntingtin revealed that BDNF increased in wt-cells, while mu-cells exhibited a significantly decreased production and release of BDNF protein. When the same measurements were conducted in 3T3 (non-neural) cells, no changes in BDNF production were observed. Moreover, decreased protein production in mu-cells was shown to be the consequence of a reduced BDNF gene transcription. It was subsequently demonstrated that both BDNF messenger and protein level was increased in a HD mouse model expressing human full-length wild-type huntingtin (YAC18) and that in mice expressing human full-length mutant huntingtin (YAC72), levels were significantly lower. These results were further supported by the analysis of BDNF mRNA and protein in fronto-parietal cortex from HD brains. Decreased cortical BDNF mRNA levels were found in the presence of the mutation leading to a reduced BDNF cortical trophic support to striatal neurons, contributing to the striatal selective vulnerability (Zuccato C, 2001). The initial finding that normal huntingtin was able to stimulate BDNF gene transcription/protein production has prompted the analysis of BDNF levels in the cortex and striatum from different transgenic HD mice models and from HD patients. BDNF mRNA levels were shown to be reduced in the cortex of transgenic mice from early pre-symptomatic stages (Hermel E, 2004). Reduced BDNF protein and mRNA levels were confirmed in the brain of R6/2 mice (Zhang Y, 2003; Luthi-Carter R, 2002a). Moreover, both a HD mouse model expressing a 517 amino acid N-terminal portion of huntingtin with 82 polyQ repeats (Duan W, 2003) and also a mutant huntingtin knock-in mouse model showed reduction in cortical BDNF mRNA and protein levels (Gines S, 2003).

A study from Saudou and collaborators has shown that wild-type huntingtin stimulates BDNF vesicle transport and that mutation in huntingtin causes a reduction in the efficiency of BDNF vesicle transport along microtubules (Gauthier LR, 2004), which may also be contributing for striatal selective vulnerability in HD.
One of the goals of this thesis was to explore the role of huntingtin in the regulation of BDNF gene transcription.

By following BDNF messengers II, III and IV levels in the cortex of mice expressing a 63 amino-acid N-terminal portion of mutant huntingtin (R6/2 mice) we have shown that mRNA levels produced from each BDNF exon are differently modulated over disease progression. Moreover, we reported that reduction in BDNF gene transcription occurs in the cortex of R6/2 transgenic mice starting from a pre-symptomatic stage, suggesting that molecular abnormalities occur in the absence of neurological signs. The different kinetics of down-regulation of the three BDNF mRNAs analyzed may be explained by the different ability of wild-type and mutant huntingtin to influence, over time, the transcriptional complexes that regulate their expression (PAPER I).

Accordingly with our results, studies from the group of Robert Friedlander have shown a significant depletion of endogenous wild-type huntingtin in R6/2 transgenic mice at 7 weeks, an event that parallels the timing of exon II mRNA level reduction (Zhang Y, 2003). These findings directed the hypothesis that reduction of wild-type huntingtin endogenous level may cause a down-regulation in the activity of BDNF promoter exon II and consequently the reduction of BDNF exon II mRNA levels in R6/2 mice. Moreover, since BDNF exon IV mRNA levels were reduced at very early symptomatic stages, when endogenous huntingtin is still present at its normal level, we suggested that reduced BDNF gene transcription starting from exon IV might be exclusively due to the toxic gained activity of the mutant huntingtin protein (PAPER I).

We went further on testing if BDNF transcription was also affected in HD periphery. We found that BDNF transcript level is reduced in blood from R6/2 mice, respect to age-matching WT littermates (PAPER II). Importantly, level of total BDNF mRNA in R6/2 mice blood decreased over disease progression from pre-symptomatic (2-4 weeks) to early- (8 weeks) and symptomatic (12 weeks) stage of HD. Together, these results indicate that BDNF mRNA level in HD blood parallels the progressive decrease of BDNF gene transcription in HD brain (Zuccato et al, 2005). BDNF has a very complex multipromoter gene structure, suggesting a high degree of regulation at the promoter level (Altiери et al., 2004; Bishop et al., 1994; Timmusk et al., 1993; Liu et al., 2005; Liu et al., 2006; Aid et al., 2007). Our data suggest that huntingtin might similarly modulate BDNF gene transcription both in HD brain and periphery. Recent reports revealed that some of mouse BDNF messengers could be detected in peripheral organs, such as spleen and kidney (Aid et al., 2007; Liu et al., 2006). We now demonstrate that although total BDNF messengers can be measured in mice blood, the only splice variant detectable in blood from WT mice was BDNF messenger III. Moreover, this BDNF splice variant was no longer present in age-matched R6/2 HD mice. From our data we cannot exclude that other BDNF splice variants, accounting for total BDNF mRNA level, are expressed in mouse blood, but under the tested conditions, only BDNF messenger III was detected. Consistently with our results, BDNF messenger III was recently reported has being the only BDNF splice variant amplified in thymus, spleen and lymph node from C57Bl/6N mice (Kruse et al., 2006).
Our findings suggest that BDNF mRNA isolated from blood might possibly be useful in monitoring progression of HD. To test this hypothesis, we carried out an analysis of BDNF messenger level in blood from R6/2 mice treated with CEP-1347, a small molecule inhibitor of the mixed-lineage kinase (MLK) family of kinases, known to exert a neuroprotective activity both in cellular systems and animal models (Reviewed in Wang, Leo H. Annu.Rev.Pharmacol. Toxic., 2004). Comparison of BDNF messenger levels between WT and R6/2 vehicle-treated mice confirmed our initial finding, as BDNF mRNA levels were reduced in blood from HD mice, respect to age-matching WT littermates. Interestingly, CEP-1347 treatment led to a rescue of BDNF mRNA level in blood from R6/2 mice (PAPER II).

Correlating BDNF mRNA changes in blood with: i) BDNF mRNA levels in cortex from R6/2 CEP-1347 treated mice; ii) the rate of neurodegeneration and iii) the behaviour of these mice is under evaluation (Collaboration with L.Thompson group).

Recently, CEP-1347 has been tested in clinical studies in Parkinson Disease but results do not show appreciable effects. To rule out the reason for these disappointing results, further investigation on the dosage and trial design and execution needs to be done. Moreover, the selected biomarker (the dopamine transporter, β-CIT) and brain imaging may not represent the ideal measurements to evaluate the success of CEP-1347 treatment in PD patients (Waldmeier P., Biochemical Pharmacology Commentary, 2006). Indeed the need of accurate biomarkers of disease is becoming one of the main research interests.

Although the CAG expansion within the HD gene serves as the ideal trait marker, there is an increasing need of state markers that measure the state of the disease and/ or the effectiveness of a treatment. A biomarker not only sharpens the disease diagnosis but also importantly, can improve the efficiency of clinical trials by monitoring treatment response and, in the best case, provides surrogate endpoints of the disease. A biomarker must follow a series of requirements: to be objectively and reliably measured, to change in a predictable manner over time, to predict the onset of symptoms, and possibly to be associated with a known mechanism of pathology (i.e. striatal and cortical neurodegeneration). Additionally, an ideal source for a biomarker must be readily accessible and blood represents one of the most easily recovered biological materials. In fact, the possibility of using peripheral blood to analyze specific genomic profiles and associate them with different brain diseases has been matter of intensive investigation. Preliminary studies reveal that there are gene expression changes in peripheral white blood cells after acute brain injuries (Sharp et al., 2006). Combining the fact that: i) BDNF mRNA level changes over disease progression both in cortex and blood, enabling discrimination of disease stages, and ii) it responds to a (CEP-1347) treatment, prompted us to propose BDNF mRNA levels (in blood) as a possible state biomarker for HD (PAPER II).

Besides this exciting idea of using BDNF mRNA level as a biomarker, the available data suggests that BDNF has a potential interest for the development of new therapeutics in neurodegenerative disease.
Accumulating data on the pro-BDNF function of wild-type huntingtin in the brain, and the fact that reduced BDNF levels affect the development of HD has conducted to new therapeutical approaches. A first approach tested was BDNF administration. Early studies showed that BDNF administration in in vitro models of HD reduced apoptotic cell-death, suggesting that BDNF may have also anti-apoptotic properties (Saudou F, 1998). Other attempts have already been done in this direction. Survival of striatal projection neurons has been observed after grafting cell lines expressing BDNF in the striatum of rat then treated with quinolinic acid, which has been well demonstrated to induce a gradual striatal projection neurons loss (Perez-Navarro E, 2000). BDNF has also been administered via intrastriatal injection of adenovirus encoding BDNF to rat then exposed to quinolinic acid. One month after the lesion, histological studies revealed that striatal neurons were protected in rats treated with BDNF adenovirus demonstrating that transfer of the BDNF gene is of therapeutic value for HD (Bemelmans AP, 1999). The observation that BDNF protein levels increased 3 to 4 fold in the striatum and cerebellar cortex of HD mice maintained on Dietary Restriction gave further support to the beneficial effect of BDNF (Duan W, 2003). It was also found that the HD phenotype in mice could be restored by exogenous striatal BDNF administration (Canals JM, 2004) and by environmental enrichment that was able to readily increase BDNF protein levels (Spires T, 2004).

However, many obstacles still remain. It is possible that difficulties intrinsic to the use of animal models mask the positive effect of BDNF. In addition, BDNF might be powerful in restoring some, but not all, of the neuronal activities. Finally, abnormalities that are typical of the disease may reduce the efficacy of neurotrophin’s delivery. For example, it is possible that the BDNF receptor and its intracellular signalling components are affected in the disease state therefore evoking a reduced biological activity. Another main obstacle in the use of BDNF, as well as of other neurotrophins, for the treatment of HD and other degenerative disorders, is their transfer across the blood-brain barrier. One proposed strategy consisted in the administration of neurotrophins via pumps or capsules (Aebischer P, 1996; Penn RD, 1997; Ochs G, 2000), but further work needs to be done.

The second goal of this thesis was to refine huntingtin functional domains through a Phylogenetic approach.

Huntingtin has many potential domains, but their boundaries and activities are far from being fully understood. The primary amino acid sequence of huntingtin reveals little, as there are only a few known sequence motifs and no structural domains with defined function. In fact, no sequence homology between huntingtin and other proteins of known function has been reported. Additionally, huntingtin shows a ubiquitous expression in humans and rodents, and a widespread subcellular localization and these properties had always rendered the identification of its physiological function an intricate topic. Lack of information on huntingtin’s three-dimensional structure, led us to propose the comparison of huntingtin homologues-Phylogenetic approach- as an alternative approach to define conserved, and/ or newly acquired functional domains in mammalian cells (Cattaneo E, 2005).
Several lines of research suggest that huntingtin is a multifunctional protein, capable of assuming specific conformations and activities depending on its subcellular location and time of maturation in a given cell (Sipione S, 2001; Harjes P, 2003; Goehler H, 2004; MacDonald ME, 2003; Marcora E, 2003). In agreement with this notion, diverse huntingtin epitopes are differently accessible by various antibodies, depending on huntingtin subcellular localization (Trettel F, 2000; Ko J, 2001), and expansion of polyQ tract can alter the protein structure and its affinity to interact with other proteins. Huntingtin has even been proposed as a scaffold protein (Perutz MF, 1994; Harjes P, 2003).

To study normal function of huntingtin, we have followed a phylogenetic approach. Briefly: i) huntingtin orthologues were isolated from key species of animal phylogenetic evolution; ii) expression constructs containing full-length or portions of huntingtin orthologues were produced; iii) biological assays were designed to identify defects of loss of wild-type huntingtin function in huntingtin-depleted mouse embryonic stem (ES) cells and iv) the ability of the first 548 amino acids from human huntingtin was tested to rescue the observed defects. This represents the first step towards the identification of huntingtin functional domains. The developed biological assays will be eventually applied in cells stably expressing each of the constructs containing full-length or a portion of huntingtin isolated from the selected ancient species.

Starting from the notion that depletion of wild-type huntingtin likely results in a detrimental additional loss of protective function, we have developed cell-based assays that could permit us to identify loss-of-function defects in the extremely useful cellular model, the huntingtin-depleted embryonic stem (ES hdh-/-) cells. Assays were developed both in ES cells kept in proliferation conditions (undifferentiated cells) and in neural differentiated ES cells (through a monolayer neural differentiation protocol).

It has been previously demonstrated by several groups, ours included, that increasing levels of wild-type huntingtin results in a resistance to apoptotic cell death and to neurodegeneration in vivo, providing further support to huntingtin neuroprotective role in a pathological condition.

Under proliferation conditions, differences between cells expressing both (ES hdh+/+), only one (ES hdh+/−) and none (ES hdh−/−) of huntingtin alleles were very subtle. Absence of huntingtin appeared to affect minimally undifferentiated ES cells. This is not totally unexpected given their prolonged viability in culture. Only under stress-induced conditions, ES hdh−/− cells revealed a lower survival. Robert Friedlander’s group has recently reported similar results (Zhang Y, 2006). Exposure to a mitochondrial complex II inhibitor resulted in a similar reduced survival in wt and huntingtin-depleted ES cells. This data suggests that the reported deficits in HD mitochondrial function might be a direct or indirect consequence of the mutation, and independent from wild-type huntingtin depletion. In agreement, studies conducted on striatal cells exposed to 3-NP revealed significantly greater cell death in cells expressing mutant huntingtin, respect to wild-type striatal cells (Ruan Q, 2004).

Neural differentiation of pluripotent ES cells provides a unique opportunity to study the role of altered sequence or expression of selected genes during neurogenesis. Work from
Martina Metzler and colleagues reported for the first time that huntingtin depleted ES cells could be differentiated into neurons, through an embryoid body-dependent differentiation protocol. In those conditions, generation of neurotransmitter-responsive postmitotic neurons was not inhibited by lack of huntingtin (Metzler M, 1999) but at the time, how huntingtin may affect proliferation of neuronal progenitor cells was not assessed. Conventional embryoid body differentiation protocols generate a chaotic mixture of differentiated cell types (Doetschman T, 1985) and even if selective regimes can be used to enrich for cell types of interest (Li M, 1998; Okabe S, 1996), the initial lineage commitment process remains obscured and uncontrolled. To identify eventual defects of huntingtin-depleted cells during intermediate stages of neural differentiation, we took advantage of the monolayer neural differentiation (Ying QL, 2003).

Here, we have applied 21-days monolayer neural differentiation protocol (Ying QL, 2003) to our cellular system, the mouse ES hdh+/+, +/- and -/- cells. Total number of cells present in each genotype monolayer culture was counted and results clearly show a reduced survival following neural induction in the absence of huntingtin. Consistently, a higher activation of caspase-3 activation was observed in huntingtin-depleted cells. Apoptosis is a mechanism known to be important for the correct neural differentiation to occur, but huntingtin depletion seems to induce an even greater caspase-3 activation in in vitro neural differentiation of ES cells. A recent work from Anna Strehlow and colleagues has also shown a significant increase in transcripts encoding proteins involved in apoptosis, as caspase-3, suggestive of elevated cellular stress in neuronal differentiated hdh/- cells (Strehlow A, 2006). Together these results indicate that a reduced dose of wild-type huntingtin results in a higher caspase-3 activation throughout neural differentiation and that the correct dose of huntingtin is critical for cell viability through different developmental stages. Coupled with this higher apoptotic cell death, huntingtin depletion seems to impair mitotic cell division, at least during the early (proliferative) stages of neural differentiation, as less cells stained positively for phospho-histone H3.

Under standard monolayer neural differentiation conditions, some cells tend to resist neural specification, as they remain as ES cells or they differentiate into non-neural lineages (Lowell S, 2006). We found that huntingtin-depleted monolayer cultures tended to maintain an abnormally higher quota of pluripotent ES (Oct-4⁺) cells throughout monolayer neural differentiation. The failure of ES cells to differentiate into the neural lineage can be reduced by performing a replating of the culture, but even in replated monolayer cultures we observed a higher presence of ES cells in huntingtin-depleted cultures, respect to hdh+/+ monolayer cultures. These results imply that depletion of huntingtin may limit some neural inductive signal, or promote anti-neural signals, leading to the failure of ES hdh/- cells to readily differentiate. The POU domain transcription factor Oct-4 is a critical regulator of ES cell pluripotency (Nichols, 1998) and down-regulation of Oct-4 is necessary for ES cell differentiation (Pesce and Scholer, 2001). The mechanisms that regulate Oct-4 expression are not fully understood but the persistent Oct-4 expression in huntingtin-depleted monolayer cultures suggest that huntingtin may
be involved in the normal down-regulation of Oct-4 that occurs in the absence of LIF and serum.

The subsequent step towards neural lineage is the transition into neural progenitors, also known as neural stem cells. While undifferentiated ES cells do not express nestin, neural rosettes express this marker gene and we captured its expression after 7 and 14 days of neural differentiation conditions. Our results indicate that in the absence of huntingtin cells produce less and smaller neural rosettes, especially in the first days of lineage transition. Interestingly, rosettes are commonly considered as the in vitro neural tube and mice embryos with complete hdh inactivation implant normally but develop a primitive streak that is not fully extended and die before the emergence at E8.0 to E8.5 of the neural tube, the precursor of the HD defect’s target tissue in humans (Duyao, 1995).

Consistent with results obtained by Metzler and colleagues, in our monolayer neural (embryoid body-independent) differentiation conditions, huntingtin-depleted ES cells could be efficiently differentiated into neurons. Furthermore, we have assessed if huntingtin depletion could interfere with astrocytes formation but a similar astrocyte quota (GFAP⁺) was observed in hdh+/+, +/ and -/- monolayer cultures. Taken together, these results highlight that neurogenesis and astrocytes formation can occur in the absence of huntingtin, at least under the described neural differentiation conditions. It is worth to notice that by supplying neural induction factors (such as retinoic acid), present in monolayer media, we may be bypassing possible huntingtin-depletion defects.

Work from our group has extensively demonstrated that huntingtin is an important regulator of BDNF expression. We have analyzed BDNF mRNA level in ES hdh-/- cells both in proliferation and even more interesting throughout their neural differentiation. Reduced BDNF transcript level was observed throughout neural differentiation of ES hdh-/- cells. Since ES hdh-/- cells were perfectly able to produce neurons, we hypothesize that the reduced BDNF level observed in the absence of wild-type huntingtin may be due to a dysfunction at the transcriptional level of the hdh-/- neurons. It is worth of note that astrocytes can also produce BDNF, but the quota of astrocytes was similar between different genotype monolayer cultures. Although, our results do not exclude that the lower level of BDNF mRNA in the hdh-/- monolayer cultures is also due to the lower total cell number is these cultures. A recent work showed that ES hdh-/- cells can be differentiated into largely functional neurons, but these neurons exhibit a more definitive dysfunction at the transcriptional level, made apparent by the altered expression of many classes of genes (Strehlow A, 2006).

As previously discussed, under standard monolayer neural differentiation conditions, some cells tend to resist neural specification, as they remain as ES cells. Besides maintaining an abnormally higher number of pluripotent cells, huntingtin-depleted monolayer cultures also tended to accumulate cells that differentiate into non-neural lineages. Many of these differentiated non-neural cells express cytokeratin-8, a differentiation marker that is not expressed in ES cells or neural lineages (Lowell S, 2006). Interestingly, cytokeratin⁺ cells were barely detectable in hdh+/+ and hdh+/- cultures but were highly represented in huntingtin-depleted monolayer cultures. This data opens the possibility that huntingtin
might have a role in vertebrate germ layer specification, by suppressing non-neural commitment.

We have further shown that the first 548 amino acids of human huntingtin can compensate some of the defects observed in huntingtin-depleted cells, but the beneficial effects of this potential functional domain depend on the cell type and stage of development. Expression of the N548 fragment of human huntingtin led to the early appearance of neural rosettes with well-organized radial arrangements of columnar cells, as observed in hdh+/+ monolayer cultures. This portion of human huntingtin was also able to reduce the activation of caspase-3, respect to huntingtin-depleted cells, but only at later stages of neural differentiation, when neurons were the predominant cellular population in the monolayer cultures. The ability of the ES cell line stably expressing the N-terminal fragment of human huntingtin to restore BDNF mRNA level was also tested. We noticed that the truncated form of human huntingtin was unable to restore BDNF expression, suggesting that this may not be the huntingtin domain involved in the regulation of BDNF gene transcription. Finally, our results suggest that the first 548 amino acids of human huntingtin may likely be involved in suppression of non-neural commitment, as its expression was able to readily rescue the higher formation of non-neural differentiated cells in hdh--/ monolayer cultures. Consistently with the data presented here, human huntingtin has been shown to compensate for the critical function of murine huntingtin during gastrulation, by rescuing mice with targeted disruption on both hdh alleles (Hodgson, 1996). With our results we present further evidence for cross-species complementarity of huntingtin. Cross-species complementation between mouse huntingtin and human huntingtin, in both protection against apoptosis and neural differentiation, most probably reflects the high degree of sequence conservation, as mouse huntingtin and human huntingtin share complete identity of nucleotides at a level of 90% similarity in amino acid structure (Lin, 1994).

Here, we provide strong evidences that wild-type huntingtin may normally have an anti-apoptotic function in adult brain cells and establish a first step towards the mapping of huntingtin functional domains. Hopefully our phylogenetic approach will offer further insight on the functional domains of wild-type huntingtin.
This section will be divided on material and methods used in each of the aims presented in this thesis.

For the accomplishment of Aim I, “Analysis of BDNF progressive loss in a Huntington’s disease mouse model” the following material and methods were used:

**Animals**

R6/2 mice and littermate mice were obtained from Charles River. The mice were fed with standard laboratory chow and tap water before experiments. Mice after 1, 4, 6, 8 and 12 weeks from birth were killed by cervical dislocation and their respective brains were removed. Cerebral cortex was dissected out, snap frozen on dry ice and stored at –80°C.
RNA Isolation from Mouse Cortex

Total RNA was isolated from cortex of R6/2 and littermate mice with TRIZOL reagent (Invitrogen). RNA was treated with RQ1 Dnase (Promega) at 37°C for 30 min.

Semi-quantitative RT-PCR

PCR for mouse and rat BDNF exons I, II, III and IV mRNA levels. PCR was performed in a total volume of 50 µl containing cDNA made from 0.25 µg of total RNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM of the common reverse primer corresponding to exon V, 0.4 µM of one of the 5' exon-specific primers, 0.4 µM of β-Actin primers and 2U Taq polymerase (Invitrogen).

Primer sequences were as follows:
- ratBDNFI FWD, 5'-GGTGGATGAGAGTTGAAGCTTGCGA-3';
- ratBDNFII FWD, 5'-GGAGCGGAGCGTTTGGAGAGCCA-3';
- ratBDNFIII FWD, 5'-CAGGAGTACATATCGGCCACCA-3';
- ratBDNFIV FWD, 5'-GGCTTTGATGAGACCGGGTTCCCT-3';
- ratBDNFW REV, 5'-GTAGGCCAAGTTGCCTTGTCCGT-3';
- mAct FWD, 5'-CTCTTTGATGTCACGCACGATTTC-3';
- mAct REV, 5'-GTGGGCCGCTCTAGGCACCAA-3'.

Each amplification cycle consisted of the following steps: 94°C for 30s, 57°C for 30s and 72°C for 30s. PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.

Radioactive RT-PCR

Total RNA extracted from the cortex of each transgenic and littermate mouse was retro-transcribed into single-stranded cDNA using Superscript II Rnase H⁻ Reverse Transcriptase (Invitrogen). This procedure was repeated twice to obtain cDNAs replicates. Each cDNA was further analyzed by three independent PCR. In total, six measurements were performed for each of the time points analyzed. In addition, for each PCR experiment, BDNF mRNA levels were quantified and normalized relatively to β-Actin mRNA level.
Radioactive PCR was performed in a total volume of 50µl containing:

- cDNA made from 0.25 µg of RNA,
- 20 mM Tris-HCl, pH 8.4,
- 50 mM KCl,
- 1.5 mM MgCl₂,
- 0.2 mM dNTPs,
- 1.7 µCi [α³²P]dCTP,
- 2 U Taq polymerase (Invitrogen)
- 0.4 µM of each primer

All BDNF promoters were amplified with multiple cycle number (25-30 cycles) to determine the appropriate conditions for obtaining semi-quantitative differences in their expression levels. PCR products were separated by non-denaturing 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

**Statistical Analyses**

Data were analyzed with one-paired t-test.
For the accomplishment of Aim II, “Investigation of BDNF mRNA levels in blood from R6/2 mice throughout disease progression and upon CEP-1347 treatment”, the following material and methods were used:

**Animals**

In this study, R6/2 mice and littermate mice were obtained from Charles River. The R6/2 mouse expresses exon 1 of the human Huntington’s disease (HD) gene carrying a 141-157 CAG repeat (Mangiarini). Mice at asymptomatic (2 weeks), presymptomatic (4 weeks), early-symptomatic (8 weeks) and symptomatic (12 weeks) stages of disease were sacrificed by CO₂ and blood from heart was collected into 1.5ml tubes (Eppendorf) containing TRIZOL (Invitrogen). Samples were stored at -80°C.

Blood samples for BDNF kinetic study were collected from 3 WT and 3 R6/2 mice at 4, 8 and 12 weeks. In order to follow the same mice over time, blood was recovered from the left and right eye (orbital venous sinus collection) from each mouse at 4 and 8 weeks, respectively. Following blood collection, the eyelids should be held closed for a few seconds to allow the punctured blood vessel to clot. A small amount of ophthalmic ointment (Optimmune, Schering-Plough) was placed into the eye following this procedure (as recommended in the Laboratory Animal Technician Training Manual). At 12 weeks, mice were sacrificed and blood was recovered by cardiac puncture. For all blood sampling, mice were anesthetized with mix composed by xylazine and ketamine (100µL/ 30g mouse weight). Blood samples were stored at -80°C.

**Blood Samples from CEP-1347 Treatment**

For the CEP-1347 treatment, a total of 48 blood samples were obtained from through collaboration with the group of Leslie Thompson at the Irvine University- California. From the acute treatment a total of 24 blood samples were analyzed: 6 wild-type administered with vehicle (0.15% Cremphor E.L. in 0.9% saline, 10 ml/kg s.c.), 6 wild-type treated with CEP-1347 (1mg/Kg in vehicle; 10ml/Kgs.c.), 6 R6/2 with vehicle and 6 R6/2 with CEP-1347 (1mg/Kg in vehicle; 10ml/Kgs.c.). After 4h from the administration, mice were sacrificed and blood was collected from heart by cardiac puncture. From the chronic treatment a total of 24 blood samples were assessed: 6 wild-type administered vehicle (0.15% Cremphor E.L. in 0.9% saline, 10 ml/kg s.c.), 6 wild-type treated with CEP-1347 (0.5mg/Kg in vehicle; 10ml/Kgs.c.), 6 R6/2 with vehicle and 6 R6/2 with CEP-1347 (0.5mg/Kg in vehicle; 10ml/Kgs.c.). This treatment was repeated once daily and after 4 weeks from the first injection, mice were sacrificed and blood was collected by cardiac puncture.
Treatments began when mice were 6 weeks of age. Blood samples were kept in TRIZOL (Invitrogen) until RNA extraction.

**RNA isolation and Dnase treatment**

Total RNA was extracted from blood of R6/2 and littermate mice with TRIZOL Reagent (Invitrogen), according to the manufacturer’s protocol. Briefly, 1ml of TRIZOL was added to each blood sample. Chloroform (Sigma) (1/5 volume) was added at room-temperature and mixed with vortex twice. A 20 minutes centrifugation (9600rpm) was performed at 4°C and supernatant was recovered. Isopropanol (Sigma) was added 1:1 volume and RNA precipitation step was done overnight at −20°C. A 10 minutes centrifugation (9600rpm) was performed at 4°C and RNA pellet was recovered. Washing of RNA was done by adding cold 75% ethanol (Sigma) and by a centrifugation (6000rpm) at 4°C for 7 minutes. RNA pellet was dried at room-temperature and resuspended in 20-30 µl of sterile water (Sigma).

RNA was treated with DNA-free DNase Treatment & Removal (Ambion) at 37°C for 10 min. After DNase treatment, 1µg of RNA from cortex and 5 ug of RNA from blood collected from R6/2 mice at different stages of disease were used for first-strand synthesis using SuperScript III First-Strand synthesis system (Invitrogen).

**RT-PCR**

The product of reverse transcriptase was denatured and amplified by PCR using 5 ug of cDNA, 50 mM KCl, 20 mM Tris-Cl, pH 8.4, 0.2 mM of each dNTPs iTaq DNA polymerase, 25 units/ml, 3 mM MgCl2, and stabilizers (iTaQ DNA Polymerase and dNTP Mix-Biorad) and 0.5 mM of forward and reverse primers. To analyze expression of BDNF transcripts, reverse primer specific for 3’ BDNF coding exon and forward primers specific for 5’ non-coding exons in mouse were used. Amplification cycles consisted of a first denaturing cycle at 95°C 3 min, followed by 45 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C for all BDNF transcripts. For amplification of mouse BDNF mRNA splice variants was performed with the oligonucleotides listed above:

- β-actin Forward 3’-GTGGGCCGCTCTAGGCCACCAA-5’
- β-actin Reverse 3’-CTCTTTTATGTGCCTACCGCATTTT-5’
- BDNF I 3’-GTGTGACCTGACAGTGGGCAGAGAAGAAGA-5’
- BDNF III 3’-GCTTTCTATCATCCCTCCCCGAGAGT-5’
- BDNF IV 3’-CTCTGCTCCTAGCTGATGAGAGCCTATC-5’
- BDNF V 3’-CTCTGTGGTGTGTTTATTATTGTGTTGC-5’
- BDNF VI 3’-GCTGGGCTGTGACCGGTCTCCATT-5’
Beta-actin was used as an internal control for blood samples. All RT-PCR reactions were performed in triplicate. PCR products were resolved in 2% agarose gel and visualized by staining with ethidium bromide.

**Real Time PCR for BDNF gene expression**

iCycler Thermal Cycler with Multicolor Real-time PCR Detection System (Biorad) was used. All reactions were performed in a total volume of 25 µl containing 5 ug of cDNA, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTPs, 25 units/ml of iTaq DNA polymerase, 3 mM MgCl2, SYBR Green I, 10 nM fluorescein, stabilizers (iQ™ SYBR Green Supermix-Biorad) and 0.5 mM of forward and reverse primers. Amplification cycles consisted of a first denaturing cycle at 95°C 3 min, followed by 45 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C for. Fluorescence was quantified during the 60°C annealing step and product formation was confirmed by a melting curve analysis (55°C-94°C). BDNF mRNA amounts were normalized to β-actin, according to M.W. Pfaffl, Nucleic Acids Research 2001, 29:2002-2007.

The oligonucleotides used for the amplification of mouse BDNF and β-actin mRNA are listed above:

- BDNF Forward, 5'-TCGTTCCCTTCAGTTGCCTCA-3'
- BDNF Reverse, 5'-TTGTGTAAGGCGCAAAAC-3'
- β-actin Forward, 5'-AGTGTACGTTGACATCCGTA-3'
- β-actin Reverse, 5'-GCCAGGACGTAACTCCTCT-3'

For each RNA sample, three retro-transcription reactions were performed and each cDNA was analysed by three independent Real Time PCR reactions. A total of nine measurements were performed for each sample. For every Real Time PCR experiments, total BDNF levels were quantified and normalized relative to β-actin mRNA level.
**Statistical analysis**

For BDNF gene expression studies, one-way analysis of variance (ANOVA) test was used for data comparison.
For the accomplishment of Aim III, “Validation of constructs expressing the N- terminus from htt orthologues in huntingtin-knockout mouse embryonic stem (ES hdh-/-) cells” the following material and methods were used:

**Cell Culture**

Mouse embryonic stem cells - mES hdh +/-, +/- and -/- (Duyao, 1995; Nasir, 1995; Zeitlin, 1995) were propagated in an undifferentiated state on gelatin-coated plates. Cells were cultured and passed every two days in proliferation medium i.e. Glasgow minimal essential medium-GMEM; Invitrogen- Life Technologies supplemented with 10% heat-inactivated fetal bovine serum (FBS- EuroClone, Italy), 2mM L- glutamine, 100U/ml penicillin- 100 µg/ml streptomycin, 100 µM non-essential amino-acids, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol (Gibco) and 1000U/ml murine leukemia inhibitor factor (LIF- ESGRO).

**Transfection methodology: Nucleofection**

To validate the expression of each of the available constructs, mES hdh -/- cells were transiently transfected with nucleofection technology (Amaxa).

The day before nucleofection, for each construct, 2x 10^6 mES hdh -/- cells were seeded in a gelatin-coated T25 flask (IWAKI, Japan), in GMEM containing 10% FBS and LIF.

One the day of transfection, cells were tripsinized and spined down for 5min. at 80xg to completely remove the culture medium. The obtained cell pellet was then resuspended in 90µl of the supplemented mouse ES cell nucleofector solution (Amaxa). For each nucleofection, one 1.5ml tube containing 10µl of the supplemented mouse ES cell nucleofector solution and 4µg of the tested construct was prepared. When two constructs were co-transfected into the same cell suspension, a total of 8µg of plasmid DNA was diluted into 10µl of the supplemented mouse ES cell nucleofector solution. Cell suspension and 4ug of DNA already diluted in 10µl of mouse ES cell nucleofection solution were mixed and introduced into the nucleofector cuvettes (Amaxa). After testing the nucleofector programs A-13, A-23, A-24, A-30 (available for mES cells), the A-24 program was chosen has the optimal one (higher transfection efficiency and lower cell death). To avoid damage to the cells, samples were immediately removed from the cuvettes after program has finished and added to 500µl pre-warmed medium using the plastic pipettes provided in the nucleofector kit to prevent damage and loss of cells.

Nucleofected cells were then plated onto gelatin-coated 4 well plates (NUNC), in GMEM containing 10% FBS and LIF, for further immunocytochemistry analysis of each construct expression in mES hdh -/- cells.
Constructs Expression Analyses: Immunocytochemistry (IC)

Cells were fixed in 4 well plates (NUNC) in 4% paraformaldehyde for 20 min, permeabilized for 20 min with 0.1% Triton X-100 and immunostained. Primary antibodies (1:500 anti-Green Fluorescence Protein, Molecular Probes; 1:250 anti-Huntingtin mAb2166, Chemicon; 1:1000 anti-HA, Sigma; and 1:100 anti-Phospho Histone H3, Cell Signaling) were incubated overnight at 4°C. Secondary antibodies (1:500, AlexaFluor 488 and AlexaFluor 568, Molecular Probes) were incubated for 2 hours at room temperature. Hoechst 33258 (1:10000, Molecular Probes) was used to visualize the nuclei. PermaFluor (Thermolab) was used as mounting solution. Fluorescent images were visualized using epifluorescence microscopy (Axiovert, Zeiss).
For the accomplishment of Aim IV, "Development of read-out assays to identify loss-of-function defects in huntingtin mouse ES hdh-/- cells" and Aim V, "Test ability of the first 548 amino acids of human huntingtin in complementing the defects found in mouse ES hdh-/- cells", the following material and methods were used:

**Cell Culture**

Mouse embryonic stem cells- mES hdh +/-, +/- and -/- (Duyao et al., 1995) were propagated as described above (material and methods section for Aim III).

**Generation of stable ES cell clones expressing the first 548 amino acids of human huntingtin (ES hdh -/- N548 human huntingtin cell line)**

The cDNA of the N-terminal part (N-548) of wild-type human huntingtin was excised from the construct pCI Htt-N548 (gift from Michael Hayden’s group- University of British Columbia, Vancouver) and subcloned into the pCAG expression vector carrying an IRES element and a puromycin resistance cassette. The huntingtin-knockout mouse ES cell line (kindly provided by Marcy Macdonald’s- Massachusetts General Hospital, Boston) was transfected with the pCAG Htt-N548 vector using Lipofectamine (Invitrogen) and selected by 15 days with 1.5ug/ml puromycin. Four puromycin-resistant clones were selected and the expression of the first 548 amino acids of human huntingtin was assayed by western blot.

**MTT Assay**

Cells were plated in triplicates into twenty-four well plates (IWAKI, Japan) at a density of 2x10^4/ well in supplemented GMEM as indicated above. After 12 hours incubation at 37°C, medium was replaced in half the wells with serum-deprived medium (composition: GMEM supplemented as described above but without serum), and in the remaining half of the wells with fresh complete GMEM. All cells were then incubated at 37°C for the following time points 6, 12, 24, 36, 48 and 96 hours. At each time point, cells were exposed to 3-[4.5-dimethylthiazol-2-phenyl]-2.5-diphenyl-tetrazolium bromide, and mitochondrial formazan release was quantified at 560nm using a plate reader (Bio-Rad). For MTT experiments with 3-nitropropionic acid (3-NP, Sigma), cells were plated in complete GMEM and after 12 hours from plating were exposed to 1mM, 5mM and 10mM of 3-NP acid. Formazan release was quantified after 24 and 48 hours incubations at 37°C.
Lactate Dehydrogenase Based (LDH) Assay ®

The kit, TOX-7 assay ® (Sigma), chosen for this assay permits two types of analysis:
1- LDH Release: assesses the membrane integrity of cells as a function of the amount of LDH leakage into the medium.
2- Total LDH: measures the total cell number. In the present studies, total LDH was used to normalize LDH release measurements.

For each tested condition, cells were plated in triplicates into twenty-four well plates (IWAKI, Japan) at a density of 2x10⁴/well in supplemented GMEM. After 12 hours incubation at 37ºC, medium was replaced in half the wells with serum-deprived medium (composition: GMEM supplemented as described above but without serum), and in the remaining half of the wells with fresh complete GMEM. All cells were then incubated at 37ºC for the following time points 6, 12, 24, 36, 48 and 96 hours.

For the LDH release, 100µl of the medium from each well was transferred into a 96 well plate (IWAKI, Japan) and incubated with 50µl of LDH Assay Mixture® (equal amounts of Lactate Dehydrogenase Assay Substrate, Enzyme and Dye Solutions) for 30 minutes at room- temperature in the dark. The reaction was stopped by the addition of 1/10 volume of HCl per well. The LDH released into the medium was quantified at 490nm and background absorbance at 690nm using a plate reader (Bio-Rad).

Total LDH measurements were performed by adding to each well 1/10 volume of LDH Assay Lysis Solution® and incubating cells for 45 minutes at 37ºC. Plates were centrifuged, at 250xg for 4 minutes, and 100µl of the solution (medium + lysis solution) were transferred into a 96 well plate (IWAKI, Japan) and proceed with the enzymatic analysis described above for the LDH release measurements.

Each experiment was independently repeated at least three times.

Caspase-Glo 3/7® Assay multiplexed with Cell Titer-Blue® Assay

For each tested condition, cells were plated in triplicates in a 96 well plate with transparent bottom (PBI) at a density of 5 000 cells/well. After 36 hours incubation at 37ºC, half wells received 100µl of freshly prepared complete GMEM and the other half 100µl GMEM lacking serum. After 3 hours incubation, 20µl of Cell Titer-Blue ® Reagent (Cell Titer-Blue ® Assay- Promega) was added to each well and plates were incubated for another 3 hours at 37ºC. Fluorescence was recorded at 550/595nm (plate reader- Bio-Rad) to measure cell viability. At this point, an opaque sticker was added to the transparent bottom of the 96 well plate and an equal volume (120µl) of Caspase-Glo 3/7 Reagent was added to each well. After 1 hour of room temperature incubation, luciferase achieved the steady state
and luminescence was recorded with a luminometer (Veritas-Microplate Luminometer, Turner Byosistems).
Each experiment was independently repeated at least three times.
Results are presented as the ratio of Fluorescence/ Luminescence recorded for each tested condition.

**Statistical analysis**
One-way analysis of variance (ANOVA) test was used for data comparison.

**Monolayer Neural Differentiation of Mouse Stem Cells**

Mouse embryonic stem cell lines: ES hdh +/+ (also called as wt or R1), ES hdh +/- and ES hdh -/- cell lines were kindly provided by Marcy Macdonald group were used for monolayer neural differentiation (Ying, 2003) experiments.

Before initiating differentiation, ES cells were plated at high density (2x10⁶ cells/ T25 flask) and cultured for 24 hours in standard ES cell medium containing LIF (as described above). Undifferentiated ES cells were dissociated using 0.025% trypsin (Gibco, Invitrogen) solution at 37°C, and then plated onto 0.1% gelatin-coated tissue culture plastic at a density of 1.0x10⁴/cm² in N2B27 serum-free medium. N2B27 consists of a 1:1 ratio of DMEM/F12 (Gibco) and Neurobasal (Gibco) media supplemented with 0.5% N2 (Gibco), 1% B27 (Gibco) and 0.2% 2-mercaptoethanol (Gibco, Invitrogen). The medium was then changed every other day. For some experiments, cells were replated after 7 days onto poly-ornitine (Sigma) and laminin (Gibco, Invitrogen) coated plastic at a density of 0.5-1.5x10⁴/cm². A supplement of 10-20ng/ml of b-FGF (PeproTech, TebuBio) in the medium used for the replating step improved cell viability. Media was then changed every 3-4 days and monolayer cultures were kept under differentiation for up to 21 days.

**Trypan Blue and Cell Coulter Counter**

To count the total number of cells present throughout monolayer neural differentiation, triplicates for each of the cell lines under study (mES hdh +/+, +/-, -/- and -/- N548 human cells) were seeded in 6 well plate (IWAKI, Japan), at each of the differentiation days analyzed (Days 0, 2, 5, 7, 14 No Replate/ Replate and 21 No Replate/ Replate). Medium was aspirated from each well, and in the cases there were too many cell debris/dead cells (later days of differentiation) cells were washed once with PBS 1X (Sigma). Trypsine was diluted 1:2 in PBS1X (500ul/ well) and used to detatch cells. Tripsinization was blocked with 2ml of PBS1X. Cell suspension was centrifuged at 1200rpm for 3min and cell pellet was resuspended in 1ml of differentiation medium.

For Trypan Blue counts, 10µl of cell suspension was mixed with 10µl of Trypan Blue® reagent (Sigma) and the average of 3 counts was calculated. For Cell Coulter Counter (ZM; Coulter Instruments), 500µl of cell suspension was mixed with 20ml of saline buffer
solution and triplicates were counted and an average was calculated. As blank, medium without cell suspension was assayed.

**Western Blot Analyses**

ES undifferentiated/ neural differentiated cells were washed with phosphate buffered saline (PBS, Sigma) and lysed for 60 minutes on ice in a buffer containing Tris HCl 10 mM, NaCl 100 mM, Triton X-100 1%, EGTA 5 mM, EDTA 5 mM, protease inhibitors (Bio-Rad). Samples were then centrifuged for 15 min at 14,000 rpm, 4 °C, supernatants were recovered, and protein concentration was evaluated using the Bradford Reagent (Bio-Rad). Equal amounts of protein were separated by 12% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad), and incubated in blocking buffer consisting of TBS (10 mM Tris, pH 7.6 and 150 mM NaCl) with 5% milk. Blots were incubated overnight at 4°C with primary antibodies in blocking buffer. Primary antibodies used were: anti-huntingtin mAb2166 (dilution 1:2000, Chemicon), anti-active caspase-3 (dilution 1:1000, Santa Cruz), anti-BLBP (dilution 1:5000, Chemicon), anti-β-III Tubulin (dilution 1:2000, Promega), anti- Cytokeratin (dilution 1:250, Development Studies Hybridome Bank) and α-tubulin (dilution 1:3000, Sigma). After washing with TBS with 0.05% Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer (dilution 1:3000, Bio-Rad), washed again in TBST and signals visualized with the enhanced chemiluminescent system (ECL Plus, Amersham). Western blot quantification analysis was done with Quantity One ® (Bio-Rad).

**Immunocytochemistry Analyses**

Cells were fixed in 6 well tissue culture dishes (IWAKI, Japan) in 4% paraformaldehyde for 20 min, permeabilized for 20 min with 0.1% Triton X-100 and immunostained. Primary antibodies were incubated overnight at 4°C. Secondary antibodies (1:300, AlexaFluor 488 and AlexaFluor 568, Molecular Probes) were incubated for 2 hours at room temperature. Hoechst 33258 (1:10000, Molecular Probes) was used to visualize the nuclei. PermaFluor (Thermolab) was used as mounting solution. Fluorescent images were visualized using epifluorescence microscopy (Axiovert, Zeiss). For each primary antibody a particular blocking solution and dilution were optimized as indicated in Table 4.
Table 4: Description of the immunocytochemistry conditions applied for each of the primary antibody used throughout this study.
**RNA Extraction from Cells and RT-PCR**

Total RNA was extracted from ES cells with TRIZOL Reagent (Invitrogen), according to the manufacturer’s protocol. RNA was treated with DNA-free DNase Treatment & Removal (Ambion) at 37°C for 10 min. After DNase treatment, 1ug of RNA from each cell line at different days of monolayer neural differentiation (0, 2, 5, 7, 10, 14 and 21 days) were used for first-strand synthesis using SuperScript III First-Strand synthesis system (Invitrogen).

**Qualitative PCR**

PCR reaction was performed in a thermocycler (Gene Amp ®, Applied Biosystems). The cycle sequence consisted of an initial denaturation step for 5 min at 95°C, followed by 25-30 cycles (20 cycles for β-actin) of 30 sec at 95°C, 30 sec at 58–64°C (see below) and 30 sec at 72°C. A final 7 min extension at 72°C was also performed. PCR products were analyzed in 1% agarose gel.

Primers Sequences and Annealing Temperatures were as follows:

- Oct4 Forward: 5’-GTGGAGGAAGCGACAACAATG-3’,
- Oct4 Reverse: 5’-GATATCAGTTTGAATGCATGGG-AGAG-3’ (60ºC);
- Nanog Forward: 5’-ATGAAGTGAAGCGGTGGCAGAAA-3’,
- Nanog Reverse: 5’-CCTGGTGGAGTCACAGAGTAGTTC-3’ (64ºC);
- β-actin Forward: 5’-GGCCCAGAGCAA-GAGAGGTATCC-3’,
- β-actin Reverse: 5’-ACGCACG-ATTTCCCTCTCAGC-3’ (58ºC).

**Quantitative PCR (Real- Time PCR)**

Quantitative PCR was performed with a Light Cycler from Bio-Rad and the SYBR green kit (Bio-Rad, Italy). Amplification included initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 sec, annealing at 57–60°C (see below) for 30 sec and extension at 72°C for 30sec.
Primers Sequences and Annealing Temperatures were as follows:

- Nestin Forward GAGAGTCGCTTAGAGGTGCA
- Nestin Reverse CCACTCCAAGACTAAGGAC (59°C)
- BDNF Forward TCGTTCCCTTTCGAGTTAGCC
- BDNF Reverse TTGGTAAACGGCACAAAAC (60°C)
- ß-actin Forward AGTGTGACGTTGACATCCGTA
- ß-actin Reverse GCCAGAGCAGTAACCTTCTTCT (60°C)

**Statistical analysis**

For gene expression studies, one-way analysis of variance (ANOVA) test was used for data comparison.
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