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Molecular Mechanisms and Potential Therapeutical Targets in Huntington's Disease

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Zuccato C, Valenza M, Cattaneo E. Molecular Mechanisms and Potential Therapeutical Targets in Huntington's Disease. *Physiol Rev* 90: 905–981, 2010; doi:10.1152/physrev.00041.2009.—Huntington's disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the gene encoding for huntingtin protein. A lot has been learned about this disease since its first description in 1872 and the identification of its causative gene and mutation in 1993. We now know that the disease is characterized by several molecular and cellular abnormalities whose precise timing and relative roles in pathogenesis have yet to be understood. HD is triggered by the mutant protein,

and both gain-of-function (of the mutant protein) and loss-of-function (of the normal protein) mechanisms are involved. Here we review the data that describe the emergence of the ancient huntingtin gene and of the polyglutamine trait during the last 800 million years of evolution. We focus on the known functions of wild-type huntingtin that are fundamental for the survival and functioning of the brain neurons that predominantly degenerate in HD. We summarize data indicating how the loss of these beneficial activities reduces the ability of these neurons to survive. We also review the different mechanisms by which the mutation in huntingtin causes toxicity. This may arise both from cell-autonomous processes and dysfunction of neuronal circuitries. We then focus on novel therapeutical targets and pathways and on the attractive option to counteract HD at its primary source, i.e., by blocking the production of the mutant protein. Strategies and technologies used to screen for candidate HD biomarkers and their potential application are presented. Furthermore, we discuss the opportunities offered by intracerebral cell transplantation and the likely need for these multiple routes into therapies to converge at some point as, ideally, one would wish to stop the disease process and, at the same time, possibly replace the damaged neurons.

I. INTRODUCTION

Huntington's disease (HD) is a dominant inherited neurodegenerative disorder that is caused by an unstable expansion of a CAG repeat within the coding region of the IT-15 gene (246). The gene encodes for a protein called huntingtin, and the mutation results in an elongated stretch of glutamine near the NH_2 terminus of the protein (246).

Prevalence of the mutation is 4–10 cases per 100,000 in populations of Western European descent, with many more at risk of having inherited the mutant gene. Over time, the consequence of carrying the HD mutation is a massive brain neurodegeneration characterized by the prevalent loss of efferent medium spiny neurons in the striatum (caudate nucleus and putamen) of the basal ganglia, which is primarily responsible for the typical HD symptoms (464). However, it is now well established that a more widespread degeneration occurs in the brain and also involves cortical structures (474, 475, 479).

Since HD is caused by a single mutation, the introduction of the mutant gene into non-human primate, mouse, fly, fish, and worm has generated disease models. This single mutation in huntingtin is the triggering event that endows the protein with new toxic functions that are deleterious for brain cells. At the same time, it also impairs the ability of normal huntingtin protein to exert molecular activities that are fundamental for the survival and functioning of the neurons that predominantly degenerate in the disease (99, 668). Although a number of molecular dysfunctions have been elucidated and contribute to explain the early deterioration of the spiny-projection GABAergic neurons of the striatum, the exact mechanisms whereby mutation in huntingtin causes the observed neuronal degeneration, despite a ubiquitous expression, are still unclear. Evidence shows that the pathophysiology of HD may arise both from cell autonomous processes within vulnerable neurons and dysfunction of interneuronal interactions, specifically at the level of the cortical-striatal afferents (101, 176, 274, 668).

The aim of this review is to outline the advances in understanding the molecular pathogenesis of HD by discussing the multiple research approaches that have been undertaken since the discovery of the HD gene in 1993. We focus on the function and dysfunction of normal huntingtin and on the known molecules and pathways that are affected in HD. Moreover, we describe how some of these molecules and downstream effectors are becoming the next targets for the development of therapeutics. State of the art therapeutical approaches will be presented as well as the emergent technologies aimed at eliminating mutant huntingtin or at replacing the lost cells. We also describe how some of these targets have been exploited in peripheral cells in the search for biomarkers allowing for the monitoring of disease progression, phenoconversion, and drug efficacy in HD sufferers.

II. GENERAL INTRODUCTION TO HUNTINGTON'S DISEASE

A. Historical Background

HD is also known as Huntington's chorea. Although an epidemic of dancing mania was described in 1374, it was Paracelsus (1493–1541) who first used the term *chorea* to define this movement disorder, suggesting its central nervous system (CNS) origin. In the following years, until the 17th century, the disease had remained obscure and its nature had not been understood. In 1600, English colonists used the name "that disorder" or "San Vitus" dance to refer to HD. In those days, people with chorea, because of the involuntary muscle jerks and twitches characteristic of HD, were often thought to be possessed by the devil. It is believed that at least one of the alleged witches executed in Salem (Massachusetts) in the 1690s had HD.

A first attempt of a medical description for HD as "chronic hereditary chorea" was made two centuries later, in the 1840s, by physicians in the United States, England, and Norway. However, the first accurate description of the disease came about 30 years later, in 1872, by a 22-yr-old American doctor, George Huntington, working in Long Island, New York, who wrote a brief, uniform, anecdotal, and entirely unreferenced paper called *On Chorea* published in the Medical and Surgical

Reporter of Philadelphia (volume 26, no. 15, April 13, 1872). A closer description of how scientists and physicians have faced HD in the 19th century as well as the long history of prejudice and misunderstanding that characterized families affected by HD is contained in a recent book by historian Alice Wexler, from the UCLA Center for the Study of Women, entitled "*The woman who walked into the sea*" that offers a ground-breaking medical and social history of this disease. One of the central themes of the book is the hereditary nature of HD and how it has profoundly changed the approach to the disease and the social consideration of HD sufferers. The genetic nature of the disease led to more than a century of attempts to identify those large communities of persons at risk to develop HD. In the early 1920s, the American eugenicist Charles B. Davenport tracked families with inherited disorders, producing what was, at the time, the largest study of families with HD. Those years were also very productive in terms of knowledge of HD neuropathology because researchers first noted the deterioration in the central region of the brains of patients as the disease progresses, identifying the caudate nucleus as the central target of brain cell death in HD. Later, in the 1950s, Dr. Amerigo Negrette diagnosed HD in a large community of people living around Lake Maracaibo, Venezuela, which 20 years later became the center of a breath-taking crusade towards the discovery of the HD gene, made possible thanks to the remarkable efforts of Nancy Wexler, a neuropsychologist at Columbia University and cofounder of the Hereditary Disease Foundation (HDF), and of the many scientists and clinicians from the Boston area and other parts of the world.

B. Neuropathology

The pathology of HD is notably brain specific with prominent cell loss and atrophy in the caudate and putamen (464, 616). The most commonly used grading system to assess the severity of HD degeneration was developed by the neuropathologist Jean Paul Vonsattel at Columbia University in 1985. It is based on the pattern of striatal degeneration in post mortem tissues and classifies HD cases into five different severity grades (0–4). Grade 0 appears indistinguishable from normal brains after gross examination. However, 30–40% neuronal loss can be detected in the head of the caudate nucleus upon histological examination. Grade 1 shows atrophy, neuronal loss, and astrogliosis in the tail and, in some cases, the body of the caudate nucleus. Grades 2 and 3 are characterized by a progressive severe gross striatal atrophy. Grade 4 includes the most severe HD cases with atrophy of the striatum and up to 95% neuronal loss (617).

A deeper neuropathological analysis of the HD striatum performed by Robert Ferrante and colleagues at Massachusetts General Hospital, Boston (181–183, 185, 615) revealed that different degrees of degeneration could be observed within the striatal neuronal population. GABAergic medium-sized spiny neurons were found to preferentially degenerate in HD, whereas medium-sized aspiny cholinergic interneurons containing somatostatin, neuropeptide Y, or NADPH diaphorase (or nitric oxide synthase) are relatively spared (615). Further immunohistochemical studies performed by Anne Young's group, originally at the University of Tennessee and now at the Massachusetts General Hospital, revealed differential loss of striatal projection neurons in HD. In early and middle stages of HD, enkephalin-containing neurons projecting to the external segment of the globus pallidus were much more affected than substance P-containing neurons projecting to the internal pallidal segment. Furthermore, substance P-containing neurons projecting to the substantia nigra pars reticulata were more affected than those projecting to the substantia nigra pars compacta. At the most advanced stages of the disease, projections to all striatal target areas were depleted, with the exception of some apparent sparing of the striatal projection to the substantia nigra pars compacta (464). However, the extreme striatal atrophy and the loss of neurons observed in grade 4 indicate that both spiny and aspiny neurons are vulnerable at the end stage of the disease.

Although the striatum is the most profoundly affected region in HD, the clinical phenotype of HD is far more complex and variable than depictions of it as a progressive movement disorder dominated by neostriatal pathology represent. Early neuropathological studies showed that in grades 3 and 4, the cerebral cortex (particularly layers III, V, and VI), globus pallidus, thalamus, subthalamic nucleus, substantia nigra, white matter, and the cerebellum could be markedly affected (616). Recent work has also indicated that the hypothalamus can be significantly atrophied in HD patients (295, 449), which is in agreement with findings of loss of somatostatin-positive neurons in the lateral tuberal nucleus (318, 319) and of orexin (hypocretin)-secreting neurons in the lateral hypothalamus (444).

Advances in neuroimaging techniques have greatly contributed to a better understanding of HD pathology, providing correlations between morphological brain changes and the development of cognitive deficits in attention, working memory, and executive functions (68, 434, 473, 478). In 2003, neurologist Diana Rosas and colleagues at the Massachusetts General Hospital adopted magnetic resonance imaging (MRI)-based morphometric analysis and confirmed that subjects with HD had significant volume reductions in almost all brain structures, specifically in the cortex (475). Further studies from the same group revealed that such changes take place before symptoms onset (474, 480). Cortical involvement contributes to important symptoms, including those ascribed primarily to the striatum, and might explain much of the

clinical heterogeneity and complexity of HD (479). More recently, neurologists from many clinical sites in the United States and Europe have begun to explore the sensitivity, reliability, and reproducibility of neuroimaging methods to serve as a biomarker of HD onset and HD progression, and its potential to enhance the efficiency of clinical trials (see sect. VIIIA).

C. Symptoms

HD symptoms comprise adult-onset personality changes, generalized motor dysfunctions, and cognitive decline. The peak age of adult-onset HD is between 35 and 50 years. A small percentage of patients (10%) develop symptoms before age 20. This is a juvenile variant of the disease usually resulting from paternal transmission. Early onset is associated with increased severity as well as with a more rapid disease progression (45, 128). In the early stages, HD is classically associated with progressive emotional, psychiatric, and cognitive disturbances (38). Commonly reported symptoms in HD include progressive weight loss, alterations in sexual behavior, and disturbances in the wake-sleep cycle that occur very early in the course of the disease and may partly be explained by hypothalamic dysfunction (449). In the later stages, HD is characterized by motor signs, progressive dementia, or gradual impairment of the mental processes involved in comprehension, reasoning, judgment, and memory (38, 481). Due to increasingly severe dementia and progressive motor dysfunction, patients with advanced HD may become unable to walk, have poor dietary intake, eventually cease to talk, and become unable to care for themselves, therefore potentially requiring long-term institutional care. Life-threatening complications may result from injuries related to serious falls, poor nutrition, infection, choking, and inflammation. Most HD patients eventually succumb due to aspiration pneumonia because of swallowing difficulties (38).

D. Gene Hunters

The search for the HD gene began in a tiny community around Venezuela's Lake Maracaibo in the early 1980s, where the highest concentration of HD sufferers was found. Starting from 1979, a group of geneticists and physicians kept medical records, took blood and skin samples, and charted the transmission of the disease within families of the Lake Maracaibo community. From that experience, after having analyzed blood samples from as many HD sufferers as they could find, in 1983 a team composed of 14 scientists led by Joseph Martin from the Massachusetts General Hospital, which included Jim Gusella from the same Hospital and Nancy Wexler, made use for the first time of restriction fragment length polymorphism (RFLP) and linkage analyses to identify a polymorphic DNA marker on the fourth human chromosome predictably linked to HD (231). Soon after, 58 scientists from all over the world joined together into a team that was collectively named the Huntington Disease Collaborative Research Group that, after 10 years and under the guidance of Gusella and several others, reported the discovery of the gene responsible for HD and of its associated mutation (246). This team found that the disease was linked to the IT15 gene that was unlike any other previously identified human gene. Furthermore, it was found that the first exon of the IT15 gene contained a repetitive DNA element consisting of three nucleotides: C (cytosine), A (adenine), and G (guanine). When researchers examined this region of IT15 in non-HD controls, they found that the number of CAG repeats varied from 6 to 35; they described this phenomenon as "instability of the trinucleotide repeat." Analysis of the same region in the IT15 gene in individuals with HD showed that these people always had 40 or more CAG repeats; in fact, the largest number of CAG repeats the researchers detected at that time was 100 (246). It was concluded that the trinucleotide repeat expansion in the IT15 gene was responsible for HD. The IT15 gene is now renamed the huntingtin gene because of the name assigned to the protein.

Further studies revealed that some individuals with no symptoms who show "intermediate-sized" CAG repeats ranging from ~27 to 35 (22, 518) are at risk of transmitting the disease to their children, because of a phenomenon known as "genetic anticipation" (458). This phenomenon is explained by the fact that the expanded CAG repeats are not stable and tend to expand from generation to generation specifically when the disease gene is inherited from the father. During mitosis, the risk of expansion is more frequent in spermatogenesis, probably caused by replication slippage compared with oogenesis (439). Therefore, individuals with HD who inherit the disease gene from their fathers may have a longer CAG repeats tract and tend to develop symptoms at an earlier age than their fathers (458). In fact, the length of the expanded CAG repeats has some relation to the age of symptomatic onset (12, 246, 484). Patients with a large number of repeats tend to develop symptoms at an earlier age. Extremely large CAG repeats of 60 or greater are often associated with a disease onset during childhood or adolescence (juvenile HD). Such a correlation is less apparent in individuals with a shorter range of CAG repeats. This CAG repeat number only explains 40–50% of the variance in the age of onset and the remaining is influenced by environmental and genetic factors as, for example, paternal inheritance (12, 77, 458, 634). A recent clinical study shows that increasing CAG repeat size in the normal allele reinforces the association between mutant CAG expansion and disease severity and progression. In

subjects having the mutant CAG expansions in the low range, increasing size of the normal repeat correlated with more severe symptoms and pathology. In patients with a long CAG repeat, increasing CAG in the normal allele did not cause an exacerbation of the disease (29). These data indicate for the first time that the normal huntingtin allele can also influence disease severity. One possibility is that wild-type huntingtin with large CAG repeats could lead to a stronger association with mutant protein fragments, promoting their coaggregation and preventing them from aberrantly interfering with other proteins (88). A strong interaction between normal and mutant huntingtin could also result in a higher degree of loss of normal huntingtin function (see sect. III, *E* and *F*), leading to detrimental effects. Further investigations will be necessary to discriminate between beneficial and toxic effects of polyQ interactions.

E. Genetic Modifiers of HD

Although there is a correlation between CAG repeats length and age at onset of motor symptoms, HD patients may differ dramatically in age of onset and disease manifestations, despite similar CAG repeat lengths. Several studies revealed that a large set of genes distinct from the HD locus itself could contribute to modify disease onset and progression.

Early studies showed that genetic polymorphisms adjacent to the CAG repeats could influence the disease onset (13, 177, 484, 536, 618). To date, several genetic modifiers of HD have been described. All of these modifiers relate to various mechanisms implicated in HD pathology (see sect. IV) as excitotoxicity, dopamine toxicity, metabolic impairment, transcriptional deregulation, protein misfolding, and oxidative stress. Genetic analyses showed that patients carrying the Δ2642 glutamic acid polymorphism (a deletion of three nucleotides encoding for glutamic acid at codon position 2642–2645) develop the disease earlier than predicted by their CAG number in the HD gene (7, 13, 484, 618). Subsequent studies revealed that polymorphisms in genes encoding for the kainate-specific glutamate receptor GluR6 (485), the apolipoprotein E $\varepsilon 2\varepsilon 3$ genotype (302), the polymorphic $(Gln\text{-}Ala)_{38}$ repeat in the transcriptional coactivator CA150 (265), the *N*-methyl-D-aspartic acid (NMDA) receptor subunit 2B (GRIN2B) (17), the ubiquitin COOH-terminal hydrolase L1 (UCHL1) (388), TP53 and hCAD (109), apoptosis signal-regulating kinase 1 (ASK1), mitogen-activated protein kinase kinase 6 (MAP2K6) (18), and PPAR-γ coactivator 1α (PGC-1α) (562, 635) may be modifiers of age of onset in HD.

Additionally, genome-wide linkage scans (as, for example, in the HD-MAPS study) revealed potential loci that may contain genes that modify age at onset. Positive linkage signals have been identified at chromosomes 4p16, 4p16.3, 6p21–23, 6q23–24, and 6q24–26 (151, 203, 337, 338, 411). The demonstration of statistically significant linkage to a potential modifier locus opens the path to cloning of a gene capable of altering HD pathogenesis, which could one day provide a validated target for the development of therapeutics.

F. Modeling HD

Beyond what is currently feasible methodologically when using post mortem human brain samples, research on HD largely depends on animal (and cellular) models. In this section we offer an overview of the wide range of HD animal models available to the HD community. These models have been successfully used to investigate pathological pathways, molecular targets, and therapeutics (see Table 1).

1. Chemical models

Before the identification of the disease gene, HD animal models were produced by injecting neurotoxins into the striatum. The initial reports demonstrating that direct intrastriatal injection of kainate, a non-NMDA glutamate agonist, could mimic in rats the axon-sparing striatal lesion observed in the human HD, represented the starting point of a wide literature on the use of glutamate analogs to produce striatal selective neurodegeneration in rodents (379). Quinolinic acid and kainic acid have been the two most commonly used agents to produce rodent and non-human primate models of HD, suggesting that excitotoxicity could participate in the cell death observed in the disease (42, 43, 129). Later studies indicated that injection of mitochondrial toxins such as 3-nitropropionic acid and malonic acid were capable of replicating some of the behavioral aspects of HD in rats, indicating that mitochondrial dysfunction may also participate in HD pathogenesis (78) (see sect. IV*F*). These chemical models were welcomed also because they replicate the regional selectivity of HD neuropathology. However, they are unable to reproduce the pathophysiological mechanisms induced by the mutant gene. Nonetheless, they still remain good models to study neuroprotection and neurorestorative therapies in HD (see sect. VII).

2. Genetic models

Thanks to the availability of several genetic models of the disease, it is now possible to monitor the actions of either normal or mutant huntingtin at tissue and subcellular levels at different time points.

In particular, HD cell lines, which allow the stable or inducible expression of wild-type or mutant huntingtin, have been useful for the dissection of disease mechanisms, and they have been recently exploited for the

TABLE 1. *Rodent genetic models of Huntington's disease*

	R6/1 (Mangiarini et al., Ref. 365)	R6/2 (Mangiarini et al., Ref. 365)	N171-82Q (Schilling et al., Ref. 513)	YAC128 (Slow et al., Ref. 533)
		General feature		
Animal	Transgenic mouse	Transgenic mouse	Transgenic mouse	Transgenic mouse
Construct	1.9-kb fragment from the 5′ of human Htt	1.9-kb fragment from the 5′ of human Htt	First 171 amino acids of human Htt	Yeast artificial chromosome expressing full-length human Htt gene
Promoter	Human Htt	Human Htt	Mouse prion protein promoter	Human Htt
CAG	113	144	82	128
Onset of symptoms	15–21 wk	5–6 wk	10 wk	8–12 wk
Survival	32–40 wk	12–15 wk	10–24 wk	Normal life span
		Neuropathology		
NIIs/cell pathology	NIIs and neuropil aggregates throughout the brain	NIIs and neuropil aggregates throughout the brain	NIIs in cortex, hippocampus, amygdala, and striatum	EM48 positive inclusions in striatal cells, no NIIs detected
Brain atrophy and cell loss	Overall brain atrophy, reduced brain volume	Overall brain atrophy, reduced brain (44%) and striatal (41%) volume at 12 wk	Overall brain atrophy, cells with degenerative morphology (toluidine blue assay) at 20 wk	Reduced striatal (10–15%) and cortical (7–9%) volume at 48 wk, reduced striatal (9.1%) and cortical (8.3%) neuron number at 48 wk
	No evidence of massive cell death	No evidence of massive cell death		
Neuronal dysfunction	Aberrant synaptic plasticity	Aberrant synaptic plasticity	Reactive astrogliosis	Increase in NMDA, AMPA, mGLURI and II receptor binding. No change in striatal dopamine, $GABA_{A/B}$ or adenosine receptor binding
	Reduced expression of mGLURs, D1-D2 and CB1 receptors	Reactive astrogliosis		
		Decrease in D1-D2, mGLURII, AMPA, kainate and A_{2A} receptors		
		Symptoms		
Motor	Clasping behavior (onset 20 wk)	Clasping behavior (onset 8 wk)	Clasping behavior (onset 15 wk)	Clasping behavior
	Rotarod deficit	Rotarod deficit (5 wk)	Rotarod deficit (15 wk)	Rotarod deficit (24 wk)
	Gait abnormalities	Hypercinetic movements Resting tremors Circling behavior	Hypokinesis Resting tremors Tremors and gait abnormalities	Hyperkinesis (12 wk) and Hypokinesis (24 wk) Gait abnormalities
		Increase in limb movements Decrease in grip strength	Loss of coordination Muscle weakness	Circling behavior Ataxia
Cognitive	Decrease anxiety	Rigidity in cognitive process Increased exploratory behavior at 4 wk that declines and ends by 8.5 wk	Deficit in working memory Rigidity in cognitive process	Depressive behavior (12 wk)
Others	Progressive weight loss	Progressive weight loss Seizures, diabetes, and cardiac dysfunction	Progressive weight loss	Weight increase

NIIs, nuclear inclusions.

screening of therapeutics (468, 528, 606). The actual effort is towards the production of novel in vitro cellular systems based on the propagation and differentiation of neural stem cells bearing the mutant gene that can be used for drug discovery and toxicology tests in short-term applications (129). More recently, the induced-Pluripotent Stem (iPS) technology was used for the pathological modeling of Spinal Muscular Atrophy (SMA) (170), and efforts are currently underway to derive iPS cells from HD patients (150, 430).

TABLE 1. —*Continued*

BACHD (Gray et al., Ref. 221)	Transgenic HD rat (Von Horsten et al., Ref. 614)	HdhQ92-111 (Wheeler et al., Ref. 636)	HdhQ140 (Menalled et al., Ref. 382)	Hdh(CAG)150 (Lin et al., Ref. 349)
Transgenic mouse	Transgenic rat	Knock-in mouse	Knock-in mouse	Knock-in mouse
BAC expressing full-length human Htt gene	1,962-bp rat Htt fragment	Replacing exon 1 of mouse Htt with a mutant human exon 1	polyQ sequence inserted into the endogenous mouse Htt gene	polyQ sequence inserted into the endogenous mouse Htt gene
Human Htt	Endogenous rat Htt promoter	Mouse Htt promoter	Mouse Htt promotor	Mouse Htt promotor
97	51	92–111	140	150
12 wk (but symptoms become robust by 6 mo of age)	40–50 wk	96 wk	12 mo	4 mo
Normal life span	98 wk	Normal life span	Normal life span	Normal life span
mHtt inclusions in neuropil and few in cortex and striatum (48 and 72 wk)	Neuropil aggregates and nuclear inclusion in striatum and less extent in cortex (72 wk)	NIIs and striatal neuropil aggregates (68 wk)	Nuclear and neuropil inclusion bodies in striatum, cortex, hippocampus, and cerebellum (16–24 wk)	Striatal NIIs in striatum (37 wk)
Brain atrophy	Enlarged lateral ventricles	No brain atrophy observed	No brain atrophy observed	Cellular dysfunction revealed by dark bodies surrounding cytoplasmic vacuoles
Cortical and striatal volume	Focal lesions in the striatum	No cell loss observed	No cell loss observed	
Degenerating darkly stained neurons (14%) in striatum	No significant cell loss	Cells (3.5%) with degenerative morphology (toluidine blue)		
Reduced excitatory neurotransmission mediated by AMPA receptors	Reduced brain glucose metabolism	Striatal gliosis		Striatal gliosis
				Axons degeneration
Clasping behavior not reported	Progressive impairment of coordination and balance	No clasping behavior	Decrease in locomotor activity	Clasping behavior
Rotarod deficit (by 8 wk and progressed by 24 wk)	Dyskinesias of the head	No rotarod deficit	Hyperactivity and hypoactivity	Rotarod deficit (100 wk)
	Gait abnormalities		Gait abnormalities	Hypoactivity Gait abnormalities
	Reduced anxiety-like behavior, emotional and cognitive decline	No symptoms	No symptoms	No symptoms
Weight increase	Weight loss	No abnormal weight loss	No abnormal weight loss	Reduced size

A wide variety of species, including the invertebrate *Caenorhabditis elegans* and *Drosophila melanogaster*, nonmammalian species as *Danio rerio* and mammals, such as mouse and rat, have also been genetically engineered to express the HD mutation.

A large number of mouse models of HD that show different degrees of similarity to the human condition have been produced. Models that express either truncated or full-length human or mouse mutant huntingtin display significant phenotypic differences that may be attribut-

able to the influence of the protein context, mouse strain, or regulatory sequences between the mouse and human huntingtin genes. To overcome these problems, some researchers are considering also the generation of large HD genetic models such as sheep, minipig, and the non-human primate. With their size, organ capacity, and physiology resembling in several aspects that of humans, these models may be well-suited for preclinical trials and long-term safety studies, although in some cases, ethical concerns have been raised.

In this section, we describe mammalian and non-mammalian HD animal models that have particularly enlightened in the search for targets and for compounds capable of interfering with mutant huntingtin toxicity.

A) TRANSGENIC MICE. In a pioneering study, Gillian Bates and her group at the Guy's Hospital in London (137, 365) created the first transgenic mouse line by inserting a 1.9-kb fragment containing the human huntingtin promoter and exon 1 of the human huntingtin gene bearing 144 CAG repeats. These mice, known as the R6/2 mouse line, exhibit both early and severe behavioral and anatomical symptoms (137, 365) (see Table 1). Evaluation with learning and memory tasks shows abnormalities as early as 3.5 wk of age, and simple motor tasks, such as the rotorod and beam walking, reveal deficits by 5 wk of age (97, 350, 547). R6/2 mice exhibit neuroanatomical abnormalities including progressive reduction in brain and striatal volume by 5 wk, substantially reduced striatal neuron number by 12 wk, and death by 12–15 wk (137, 365). A second mouse line, known as R6/1, that shows a less dramatic phenotype was also generated. The R6 lines are characterized by the presence of widespread nuclear inclusions of mutant huntingtin in brain neurons (see sect. IV*D*) that increase steadily in number, size, and distribution as disease progresses (137, 400, 547). Striatal dopamine D1 and D2 receptors, which are widely distributed on the dendrites of striatal projection neurons, are decreased as early as 8 wk of age, consistent with both early striatal neuronal dysfunction and neurodegeneration (103, 104). Due to their extended polyQ, the R6 lines are considered more representative of the juvenile than the adult human HD phenotype. The early onset of symptoms and a fast progression of the disease make this mouse line particularly useful for therapy screening, but less suitable for the investigation of early disease mechanisms (208). The R6/2 line has been the major tool for preclinical pharmacology studies for HD, and a substantial number of interventions have been evaluated with this mouse line (208).

A similar neuropathological and behavioral phenotype was characterized in a transgenic line, N171–82Q, later obtained by David Borchelt's laboratory at the Johns Hopkins University. This model expresses 171 amino acids of the human huntingtin with 82 CAG repeats under the control of the mouse prion protein promoter that restricts the expression of the mutant protein to brain neurons (see Table 1; Ref. 513). Intranuclear inclusions and neuritic aggregates were found in the brain of N171–82Q mice, resembling the human phenotype. Compared with the R6 mice, the N171–82Q model has fewer polyglutamine repeats resulting in a later onset of symptoms, which makes it an attractive model for the study of presymptomatic therapies, also allowing for a longer experimental window during which therapies can be administered before the pathological sequelae of the disease commence.

One of the questions in the field is whether dysfunctions in HD depend on cell autonomous mechanisms affecting the striatal neurons and/or on defects in the brain circuitries (non-cell-autonomous mechanisms). To address this question, mutant huntingtin expression was confined in the forebrain by driving the expression of the transgene under the control of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα) and neuron specific enolase (NSE) promoters (326, 648). Subsequent studies aimed at an even more selective expression of the mutant protein in cortical or striatal neurons (224, 226). Out of this set of studies, one of the best results in favor of cell-autonomous mechanisms comes from the work of Ai Yamamoto, Jose Lucas, and Rene Hen (648), who produced the first conditional mouse model of HD (see Table 1). This transgenic mouse for huntingtin exon 1 contains 94 CAGs (HD94) in which the bidirectional transgene was activated in the forebrain by doxicycline removal and developed neuropathological and progressive motor dysfunction. Importantly, when the expression of the transgene was switched off, amelioration of motor signs and neuropathology were observed. The improvement was mainly attributed to the disappearance of mutant huntingtin aggregates from brain neurons, pointing at aggregates as an important cell-autonomous mechanism of toxicity (see sect. IV*D*). On the other hand, by using a constitutive NSE promoter that directed the expression of an NH_2-terminal 3-kb portion of human huntingtin cDNA bearing 100 CAG repeats, Neil Aronin's group at the University of Massachusetts Medical School (326) demonstrated the involvement of the corticostriatal pathway in developing behavioral phenotypes by analyzing NMDAR activation in electrophysiological tests. In line with these findings, William Yang and colleagues at the University of California Los Angeles (224, 226, 326) showed that the selective expression of mutant huntingtin in either cortical or striatal neurons is insufficient to cause a disease phenotype. These studies demonstrated that beyond cell autonomous mechanisms, cell-cell interactions mechanisms are critical to elicit HD pathogenesis in vivo (224, 226, 326). More recently, Michelle Ehrlich's team at the Mt. Sinai School of Medicine, New York, (80) has produced a transgenic mouse that selectively expresses mutant huntingtin in the medium spiny neurons (MSNs), specifically excluding the

neocortex. The observation that these mice develop a number of abnormalities characteristic of pan-cellular HD mouse models, including intranuclear inclusion bodies, motor impairment, and changes in striatal gene expression, raises the point that cell-autonomous events intoxicate neurons in HD. However, this evidence hardly provides explanation for the selective neuronal vulnerability which is typical of HD. Mutant huntingtin aggregates are also found in peripheral cells of transgenic mice (61, 397, 504), suggesting that either aggregates are not as deleterious in non-CNS tissues or that, in brain, additional components come into play to trigger neuronal cell death.

Transgenic mice expressing full-length huntingtin have in some cases been more successful than NH_2-terminal fragment models in terms of neuronal loss and capability to recapitulate more faithfully the sequence of events leading to HD. Four full-length HD mouse models have been produced so far (see Table 1). The first mouse model expressing a full-length IT15 cDNA clone with a CAG repeat tract in the pathological range driven by the cytomegalovirus (CMV) promoter was produced in 1998 (463). An HD-like behavioral phenotype was observed in these mice, which was accompanied by selective neuronal loss in the striatum. This mouse line was then discontinued. Michael Hayden's group at the University of British Columbia (262) created yeast artificial chromosome (YAC) transgenic mice expressing a full-length genomic HD gene transcript with a 25 kb of upstream sequence and 120 kb of the downstream sequence to ensure the presence of all endogenous regulatory regions. The remarkable battery of YAC mice thus generated included mice carrying 18, 46, 72, and 128 CAG repeats (named YAC18, YAC46, YAC72, and YAC128 mice, respectively). YAC128 mice are especially interesting because they show a uniform phenotype with age-dependent striatal and subsequent cortical neurodegeneration, and development of well-characterized progressive motor and cognitive deficits (598, 602). YAC128 mice exhibit motor abnormalities as early as 3 mo of age with increased open field activity, followed by rotarod performance abnormalities at 6 mo of age. Behavioral deficits are progressive, and by 12 mo, open field activity is diminished significantly compared with controls (602). Diffuse mutant huntingtin nuclear immunoreactivity is abundant in striatal neurons at 3 mo of age and then becomes more widespread in cortical, hippocampal, and cerebellar neurons at 12 mo of age while no nuclear inclusions (NIIs) were detected (598). Slow and colleagues in Hayden's laboratory (533) produced serendipidously the so-called "Short-Stop" mouse bearing a CAG expanded huntingtin gene truncated after intron II. Compared with YAC128, this model displayed no clinical evidence of neuronal dysfunction and degeneration as determined by brain weight, striatal volume, and striatal neuronal count despite the presence of aggregates. This finding suggests that inclusions are not pathogenic in vivo and that soluble fragments of mutant huntingtin may be more toxic. Notably, YAC128 mice containing a selective mutation of the caspase-6 cleavage site are protected from neuronal dysfunction and neurodegeneration in vivo (see sect. IV*C*) (220). Despite the presence of behavioral abnormalities and evidence of striatal neuron loss, YAC128 mice do not exhibit any decrease in a wide array of striatal neurotransmitter receptor binding sites that have been described in other murine genetic models and in human HD (51). In the same years, Lisa Ellerby's group at Buck Institute, Novato, California (566) has contributed to enlarge the number of available full-length models by producing a conditional HD mouse in which full-length human huntingtin is expressed in the brain under the control of the tet-transactivator (tTA) driven by the prion promoter PrP. In the absence of doxicycline, these mice display a progressive behavioral phenotype consisting of slowed and irregular voluntary movements, gait ataxia, tremor and jerky movements, uncoordination, weight loss, and a shortened life span. Neuropathology included prominent intranuclear inclusions in cortex and striatum as well as cytoplasmic aggregates (566). In particular, an ~60-kDa fragment, which appears to represent an NH_2-terminal cleavage product, accumulates in nuclei, indicating that proteolytic processing is part of HD pathogenesis (see sect. IV*C*). More recently, mice bearing a bacterial artificial chromosome (BAC) with the human full-length gene carrying 97 CAG repeats have been generated in William Yang's laboratory (221). These mice exhibit progressive motor deficits and selective late-onset neuropathology in cortex and striatum, thereby representing a novel and robust in vivo model for HD pathogenesis and treatment studies. BACHD mice show progressive motor deficits in rotarod performance starting from 2 mo of age, neuronal synaptic dysfunction, and late-onset selective neuropathology, which includes significant cortical and striatal atrophy and numerous darkly degenerating neurons in striatum. Unlike previous full-length mutant huntingtin mouse models, BACHD mice do not show early and diffuse nuclear accumulation of aggregated mutant huntingtin in striata or cortices (221). By 12 mo of age, BACHD brains have only a few small aggregates predominantly in the neuropil in the cortex and very tiny aggregates in the striatum (221), suggesting that diffuse nuclear accumulation of aggregated mutant huntingtin in striata or cortices is not necessarily associated with the slowly progressive and selective pathogenic process in the BACHD mice.

B) KNOCK-IN MICE. In classic HD transgenic mice, the exogenous huntingtin gene inserts randomly, which implies a risk of interference with the activity of other genes not related to HD. Moreover, the transgene expression driven by artificial promoters may lead to a phenotype that does not correctly mimic the disease, also as a consequence of the fact that the transgene is expressed above

physiological concentrations. Genetically precise mice that carry the mutation in the appropriate genomic and protein context and at a physiological concentration have been generated aiming at producing animals that more reliably replicate the pathogenesis of HD (see Table 1).

Knock-in mice have been produced by introducing pathogenic CAG repeats into the endogenous mouse HD gene (*Hdh*) located in chromosome 5 (349, 522) and/or by replacing mouse exon 1 with human exon 1 carrying expanded CAG repeats (275, 382, 636). Initial results were disappointing because these knock-in mice appeared to have a normal life span and did not show neuropathological signs of HD (522). No overt disease phenotype was observed in knock-in mice with 48, 90, and 109 CAG repeats in the endogenous *Hdh* locus (named, respectively, Hdh50, HdhQ92, and HdhQ111) (636). However, a closer examination of one of these models produced by Marcy MacDonald's group at the Massachusetts General Hospital (637) revealed subtle behavioral abnormalities at an early age and moderate striatal pathology at 2 years of age. More pronounced cellular dysfunction and progressive motor behavioral abnormalities were detected also in two other knock-in models characterized by the presence of longer CAG tracts (349, 382). The Hdh(CAG)150 mice produced by Peter Detloff's laboratory at the University of Alabama at Birmingham exhibit mutant huntingtin aggregates at ~9 mo of age (655) and weight loss, diminished activity, abnormal rotarod performance as well as a clasping phenotype that is indicative of neurological deficits at 2 yr of age (349). Similar phenotypes have been described in HdhQ140 mice (382, 383), which show that early behavioral abnormalities exist in a wide range of motor and nonmotor functions starting at 1–4 mo of age followed by progressive gliosis (12 mo) and loss of striatal neurons at 2 yr of age (256).

These studies showed that knock-in mice reproduce canonical characteristics of HD, preceded by deficits that may correspond to the protracted premanifest phase of the disease in humans. These models can be very important for the study of the early and mild neuronal abnormalities that might be primarily responsible of early functional deficits. Thus knock-in models may become useful to evaluate the ability of potential treatments to delay the onset of early abnormalities.

c) RAT MODELS OF HD. Research performed in rats over mice benefits from the availability of a larger set of behavioral and imaging tests that are suitable to identify neurological deficits such as those occurring in neurodegenerative diseases. Rats therefore become an ideal model for the evaluation of novel therapeutic approaches in longitudinal in vivo studies. In a first attempt to model HD in rats, the first 171, 853, and 1,520 amino acids of mutant huntingtin with 44, 66, and 82 CAG repeats, respectively, driven by either the phosphoglycerate kinase 1 (PGK) or the CMV promoters, were delivered to the rat striatum via lentiviral vectors (138). This strategy appeared to provide a robust acute in vivo model for selective neurodegeneration. However, the discrete, locally and temporally confined expression of mutant huntingtin represents a limit for testing drugs in longitudinal studies. To overcome local transduction of the transgene and allow a constitutive widespread expression of mutant huntingtin, Olaf Riess' group at the University of Tübingen (614) produced the first transgenic rat model of HD, bearing a 1,962-bp rat HD cDNA fragment carrying a 51 CAG repeat expansion under the control of the endogenous rat HD promotor (see Table 1). Behavioral and neuropathological analyses showed that the HD rat is characterized by adult-onset disease with behavioral phenotypes that are paralleled by histopathological alterations in the brain. The HD transgenic rat represents a valuable model for the investigation of disease phenotypes, their exploitation in longitudinal studies, and testing the efficacy of pharmacological treatments. Recent advancements in the stem cell field have led to the isolation of rat embryonic stem cells (87). It is thus expected that a number of knock-in rats for a number of human neurodegenerative diseases will be produced, providing for the first time the remarkable possibility of working with a genetically precise rat model of HD.

d) LARGE-ANIMAL MODELS OF HD. Non-human primates hold great promise for the study of human neurological disorders, for which currently available experimental models are still imperfect. Anthony W. S. Chan and his research team at Emory University (651) produced the first HD transgenic rhesus macaques, showing the feasibility of generating valuable non-human transgenic primate models of HD. The Emory research team developed this transgenic monkey model by introducing altered forms of the huntingtin gene into monkey eggs using a viral vector expressing exon 1 of the human huntingtin gene with 84 CAG repeats and green fluorescent protein. The eggs were fertilized and the resulting embryos were introduced into surrogate mothers, resulting in five live births (651). The transgenic monkeys exhibit important clinical features of HD, including dystonia and chorea (651).

Miniature pigs and sheep are also steadily gaining importance as large-animal models. With their size, organ capacity, and physiology resembling in several aspects that of humans, these animals are well suited for preclinical experiments and long-term safety studies. Russell Snell at the University of Auckland (in collaboration with the South Australian Research and Development Institute and Massachusetts General Hospital) (279) has generated the first sheep model of HD expressing the full-length human huntingtin gene with 73 CAG repeats. Jan Motlik and his team at the Academy of Sciences of the Czech Republic (268) are in the process of generating the first HD transgenic minipig.

E) NONMAMMALIAN MODELS. Genetic manipulations in the mouse, rat, sheep, minipig, and non-human primate are costly and time consuming. In addition, ethical concerns may be associated with the use of large-animal models. This generated the need for simpler, faster (in time course), and lower costing models.

A first example is represented by yeast. Although yeast is unicellular, the simplicity and genetic manipulability of the system have provided some mechanistic insights (320, 386) together with a very useful drug discovery platform. HD has been also modeled in *C. elegans* where neurodegeneration was found to be dependent on both age and polyglutamine tract length (174, 432). *C. elegans* allows the development of rapid and inexpensive in vivo assays to evaluate the efficacy of numerous candidate compounds identified in high-throughput screens (613). The fly, in particular *Drosophila melanogaster*, is an excellent choice for modeling neurodegenerative disease because it owns a fully functional nervous system. Most importantly, in *Drosophila*, foreign genes can be engineered and expressed in tissue-specific and temporal regulated patterns, and an impressive array of genetic tools are available. HD has been one of the first genetic diseases to be modeled in *Drosophila*. The expression of the polyQ repeat-expanded NH_2-terminal fragment of human huntingtin resulted in progressive neurodegeneration of the fly eye, progressive loss of motor coordination, and reduced viability (230, 369). Similar to the human situation, the age of onset and severity of neuronal degeneration correlated with the repeat length. All these features make HD *Drosophila* models useful in investigating molecular mechanisms of the disease and also in drug-screening strategies. HD has also been modeled in *Danio rerio* (zebrafish) that replicates two central features of the human disease: polyQ length-dependent toxicity and aggregation (393). Although these features are also readily recapitulated in other model systems, zebrafish embryos offer certain advantages for modeling polyQ diseases: they develop rapidly and externally, can be produced quickly in large numbers, and are transparent, permitting direct analysis of organs, tissues, and fluorescently tagged proteins. Moreover, HD zebrafish models could be used for drug-screening purposes.

Although many studies have documented the positive aspects associated with nonmammalian models, one should consider that these models are distant from the human pathology, and the risk that the molecular pathways implicated may be very different should not be underestimated.

The evidence described above indicates that many HD models are available, but a model that reproduces the full constellation of changes that compose the characteristic neuropathological phenotype seen in human HD is still missing. Therefore, the choice of a particular model depends on the question under investigation. Toxin-based models still have a role in investigating cell replacement approaches to HD, but most experimental hypotheses, especially those involving therapeutic interventions, require access to a genetic model, the choice of which, again, is strongly dependent on the experimental question. One crucial problem that has arisen in the last year is that behavioral and neuropathological phenotypes of HD genetic models have sometimes been plagued by inconsistent results across laboratories. This may stem from the lack of standardized husbandry and testing conditions, in addition to the intrinsic differences between the models. To overcome this problem, a first extensive standardized cross-characterization of several HD animal models has been performed (381), which paves the way for the use of standardized behavioral testing of motor and sensory-motor function. This is a critical step to obtain comprehensive, reproducible, and comparable results across HD models in preclinical research.

III. THE NORMAL FUNCTION OF HUNTINGTIN

Although it is well established that the disease occurs as a consequence of an expanded polyQ above 35 and that the polyQ length accounts for the disease onset, some evidence now also points to the loss of physiological activities of the normal protein as contributing to disease pathogenesis and, in particular, to its selectivity. Because the polyQ itself is present also in other proteins that cause at least eight different neurodegenerative diseases characterized by the loss of different types of neurons, in 2001 we proposed that it was the different proteins in whose backbones the CAG is expressed to identify the neurons that die. If such proteins have crucial functions for the selectively dying neurons in the disease, the resulting death may be directly attributable to the loss of those functions (98).

Accordingly, a number of findings now indicate that the ubiquitously expressed huntingtin protein has physiological functions that are particularly important for the brain neurons affected in HD. As a consequence of the mutation of this protein, reduced wild-type huntingtin physiological activity may render striatal neurons particularly vulnerable (99, 667). Importantly, there is evidence that reduction in wild-type huntingtin levels or of its activities can contribute to the pathogenesis of HD, thus affecting the development of therapeutic strategies.

In this section, we first describe the available data defining the structure and evolution of huntingtin, with a particular emphasis on how this study contributes to the understanding of huntingtin function. We then review the studies investigating the biological function of huntingtin during development and in the adult brain neurons. Finally, we describe the evidence indicating that loss of normal huntingtin function contributes to the pathogenesis of HD.

A. Huntingtin Through Evolution

Most of the known huntingtin protein homologs belong to vertebrates and show a high degree of conservation throughout their length, thus offering limited insights into the understanding of the protein function. The gene encoding for huntingtin in vertebrates is composed of 67 exons and covers a region of ~170 kb. The most divergent vertebrate species (i.e., *Homo sapiens* and the *Fugu* fish) show 80% conservation. Interestingly, because of the presence of shorter introns, the *Fugu* gene contains 67 exons as the human gene, but it spans over a region of 22 kb (503). The only entirely known invertebrate amino acid sequence is from *Drosophila melanogaster*, in the protostome branch, which is characterized by five 20–50% conserved regions distributed throughout the length of the protein (344, 660). We therefore speculated that *Drosophila* huntingtin represents a residue of the ancestral huntingtin molecule at the origin of the protostoma-deuterostoma branches, suggesting that huntingtin is, evolutionarily, an old gene (99). In line with this hypothesis, huntingtin is present in an old deuterostome, the tunicata *Halocynthia roretzi* (sea pineapple) and in the echinodermata *Heliocidaris herithrogramma (*sea urchin) (296). More recently, work from the group of Miguel Andrade and colleagues at the Max-Delbruck Center for Molecular Medicine in Berlin (424) has predicted the presence of the protein in ancient organisms such as the amoeba *Dyctostelium discoideum* and nematode *C. elegans*, but not in the *Saccharomyces cerevisiae* or in previously divergent plants, thus confirming the ancient origin of huntingtin (424).

In an attempt to infer information about functions of the protein that have evolved over time as well as the presence of possible domains in the protein, in 2004 we started collecting huntingtin protein sequences from the available databases and began the sequencing and cloning of the complete huntingtin cDNA from different ancient organisms that represent, especially for the development and structure of the central nervous system, key points of the evolutionary tree. Particularly, we reconstructed the huntingtin amino acid sequence of echinodermata (*Strongylocentrotus purpuratus*, sea urchin), chephalocordata (*Branchiostoma floridae*, lancelet), and tunicata (*Ciona intestinalis)* (93, 213, 571). Ciona and lancelet were chosen because they represent important steps in the evolution of the nervous system, which becomes here structured with an anterio-posterior polarity in the dorsal part of the organism. Sea urchin, the oldest still living deuterostome, is particularly informative because of the presence of a very primitive nervous system that is organized into radial nerves in the adult, joined at their proximal ends by a series of commissures to form a circumoral nerve ring. The nervous system of lancelet instead, being preferentially organized in the anterior part of the body, is particularly informative because it represents a first attempt at cephalization.

By bioinformatic multialignment of huntingtin protein sequences, we found a more progressive and linear evolution of the sequence of huntingtin along the deuterostome branch, while a more heterogeneous evolution of the protein emerged in the protostome branch (571). We also reported that, surprisingly, huntingtin from the oldest deuterostome sea urchin is much more similar to that of vertebrate huntingtin than it is to that of the divergent tunicate *Ciona intestinalis* (213, 571). Also, huntingtin from lancelet is very similar to vertebrate huntingtin, probably because of the structure and organization of the lancelet nervous system, which is closer to the one of vertebrates by being preferentially organized in the anterior part of the body (chephalization) (93).

Three putative domains of huntingtin have been identified in our multialignment corresponding to human huntingtin amino acids 1–386 (htt1), 683–1,586 (htt2), and 2,437–3,078 (htt3). In particular, comparison of more divergent orthologs and quantification of evolutionary pressure on the three blocks revealed that the NH_2-terminal fragment (htt1) is the most recently evolved part of huntingtin, while the COOH-terminal part represents the most conserved portion among all animals, from sea urchin to insects and mammals. This fact, together with the presence of conserved functional amino acidic residues along the deuterostome branch, allows us to define the NH_2-terminal fragment as a possible recently evolved domain. We hypothesized that this evolving portion may be endowed with newly acquired neuronal activities that have emerged in the deuterostome branch (571). In contrast, the COOH-terminal portion may be endowed with primordial activities in nonneuronal tissues (571).

B. Structure

Huntingtin is a 348-kDa protein. This high molecular weight hampers the production of crystals and mass spectrometry studies to elucidate its structure. The consequence is that 17 years after the cloning of the IT15 gene, there are no clear data on the structure of the corresponding protein.

An obvious feature of the huntingtin protein is the polyQ at its NH_2 terminus. Huntingtin is also enriched in consensus sequences called huntingtin, elongation factor 3, protein phosphatase 2A, and TOR 1 (HEAT) repeats that are organized into protein domains important for protein-protein interactions. Here, we describe also posttranslational modifications of the protein and the presence of consensus sites for proteolytic enzymes (see Fig. 1).

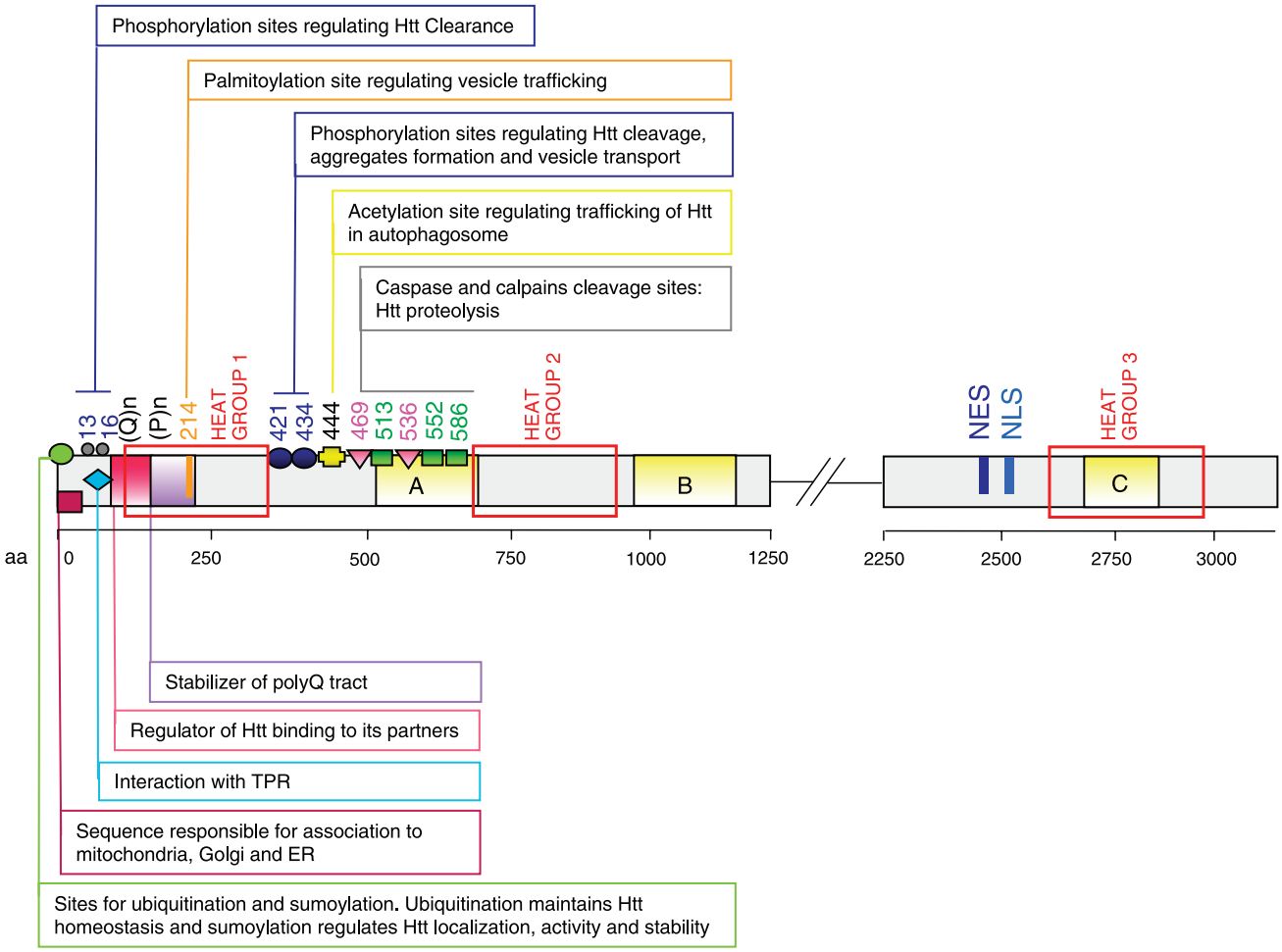


FIG. 1. Schematic diagram of the huntingtin amino acid sequence. (Q)*n* indicates the polyglutamine tract, which is followed by the polyproline sequence (P)*n*; the red emptied rectangles indicate the three main groups of HEAT repeats (HEAT group 1, 2, 3). The small green rectangles indicate the caspase cleavage sites and their amino acid position (513, 552, 586), while the small pink triangles indicate the calpain cleavage sites and their amino acid positions (469, 536). Boxes in yellow: B, regions cleaved preferentially in the cerebral cortex; C, regions of the protein cleaved mainly in the striatum; A, regions cleaved in both. Posttranslational modifications: ubiquitination (UBI) and/or sumoylation (SUMO) sites (green); palmitoylation site (orange); phosphorylation at serines 13, 16, 421, and 434 (blue); acetylation at lysine 444 (yellow). NES is the nuclear export signal while NLS is the nuclear localization signal. The nuclear pore protein translocated promoter region (TPR, azure) is necessary for nuclear export. Htt, huntingtin. ER, endoplasmic reticulum.

1. The polyQ

The polyQ stretch in human huntingtin begins at the 18th amino acid and, in unaffected individuals, contains up to 35 glutamine residues (246). In 2008, by providing the first huntingtin orthologs multialignment, we showed that the polyQ is an ancient acquisition of huntingtin, being a characteristic typical of the deuterostome branch (571). Its appearance dates back to sea urchin in which a NHQQ sequence is present, which consists of a group of four hydrophilic amino acids that can be considered biochemically comparable to the four glutamines (QQQQ) found in fish, amphibians, and birds. This finding has led us to speculate that at the base of the protostome-deuterostome divergence, the huntingtin ancestor possesses a huntingtin protein with a single Q or no Q in the corresponding position. The polyQ has then expanded gradually in mammals to become the longest and most polymorphic Q in humans. Quite interestingly, rodents show a shorter polyQ (7 and 8 Q in mouse and rat, respectively) inverting the evolutionary trend (see Fig. 2). In higher vertebrates, and specifically in mammals, the polyQ region is followed by a polyproline (polyP) stretch that is therefore a recent and sudden acquisition in huntingtin evolution. It was suggested that the polyP function may reside in the stabilization of the polyQ tract by keeping it soluble, and it is interesting to note that it has emerged in concomitance with longer polyQ stretches (549).

In 1994, Nobel Laureate Max Perutz and his team at the Medical Research Council Laboratory of Molecular Biology in Cambridge (442) showed that the polyQ forms a polar zipper structure and suggested, for the first time, that its physiological function was to bind transcription

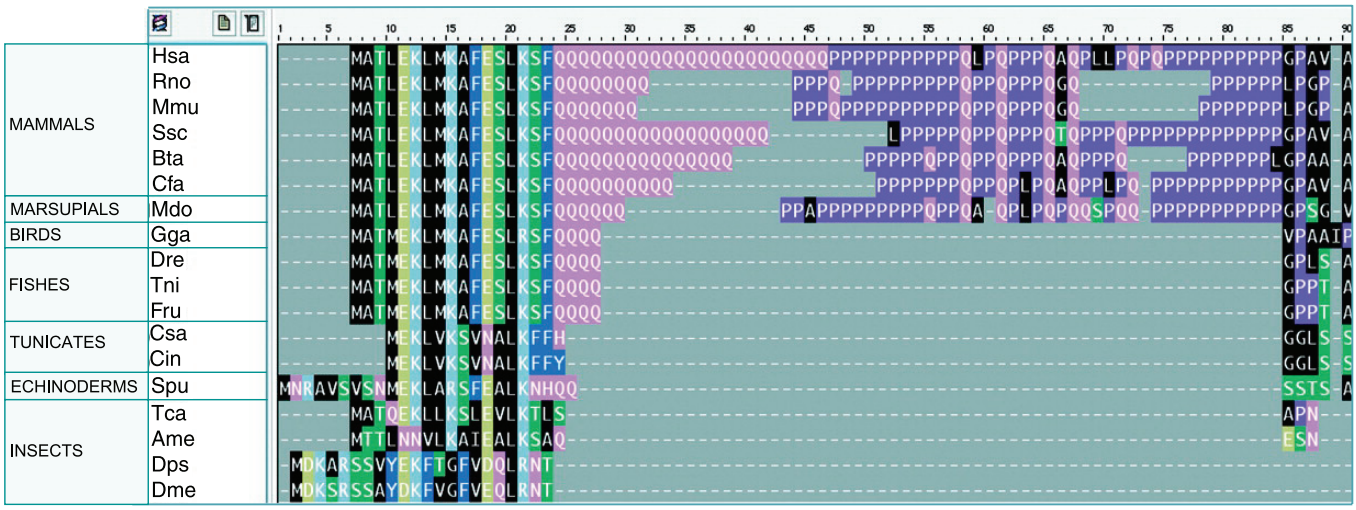


FIG. 2. The evolution of the polyQ region in huntingtin. Details of the multiple alignment (NH_2 terminus amino acid sequences) are listed following the phylogenetic tree. At the base of the protostome-deuterostome divergence, the ancestor possessed one htt with a single Q or no Q in the corresponding position, and only deuterostome homologs show a double Q that is maintained until the vertebrates, in which the first real "polyQ" tract was established (QQQQ in fish). The *Ciona* genus lost this characteristic (*Ciona huntingtin* has no polyQ tract, which is replaced by an aromatic group) and also evolved other specific and typical tracts. The four glutamines in vertebrates are stably maintained in fish, amphibians, and birds. The polyQ expands gradually from opossum to *Sus scrofa* to join the longest and most polymorphic Q in humans (which spans from 15–21 to 35 in normal huntingtin). Species abbreviations are as follows: *Homo sapiens* (Hsa); *Rattus norvegicus* (Rno); *Mus musculus* (Mmu); *Sus scrofa* (Ssc); *Bos taurus* (Bta); *Canis familiaris* (Cfa); *Monodelphis domestica* (Mdo); *Gallus gallus* (Gga); *Danio rerio* (Dre); *Tetraodon nigroviridis* (Tni); *Fugu rubripes* (Fru); *Ciona savignyi* (Csa); *Ciona intestinalis* (Cin); *Strongylocentrotus purpuratus* (Spu); *Tribolium castaneum* (Tca); *Apis mellifera* (Ame); *Drosophila pseudoobscura* (Dps); and *Drosophila melanogaster* (Dme).

factors that contain a polyQ region. It has now been shown that the polyQ tract is a key regulator of huntingtin binding to its partners and that huntingtin interacts with a large number of partners (242). One possible hypothesis is that wild-type huntingtin function during development and in adults may arise from the binding of different sets of interactors. This hypothesis is supported by the presence of HEAT repeats along huntingtin sequence that favors protein-protein interaction (9). Therefore, huntingtin might have flexible or multifunctional structures capable of assuming specific conformations and activities depending on its binding partners, subcellular location, and time of maturation in a given cell type and tissue. Bezprozvanny and colleagues at the University of Texas Southwestern Medical Center (309) used X-ray crystallography at atomic resolution to show that polyglutamine in huntingtin adopts multiple flexible conformations (α-helix, random coil, and extended loop). The structural data provided also hint that the polyQ repeat in huntingtin exon 1 may be influenced by the COOH-terminal polyproline region. In fact, the authors suggest that the polyproline region may serve both its known function as a protein-interaction domain and a less appreciated function as a protector against polyQ conformational collapse.

To determine the contribution of the polyQ stretch to normal huntingtin function, Erin Clabough and Scott Zeitlin at the University of Virginia School of Medicine (122) have generated mice with a precise deletion of the short CAG triplet repeat encoding 7Q in the mouse HD gene Hdh(DeltaQ/DeltaQ). Hdh(DeltaQ/DeltaQ) mice are born with normal Mendelian frequency and exhibit only subtle phenotypes, i.e., defects in learning and memory test. The authors suggest that the polyQ tract is not required for essential function of huntingtin but instead may modulate a normal function of huntingtin. In vitro studies on Hdh (DeltaQ) fibroblasts indicated that the polyQ contributes in modulating longevity and energy status. Further observations are needed to determine whether signs of senescence accumulate also in vivo.

2. *The HEAT repeats, a route towards huntingtin structure*

Downstream of the polyQ, the so-called HEAT repeats are found. They are ~40-amino acid-long sequences that occur multiple times within a given protein and are involved in protein-protein interactions (9, 406). Bioinformatic analyses have found 36 putative HEAT repeats in huntingtin (564). Subsequently, three main clusters of HEATs have been identified (363). A more recent study of HEAT repeats number and distribution revealed a total of 16 HEAT repeats in huntingtin, which are organized into 4 clusters (571). The repeats are well conserved in huntingtin from deuterostomes, suggesting that they are an ancestral feature in huntingtin evolution. In the protostome branch, *Drosophila melanogaster* huntingtin shows 28 putative consensus repeats whose degree of conservation with respect to humans has yet to be fully defined (564).

In 2001, Andrade et al. (10) reported that HEAT repeats could be organized in three-dimensional structures

called α-ROD. More recently, a new neural network for the prediction of α-rod repeats has been applied to huntingtin, and three domains of α-ROD have been found that defined H1 covering from amino acids 114 to 413, H2 comprised between 672 and 969, and H3 between 2667 and 2938 (424). The study revealed also for the first time the presence of intramolecular interactions between single α-RODs of human huntingtin, suggesting the possibility of homodimerization of huntingtin through inter- and intramolecular association of the α-RODs domain. These findings are in line with previous bioinformatics studies from our group that predicted the presence of three major conserved blocks in huntingtin (571). The presence of HEAT repeats suggests that huntingtin may exert its physiological function by using different protein partners (see sect. III*D*).

3. *Other consensus sites and critical sequences in huntingtin*

Huntingtin contains well-characterized consensus cleavage sites for proteolytic enzymes that cleave the protein and generate a wide range of fragments. Caspases, calpain, and aspartyl proteases are all involved in this process (see Fig. 1). Huntingtin is cleaved by caspase-3 and caspase-7 at amino acids 513 and 552, caspase-6 at amino acid 586, and caspase-2 at amino acid 552 (253, 630–632). Two specific calpain cleavage sites have been identified in huntingtin protein at residues 469 and 536, in the same region as the caspases cleavage sites (310). Other sites, whose exact amino acid positions are not well defined, seem preferentially active in some brain regions (169). In addition, huntingtin fragments produced by caspase-independent cleavage accumulate in the cytoplasm and nucleus (355, 460). The exact contribution of huntingtin proteolysis to cell functioning is unclear. However, as extensively reviewed in section IV*C*, modifications in the activity of caspases and calpains that reduce huntingtin proteolysis also diminish toxicity of the mutant protein and delay disease onset and progression.

A functionally active COOH-terminal nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS) are present in huntingtin, which might indicate that the protein (or a portion of it) is involved in transporting molecules from the nucleus to the cytoplasm (647). This hypothesis is supported by huntingtin's perinuclear and nuclear distribution and by the demonstration that the 17 amino acids preceding the polyQ region interact with the nuclear pore protein TPR (translocated promoter region), which exports proteins from the nucleus. Removal of these amino acids causes huntingtin to accumulate in the nucleus (131).

A closer examination of the first 17 NH_2-terminal amino acids of huntingtin revealed that the sequence forms an amphipathic α-helical membrane-binding domain that is required and is sufficient for huntingtin association to mitochondria and for its colocalization with Golgi and endoplasmic reticulum (ER) (23, 470). This sequence also enhances the formation of visible aggregates and, together with the expanded polyQ repeat, promotes acute calcium dyshomeostasis (470).

4. *Huntingtin is subjected to posttranslational modification*

Early studies showed that the protein is ubiquitinated at the NH_2-terminal lysines K6, K9, and K15 and targeted to the proteasome (148, 291). Ubiquitination controls the stability, function, and intracellular localization of huntingtin, thus contributing to maintain huntingtin homeostasis in the cells. When an expanded polyglutamine expansion is present, this process is impaired causing proteosomal dysfunction and accumulation of huntingtin fragments that become toxic to neurons.

Several reports show that huntingtin can be phosphorylated on serine-421 by protein kinase B or Akt (271, 459, 625) and at serine-434,-1181, and -1201 by the cyclin-dependent kinase 5, and this phosphorylation reduces caspase-mediated huntingtin cleavage at residue 513 and attenuates aggregate formation and toxicity (356) (see Fig. 1). In a recent study, the phosphorylation sites of full-length huntingtin were mapped by mass spectrometry, and six novel serine phosphorylation sites were identified. The mutation of one of these sites, which lies in the proteolytic susceptibility domain (serine 536), inhibited calpain-mediated cleavage and reduced toxicity of mutant huntingtin (512). Phosphorylation of specific amino acids seems to confer neuroprotective properties to wild-type huntingtin and is responsible for huntingtin-mediated transport of vesicles in neurons. In a very recent paper, Joan Steffan and colleagues at the University of California Irvine (579) have demonstrated that the IkappaB kinase (IKK) complex, previously shown to directly interact with huntingtin (306), phosphorylates huntingtin serine-13 and may activate phosphorylation of serine-16. Phosphorylation of these residues promotes modification of the adjacent lysine residues and target wild-type huntingtin clearance by the proteosome and the lysome (579) (see also sect. IV*D*). The authors found also that the presence of a polyQ expansion reduces the efficiency of this phosphorylation and mutant huntingtin clearance. To address the importance of serine-13 and -16 phosphorylation in vivo, William Yang and colleagues (225) have developed BACHD mice expressing full-length mutant huntingtin with serine-13 and -16 mutated to either aspartate or alanine. Disease pathogenesis, including motor and psychiatric deficits as well as mutant huntingtin aggregation, is abolished when both serines are mutated to aspartate, but preserved when mutated to alanine. These studies indicate that serine-13 and -16 are critical determinants of

disease pathogenesis and suggest that targeting of the two residues may represent a novel therapeutic strategy for HD (225).

Sumoylation of wild-type huntingtin on the first 17 amino acids has been described by Lawrence Marsh, Joan Steffan, and Leslie Thompson's group at the University of California Irvine. Sumoylation modulates its subcellular localization, activity, and stability (204, 549) (see Fig. 1). Michael Hayden's team at the University of British Columbia (649) was the first to show that huntingtin is also palmitoylated at cysteine-214 by its copartner, huntingtin-interacting protein 14 (HIP14, a palmitoyl transferase). The palmitoylation of huntingtin is consistent with its proposed role in regulating vesicular trafficking, since palmitoylated proteins are often involved in the dynamic assembly of the components that control vesicle trafficking and synaptic vesicle function. Expansion of the polyQ tract in huntingtin results in decreased palmitoylation, which contributes to the formation of inclusion bodies and enhanced neuronal toxicity (649) (see Fig. 1). Dimitri Krainc at the Massachusetts General Hospital (284) suggested that huntingtin is also acetylated at lysine residue 444 and that acetylation is required to target the protein to the macroautophagy pathway (see Fig. 1). In fact, when mutant huntingtin is rendered resistant to acetylation, it dramatically accumulates and leads to neurodegeneration (284).

C. Cellular and Tissue Distribution

Huntingtin is expressed ubiquitously in humans and rodents, with highest levels in the neurons of the CNS (147, 181, 196, 583, 607). Particularly, huntingtin is enriched in cortical pyramidal neurons in layers III and V that project to the striatal neurons (196).

Humans and rodents have two mRNAs that are generated by alternative cleavage and polyadenylation of the primary transcript, producing a long and a short 3-untranslated region (UTR) that differ by 3 kb (347, 348). In both organisms, the long-UTR transcript is predominantly expressed in the brain, whereas the short-UTR transcript is more widely expressed (348).

Within the cell, mammalian huntingtin is associated with a variety of organelles, including the nucleus, endoplasmic reticulum, Golgi complex, and mitochondrion (257, 263, 301, 427, 554). It is also found within neurites and at synapses, where it associates with various vesicular structures such as clathrin-coated vesicles, endosomal compartments or caveolae, as well as microtubules (147, 257, 263, 607). This widespread subcellular localization does not facilitate the definition of its function.

The analysis of huntingtin tissue distribution in species of the phylogenetic tree that represent crucial points in evolution has allowed us to propose a new hypothesis on the function of the protein. For example, interesting information emerges from the expression of the huntingtin gene in two basal deuterostomes. Deuterostomes consist of two primary clades: one, the chordates, which contains the ascidian, cephalochordates, and vertebrates; and a second clade, which contains the hemichordates and echinoderms. Preliminary analyses of huntingtin mRNA expression and distribution in the primitive chordate *Halocynthia roretzi* and in the echinoderm *Heliocidaris herithrogramma* (sea urchin) detected huntingtin mRNA at all stages of development. Interestingly, COOH-terminal huntingtin sequence seems confined to nonneural tissues in the echinoderm *Heliocidaris herithrogramma*. On the contrary, huntingtin has a ubiquitous distribution in the primitive chordate *Halocynthia roretzi* with increased levels of expression in the nervous system. This work suggested for the first time that in ancestral deuterostomes, the huntingtin expression pattern is prevalently nonneural and that neural expression of the HD gene may be a chordate evolutionary novelty (296).

More recently, we have investigated huntingtin expression during development of the cephalochordate lancelet (*Branchiostoma floridae*) (93). The development of the nervous system of lancelet is in fact particularly close to that of vertebrates as it includes vertebrate-like anatomical characteristics such as cephalization and a dorsal nerve cord. We found that in lancelet, huntingtin expression is detectable by in situ hybridization starting from the early neurula stage, where it is found in cells of the neural plate. At later stages, it is retained in the neural compartment but also it appears in limited and well-defined groups of nonneural cells. At subsequent larval stages, huntingtin expression is detected in the neural tube, with the strongest signal being present in the most anterior part (93). This study shows for the first time a subregionalization of huntingtin's expression in the nervous system (93).

When huntingtin distribution has been analyzed in the protostome branch, in the divergent organism *Drosophila melanogaster*, an ubiquitous expression of huntingtin has been revealed which does us not allow to draw any conclusion on its role in the fly (344, 660). Furthermore, when huntingtin has been genetically inactivated in *Drosophila*, the larva developed without any defect in the gastrulation process, suggesting that, in contrast to what happens in the deuterostome branch, the protein is not involved in controlling embryo development (see sect. III*E1*). On this basis, as well as on the basis of a very divergent amino acid sequence of *Drosophila* huntingtin, we concluded that huntingtin function in *Drosophila* might be very different with respect to the function of huntingtin in mammals.

Based on these studies, we speculated that huntingtin has developed novel neuronal activities in the deuterostome branch (99). These activities may have become

progressively more specialized in higher species, and specifically in vertebrates, in coincidence with the accumulation of huntingtin in neurons and the acquisition of an increasingly more complex CNS.

D. Huntingtin Interactors

The identification of huntingtin interacting proteins can provide us with a better understanding of the normal function of huntingtin and on how a polyQ expansion affects this normal function.

By using yeast two-hybrid (Y2H) screenings, affinity pull-down assays, Western blotting, and immunoprecipitation, nearly 50 proteins capable of interacting directly with huntingtin or its fragments have been described. Most proteins were found to interact with NH_2-terminal polyQ-containing huntingtin fragments and, in some cases, the strength of these interactions has been shown to be sensitive to the length of the polyQ tract (242, 342). Huntingtin-interacting proteins cover diverse cellular roles including clathrin-mediated endocytosis, apoptosis, vesicle transport, cell signaling, morphogenesis, and transcriptional regulation (242, 342). Relevant examples of huntingtin interactors include huntingtin-associated protein 1 (HAP1), a novel protein with at least two isoforms (HAP1-A and HAP1-B), which is expressed in several tissues including the brain and which interacts with the p150 subunit of dynactin, thus being involved in intracellular transport. A second protein is huntingtin-interacting protein 1 (HIP1), which binds to α-adaptin and clathrin, and is implicated in cytoskeleton assembly and in endocytosis. Huntingtin binds also to proteins of the synaptic complex such as protein kinase C and casein kinase substrate in neurons 1 (PACSIN-1) and postsynaptic density 95 (PSD95), thus participating in the regulation of synaptic activity (535). Earlier studies also showed its ability to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (342). Many transcription factors bind to huntingtin, indicating a role of the protein in the control of gene transcription (102). In some cases, the functional relationship between wild-type huntingtin and its interacting proteins has been established. Wild-type huntingtin interacts with PSD95, a protein located in the postsynaptic membrane, through its Src homology-3 (SH3) sequence (proline-x-x-proline), regulating the anchoring of NMDA and kainate (KA) receptors to the postsynaptic membrane (558). Wild-type huntingtin promotes the expression of brain-derived neurotrophic factor (BDNF) by interacting through REST-interacting LIM domain protein (RILP) and HAP1 with the repressor element-1 transcription factor/neuron-restrictive silencer factor (REST/NRSF) in the cytoplasm, and preventing this complex from translocating into the nucleus and from binding to the repressor element 1/neuron-restrictive silencer element (RE1/NRSE) present in the promoter of the BDNF gene as well as of many other neuronal genes (524, 672). Wild-type huntingtin also promotes the vesicular transport of BDNF along the microtubules through a mechanism that involves HAP1 and the p150 subunit of dynactin (202). The interaction of wild-type huntingtin with both HAP1 and mixed-lineage kinase 2 (MLK2) has been shown to promote the expression of NeuroD (368), a helix-loop-helix transcription factor that is crucial for the development of the dentate gyrus of the hippocampus and for the morphogenesis of pancreatic islets. Wild-type huntingtin also prevents the activation of caspase-8 by interacting with HIP1 and preventing the formation of the caspase-activating complex HIP1-HIP1-protein interactor (HIPPI) (206).

In 2004, Erich Wanker's team at the Max Delbrück Center for Molecular Medicine in Berlin (216) produced the first protein-protein interaction network for HD that uncovered 15 novel interacting proteins, including the G protein-coupled receptor kinase interactor 1 (GIT1), an enhancer of polyQ aggregation. In 2007 a similar approach has been followed by the group of Robert Hughes at the Buck Institute for Age Research (292), which used yeast two-hybrid and affinity pull-down/mass spectrometry protein interaction screens combined with genetic validation in a *Drosophila melanogaster* model of HD (292). This study identified 17 novel associations between huntingtin fragment and components of the vesicle secretion apparatus that were loss-of-function suppressors of neurodegeneration in *Drosophila* (292). These observations demonstrate the utility of combining protein-interaction screening with genetic-interaction screening to provide insight into disease mechanisms and identify novel potential targets for therapeutic intervention.

Recently, the group of Solomon Snyder at the Johns Hopkins University School of Medicine (555) has discovered a new huntingtin interactor that has not been identified in proteomic and Y2H studies. This protein is called Rhes, a small G protein localized to the striatum, but also expressed in other forebrain areas that are affected in HD (555). Rhes binds to mutant huntingtin, promotes its sumoylation, and augments its neurotoxicity in vitro in nonneuronal cells. Its potential involvement in causing selective striatal degeneration remains to be demonstrated (555).

E. Huntingtin Functions

1. Huntingtin in embryonic development

Two years after the cloning of the gene, huntingtin was shown to be essential for embryonic development as its complete inactivation in huntingtin knock-out mice (*Hdh*$-/-$) causes embryonal death before day 8.5, i.e., before gastrulation and the formation of the nervous sys-

tem (168, 404, 656). The basis of this effect appears to be increased apoptosis in the embryonic ectoderm shortly after the onset of gastrulation. It is known that the defect in development observed in the huntingtin knock-out mice embryos derives from a defect in the organization of extraembryonic tissue, possibly as a consequence of an alteration in the nutritive function of the visceral endoderm (156). Intriguingly, the inactivation of huntingtin gene does not reveal a phenotype in *Drosophila melanogaster* embryos, further reinforcing the evidence that the protein in the protostome branch may have different functions (660). Unexpectedly, *Drosophila* huntingtin is required for maintaining the mobility and long-term survival of adult animals, and for modulating axonal terminal complexity in the adult brain (660).

With the progression of embryonic development, experimental reductions of huntingtin levels to below 50% cause defects in the epiblast, the structure that will give rise to the neural tube, and profound cortical and striatal architectural anomalies (25, 638). Defects in the formation of most of the anterior regions of the neural plate, specifically in the formation of telencephalic progenitor cells and the preplacodal tissue, have been recently found by Henshall et al. (251) that used morpholinos to reduce huntingtin levels in the developing zebrafish. These data indicate that, in addition to its early extraembryonic function, at postgastrulation stages huntingtin participates in the formation of the CNS. To further investigate the role of huntingtin in development, Ioannis Dragatsis and colleagues at the University of Tennessee (144) have inactivated the *Hdh* gene in Wnt1 cell lineages using the CreloxP system of recombination, demonstrating that conditional inactivation of the *Hdh* gene in the midbrain and hindbrain results in congenital hydrocephalus. These results implicate huntingtin also in the regulation of cerebral spinal fluid (CSF) homeostasis (144).

Analyses of chimeras created by blastocyst injection of *Hdh*−/− ES cells have provided further insights into the role of wild-type huntingtin in brain maturation by showing that it is critical for establishing and maintaining especially cortical and striatal neuronal identity (465). Although some brain regions were appropriately colonized by *Hdh*−/− neurons (however, their functional activity has not been evaluated), few donor cells were found in cerebral cortex, striatum, basal ganglia, thalamus, and the Purkinje cell layer of the cerebellum, thus suggesting that huntingtin plays a specific role in neuronal survival in these brain regions. Preliminary analyses of blastocysts injected chimeras at E12.5 showed ongoing *Hdh*−/− cell degeneration specific to the striatum, cortex, and thalamus, thus supporting the view that neuroblasts in these areas need to synthesize huntingtin if they are to progress in development and differentiation (466).

These data indicate that huntingtin is required at different steps of embryonic development and that its total absence or >50% reduced presence generates a very early phenotype in mice. Moreover, the HD mutation does not seem to abrogate the developmental functions of huntingtin, as HD patients pass development and symptoms only start to manifest several years after birth. Human mutant huntingtin can compensate for the absence of endogenous huntingtin, by rescuing the embryonic lethality of mice homozygous for a targeted disruption of the endogenous *Hdh* gene (328). Thus it is reasonable to suggest that huntingtin's function during embryonic development is independent of the length of the polyQ. Mice carrying 50% full-length wild-type huntingtin (i.e., one allelic dose) reach normal adult life (168, 404, 656). However, one of three generated heterozygous knock-out mice, which still expresses a 20-kDa NH_2-terminal portion of wild-type huntingtin, at adulthood shows behavioral abnormalities, cognitive deficits, and significant neuronal loss in the subthalamic nucleus (404, 415). This phenotype may be ascribed to reduced huntingtin function(s) and/or to a dominant negative effect driven by the remaining NH_2-terminal fragment over the full-length wild-type protein, regardless of the CAG tract.

Although HD mice are born with no apparent defects, a recent study from Mark Mehler's group at the Albert Einstein College of Medicine in New York (398) suggests that developmental abnormalities occur in a knock-in mouse model of HD (HdhQ111) compared with a knock-in mouse model expressing only 18 CAG (HdhQ18). By analyzing the expression of markers of MSNs such as Islet1, dopamine and cAMP-regulated phosphoprotein, 32-kDa (DARPP32), and mGluR1 in the striatum of HdhQ111 embryos at E17.5, the authors report an impairment in the acquisition of the cytoarchitecture of striatal subcompartments, suggesting abnormalities in MSN specification and maturation. They also show that neural progenitor cells from embryonic striatum exhibit in vitro reduced proliferative potential, enhanced late-stage self-renewal, and impaired generation of MSN subtypes. Yet, HdhQ111 mice show a very mild disease phenotype and no striatal neurons degeneration (see sect. II*F2*B). Although more work is required, the hypothesis that HD may be a developmental disease is of interest, especially given that recent preliminary functional MRI analyses (fMRI) studies indicate that subtle brain structure abnormalities may be present in children at risk for HD (age 7–18) who are estimated to be decades from diagnosis and may be indicative of developmental defects (410).

2. Huntingtin is antiapoptotic

In vitro and in vivo studies have shown that wild-type huntingtin has a prosurvival role. The first in vitro demonstration of an antiapoptotic role of wild-type huntingtin came from experiments performed by our group on immortalized cell lines. We found that overexpression of

wild-type huntingtin in brain-derived cells protects them from toxic stimuli (467, 469). One year later, similar results have been obtained by David Rubinsztein and colleagues at the University of Cambridge (258) who showed that wild-type huntingtin can protect neuroblastoma and kidney cell lines from death triggered by the mutant protein.

Additional confirmation of an antiapoptotic role of huntingtin came from studies by Michael Hayden's group. They found that primary striatal neurons from YAC18 transgenic mice overexpressing full-length wild-type human huntingtin were protected from apoptosis compared with cultured striatal neurons from nontransgenic littermates and YAC72 mice expressing mutant human huntingtin (329). On the contrary, cells depleted of wild-type huntingtin were more sensitive to apoptotic cell death and showed increased level of caspase-3 activity, with respect to control cells (663).

Some of the mechanisms by which wild-type huntingtin protects cells from apoptotic cell death have been elucidated. We found that wild-type huntingtin blocks the formation of a functional apoptosome complex and the consequent activation of caspase-3 and caspase-9 (467, 469). In line with these findings, a study from Robert Friedlander and colleagues at the Harvard Medical School (663) showed that wild-type huntingtin blocks apoptosis by physically interacting with active caspase-3, thereby inhibiting its proteolytic activity. Other authors found that huntingtin inhibits the formation of the proapoptotic HIPPI-HIP1 complex, thus interfering with procaspase-8 activation and apoptotic cell death (206, 236). Further studies revealed that wild-type huntingtin is a substrate for Akt, a serine/threonine kinase that activates prosurvival pathways, and may participate in the phosphoinositide 3-kinase (PI3K)-Akt pathway by stimulating the expression of prosurvival genes, whereas death genes such as BAX or Bcl-2 are repressed (459).

In 2000, in parallel to our demonstration of an antiapoptotic role of huntingtin in vitro, work from Scott Zeitlin's group at the University of Virginia showed that neuronal inactivation of the huntingtin gene in adult mice by means of a Cre/LoxP site-specific recombination system driven by the neuronal-specific α-subunit of the calcium-dependent calmodulin kinase-2 (Camk2a) promoter, causes apoptotic cells in the hippocampus, cortex, and striatum, and a lack of axon fibers (157, 467). This animal model showed a progressively more severe limb-clasping-upon-tail suspension phenotype, a feature also observed in transgenic HD mouse mutants. Motor deficits were also present, and at 10–12 mo of age, the animals were hypoactive and exhibited a slight tremor. Mutant mice survived for, at most, 13 mo. This phenotype is similar to those in currently used models of HD, suggesting that loss of wild-type huntingtin function may contribute to the disease phenotype (157).

Earlier morphometric and ultrastructural analyses of one heterozygous knock-out mouse strain revealed similar results, with apoptotic cell death in the basal ganglia and adult brain, cognitive dysfunction, and behavioral abnormalities (404, 415). Further studies provided in vivo evidence that huntingtin regulates the balance between neuronal survival and death and that the levels of huntingtin modulate neuronal sensitivity to excitotoxic neurodegeneration (329). In fact, the overexpression of wild-type huntingtin in YAC18 transgenic mice was found to confer significant protection against apoptosis triggered by excitotoxicity (329). Moreover, endogenous huntingtin is reduced following ischemic injury through a caspase-mediated process, while overexpression of wild-type huntingtin two to three times with respect to levels in wild-type mice protects against ischemic injury in an huntingtin dose-dependent manner (664).

More recently, the antiapoptotic role of huntingtin has been highlighted also in nonmammalian models. In fact, apoptotic cell death has been found in zebrafish embryos in which huntingtin has been knocked down by morpholino technology (142). Huntingtin morpholino-injected zebrafish showed a massively increased cell death as indicated by caspase-3 activity especially in the midbrain/hindbrain region of the developing zebrafish embryo. This increased apoptosis was accompanied by a severe underdevelopment of the CNS (142).

3. A hint towards neuronal vulnerability

A) NORMAL HUNTINGTIN CONTROLS BDNF PRODUCTION. Biological and molecular findings have linked wild-type huntingtin to BDNF, a neurotrophin that is particularly important for the survival of striatal neurons and for the activity of the cortico-striatal synapses (668). BDNF colocalizes with huntingtin in cortical neurons that project to the striatum and, despite some reports of BDNF mRNA transcription in adult striatal neurons, it is well established that most of the striatal BDNF is produced in the cerebral cortex and delivered to the striatal neurons via the cortico-striatal afferents (8, 35, 196). Several findings have led to the conclusion that wild-type huntingtin contributes to the pool of BDNF proteins produced in the cerebral cortex and that a loss or reduction in wild-type huntingtin activity diminishes BDNF production and delivery to striatal targets, thus likely contributing to selective degeneration of those neurons (202, 668, 669, 671, 672).

Wild-type huntingtin stimulates cortical BDNF protein production by acting at the level of BDNF gene transcription as shown by in vitro and in vivo data. In fact, cultured brain cells overexpressing wild-type huntingtin produce increased BDNF mRNA and protein levels. Studies on YAC mice expressing increased full-length wild-type huntingtin levels (YAC18) also show high BDNF protein levels in the cerebral cortex, as a consequence of

the positive regulation by wild-type huntingtin of the BDNF gene transcription (669). Therefore, higher striatal BDNF levels are found in the mice (669). Conversely, reduced BDNF mRNA levels are found in brain samples from wild-type huntingtin-depleted mice as well as in heterozygous huntingtin knock-out mice (666, 672). A similar 50% BDNF reduction is found in whole zebrafish embryos in which huntingtin has been knocked down (142). Reduced huntingtin levels did not impact gastrulation and early development of huntingtin knockdown zebrafish but caused massive apoptosis of neuronal cells 24 h postfertilization. Small eyes, heads, and enlargement of brain ventricles were observed. Notably, these phenotypes are significantly rescued when BDNF protein is administered to the embryos (142).

B) RE1/NRSE, A GENOMIC TARGET OF WILD-TYPE HUNTINGTIN ACTIVITY. A more thorough assessment of the molecular mechanism by which wild-type huntingtin affects BDNF gene transcription has shown that the normal protein specifically regulates the activity of one of the BDNF promoters. The rodent BDNF gene is now known to contain 9 exons (I, II, III, IV, V, VI, VII, VIII, and IX), which are linked to separate promoters enabling the production of different transcripts. The functional BDNF protein is produced following splicing at the 3′ end of exon IX, which contains the coding region. The different promoter-like sequences upstream of exons I-IX act independently and modulate the transcription of mRNA I-IX in a stimulus- and developmental-specific manner (2, 453, 580, 581).

The use of promoter reporter assays and PCR for the specific mRNAs has allowed us to show that the increased BDNF level found in the presence of wild-type huntingtin is due to enhanced transcription from BDNF promoter II, whereas transcription from other promoters, as for example promoters IV and VI [nomenclature according to the new description of the BDNF gene by Aid et al. (2)], is not affected by wild-type huntingtin (669, 672).

Studies carried out in cells and mice overexpressing or depleted of the wild-type protein showed that levels of BDNF mRNA transcribed from promoter II are increased or decreased, respectively, compared with the respective controls. All these studies therefore identify the BDNF promoter II as a target of wild-type huntingtin. In line with this finding, transcription from the other BDNF promoters (IV and VI) is affected only in the presence of the mutant protein, suggesting that, in HD, a gain of (toxic) function mechanism is operating at the level of BDNF promoters IV and VI (666, 672).

The investigation of the mechanism by which wild-type huntingtin stimulates BDNF gene transcription has concentrated on BDNF promoter exon II, within which a conserved 21- to 23-bp DNA response element (RE1/NRSE) is recognized by REST/NRSF transcriptional regulator. REST/NRSF was identified in 1995 as a protein that binds the RE1/NRSE silencing sequences present in the rat *Scn2a2* and *Stmn2* (*SCG10*) genes. Since RE1/NRSE sites had been identified in several neuron-specific genes, REST/NRSF was initially thought to repress the transcription of neuronally expressed genes in nonneuronal tissues. This idea was then confuted by the discovery of the modulation of REST/NRSF on the transcription of neuronal genes in neuronal cells by different mechanisms and cofactors. Furthermore, the data now available suggest that REST/NRSF also plays distinct roles in regulating gene expression during transition from immature to mature neurons (419).

We have found that the RE1/NRSE silencing activity is inhibited in the presence of wild-type huntingtin, through recruitment and sequestration of REST/NRSF into the cytoplasm; REST/NRSF is therefore prevented from binding to, and activating, the nuclear RE1/NRSE regulatory elements. Huntingtin does not seem to interact with REST/NRSF directly, but rather seems to be part of a complex that contains HAP1 and RILP, a protein that directly binds REST/NRSF and promotes its nuclear translocation (524). The complex disassembles in the presence of mutant huntingtin and causes the pathological entry of REST/NRSF into the nucleus, thus leading to the repressor complex formation and reduced transcription of the BDNF gene.

Findings from Noel Buckley's group at the King's College in London indicate that the RE1/NRSE is present in more than 1,300 genes encoding both neuronal and nonneuronal proteins (84, 288). As a consequence, we have reported that wild-type huntingtin plays a broader role than expected in regulating neuronal gene transcription because cells and mice expressing increased wild-type but not mutant huntingtin levels also show higher levels of the mRNAs transcribed from many other RE1/NRSE-controlled neuronal genes (672). Consistently, depletion of endogenous huntingtin in cells and mice is associated with reduced transcription from neuronal genes carrying the RE1/NRSE silencer (666, 672). Huntingtin may therefore act in the nervous system as a general facilitator of neuronal gene transcription for a subclass of genes.

4. Huntingtin role in axonal and vesicle transport

Huntingtin is found predominantly in the cytoplasm of neurons and is enriched in compartments containing vesicle-associated proteins (607). In line with this finding, it is retrogradely transported in the rat sciatic nerve where it associates with vesicles and microtubules (65). Further evidence of a role of huntingtin in intracellular transport came from a study by Lawrence Goldstein's group at the University of California in San Diego (230). They found that reduction in *Drosophila* huntingtin disrupts axonal transport (230). Wild-type huntingtin is also

involved in fast axonal trafficking of mitochondria in mammalian neurons (584). In primary striatal neurons taken from mice expressing only one copy of the wild-type allele or <50% of normal huntingtin levels upon CRE-mediated recombination (knock-out), mitochondria became progressively immobilized (584). This effect was significantly stronger in complete knock-out neurons than in those with a 50% loss of huntingtin, which points to a dose-dependent effect. Several reports indicate that wild-type huntingtin regulates axonal transport by participating in the assembly of the motor complex on microtubules. It has been proposed that huntingtin associates with motor proteins via HAP1, a protein that has been shown to interact with both huntingtin and the p150 subunit of dynactin, thereby enabling retrograde transport and perhaps anterograde transport (229). Further evidence implicating huntingtin, HAP1, and p150(glued) comes from studies that have highlighted huntingtin's role in the control of BDNF vesicle transport (202) (see also sect. IV*A2*).

A) HUNTINGTIN AND BDNF VESICLE TRANSPORT. In 2004, the French group led by Frederic Saudou at the Centre Universitaire Orsay in Paris (202) showed that full-length wild-type huntingtin stimulates BDNF vesicular trafficking in neuronal cells and that its transport can be attenuated by reducing the levels of wild-type huntingtin using RNAi. In the same study the authors reported that the ability of wild-type huntingtin to enhance vesicular transport involves HAP1 and the p150(Glued) subunit of dynactin, which are essential components of molecular motors. Huntingtin was found to interact with the p150(Glued) subunit via HAP1, thereby stimulating BDNF transport (202). The phosphorylation of huntingtin at Ser-421 regulates BDNF vesicle retrograde and anterograde transport (125). In particular, when huntingtin is phosphorylated, BDNF anterograde transport is favored, whereas when the phosphorylated status is reduced, BDNF vesicles undergo retrograde transport (125).

However, results from a study conducted by Her and Goldstein on a knock-in mouse model of HD, which carries a 150 CAG triplet repeat expansion in huntingtin showed that the movement of BDNF vesicle is impaired in striatal and hippocampal primary neurons, but not in cortical neurons, the main source of striatal BDNF (252). Moreover, in opposition to the previous report from Gauthier and colleagues (202), this paper shows that the observed alteration in BDNF vesicle transport in HD is not attributable to a disruption of motor protein complexes that drive retrograde and anterograde transport, but rather may result from altered regulation of intact complexes (252). According to these authors, the mechanisms that underlie impaired BDNF transport in cortical neurons in HD require further investigation.

5. Huntingtin and synaptic activity

Normal communication between neurons is regulated by a number of proteins in the synapse. Normal huntingtin interacts with cytoskeletal and synaptic vesicles proteins essential for exo- and endocytosis at the synaptic terminals, thus participating in the control of synaptic activity in neurons (535). One early finding shows that wild-type huntingtin directly binds the SH3 domains of PSD95 (558). PSD95 is a key molecule in synaptic transmission and a component of the membrane-associated guanylate kinase (MAGUK) protein family that binds the NMDA and kainate receptors at the postsynaptic density (377). A decreased interaction of mutant huntingtin with PSD95 has been described in HD, suggesting that more PSD95 is released in HD, thus affecting the activity of NMDA receptors, and possibly leading to their overactivation/sensitization and to excitotoxicity (558). More recent data show that huntingtin may also take part in the presynaptic complex through its interaction with HIP1, which has been recently associated with the presynaptic terminal (433). Furthermore, huntingtin can bind to PACSIN1/syndapin, syntaxin, and endophilin A, which collectively play a key role in synaptic transmission, as well as in synaptic vesicles and receptor recycling. These interactions depend on the length of the polyglutamine repeat and are enhanced by the presence of an expanded CAG, leading to impairment of synaptic transmission in HD (535).

F. Loss of Wild-Type Huntingtin Function in HD

The above data indicate that wild-type huntingtin has beneficial activities in the mature brain. It is therefore possible that its loss in human HD reduces the ability of neurons to survive and to counteract the toxic effects of the mutant protein. In some mouse models, homozygosity for the HD mutation leads to a more severe phenotype than heterozygosity for an equivalent CAG expansion in the HD gene (463, 636). Similarly, a small cohort of HD patients homozygous for the CAG expansion seems to show a more severe disease progression than those who were heterozygous for the mutation (543). Further studies in lymphoblastoid cell lines revealed that homozygotes have a more aggressive molecular phenotype than heterozygotes (364, 542). However, these studies were unable to determine the relative contribution of the loss of wild-type huntingtin to HD pathogenesis and were limited to a few patients. We discuss here the set of rigorous genetic experiments carried out to evaluate the impact of the loss of wild-type huntingtin function in HD. In particular, we focus on the experiments aimed at genetically manipulating the level of wild-type huntingtin in animal models of HD.

1. Reduced huntingtin activity in HD

A first experiment to evaluate the in vivo contribution of the loss of wild-type huntingtin function in HD was performed by Michael Hayden's team in Vancouver. YAC128 mice were initially crossed with mice heterozygous for the targeted inactivation of the mouse HD gene (*Hdh*+/− mice) to generate YAC128 mice heterozygous for the targeted inactivation of *Hdh* (YAC128+/−). These mice were then crossed with *Hdh*+/− mice to generate YAC128 mice homozygous for the targeted inactivation of *Hdh* (YAC128−/− mice) (600). The phenotypic severity of YAC128+/+ mice (carrying extra copies of mutant huntingtin with 128Q repeats in normal huntingtin background) was compared with that observed in double-mutant YAC128−/− mice that do not express endogenous wild-type huntingtin but express the same amount of the mutant protein with 128Q. The complete loss of wild-type huntingtin in the YAC128−/− mice led to a slight worsening of striatal atrophy and neuronal loss and a small but significant decrease in the neuronal cross-sectional area. YAC128−/− mice also showed behavioral and motor abnormalities because they performed worse than YAC128+/+ mice in the rotarod test of motor coordination and were hypoactive at 2 mo of age. In addition, testicular atrophy and degeneration were markedly worsened in the absence of wild-type huntingtin. YAC128+/+ mice showed a male-specific deficit in survival at 12 mo of age, which was exacerbated in YAC128−/− mice. These data suggest that the elimination of wild-type huntingtin expression in YAC128 mice results in the exacerbation of behavioral deficits and survival, with a mild worsening of neuropathology at 12 mo of age. The absence of severe striatal abnormalities led to the suggestion that the striatal phenotype is primarily dependent on mutant huntingtin toxicity. Accordingly, Rhes, a supposedly striatal-specific protein, was recently suggested to trigger mutant huntingtin toxicity (555). However, there are no indications showing that Rhes causes cell death in the presence of mutant huntingtin in a striatal specific manner. With respect to the YAC crossing experiment, it might be important to characterize the outcome at molecular levels and at later time points. In addition, it is possible that YAC128−/− mice carry a small fragment (~20 kDa) of wild-type huntingtin not deleted in the original *Hdh*+/− mice generated by Nasir et al. (404). In fact, these mice were generated by a mutation that led to the skipping of exon 5 and to a frameshift resulting in a stop codon immediately downstream of the targeting event at nucleotide 673, resulting in a truncated protein of ~20 kDa. Therefore, we cannot exclude the possibility that this fragment still retains physiological properties of the full-length protein.

Considering a different experimental approach with nonmammalian models of HD, Zhang et al. (660) have recently shown that the removal of endogenous *Drosophila* huntingtin significantly accelerates the neurodegenerative phenotype associated with a *Drosophila* model of polyglutamine huntingtin toxicity (HD-Q93), further suggesting that disrupting the normal function of huntingtin might contribute to HD pathogenesis.

2. To increase wild-type huntingtin activity in HD

Several studies have evaluated the impact of wild-type huntingtin overexpression in HD. In 2001, Michael Hayden's team showed that expression of mutant huntingtin in the absence of wild-type huntingtin results in massive apoptotic cell death in the testes of male mice, but that the observed cell death can be modulated by the expression of normal huntingtin. In fact, no evidence of apoptosis is seen in the testes of mice expressing human mutant huntingtin when endogenous wild-type huntingtin is expressed from both *Hdh* alleles (328). These data indicate that wild-type huntingtin reduces the cellular toxicity of mutant huntingtin in vivo in the testes of mice (328). Similar results were obtained in in vitro experiments involving nonneuronal cells, showing that overexpression of wild-type huntingtin reduces the polyQ toxicity induced by an exogenous mutant huntingtin construct (258). Moreover, overexpression of wild-type huntingtin rescued the activity of a tk-RE1/NRSE-cat construct in 109/7Q knock-in cells, indicating that the wild-type protein inhibits the silencing activity of the RE1/NRSE and promotes BDNF gene transcription in HD (672). Similarly, the expression of wild-type huntingtin in 109/109Q knock-in cells seems to be able to rescue the decreased transport and release of BDNF, although the number of analyzed cells was a limiting factor in the experiment (202).

More recently, Van Raamsdonk and colleagues in Hayden's lab set up an in vivo experiment to evaluate whether overexpression of wild-type huntingtin is beneficial in HD. YAC128 mice were crossed with YAC18 mice (which bear wild-type huntingtin levels that are two times the level of wild-type huntingtin expression in wild-type mice) to generate YAC18/128 mice carrying human mutant huntingtin with 128Q and overexpressed normal huntingtin (599). This resulted in a mild improvement in striatal neuropathology with no improvement in motor dysfunction. The presence of a mild phenotype suggested that huntingtin function may be important in maintaining neuronal health and that mutant huntingtin toxicity is primarily responsible for the pathognomic striatal neuropathology. It was therefore proposed that treatment of HD with wild-type huntingtin might not be sufficient to ameliorate the symptoms of the disease. This may be especially true if one considers that the protein is cleaved by proteolytic enzymes or recruited into huntingtin aggregates (88, 217, 310). However, one should expect that higher levels

of wild-type huntingtin may be required to overcome the dysfunctions caused by mutant huntingtin. Consistently, a preliminary report by Ioannis Dragatsis at the University of Tennessee highlights that R6/2 mice, overexpressing the NH_2-terminal portion of mutant huntingtin, crossed with a mouse bearing the *Hdh* gene under the control of a strong promoter, phosphoglycerate kinase (PGK), leads to a significant delay in the onset of clasping, as well as increased survival. Preliminary histological examination also showed that brain pathology was less prominent in the context of huntingtin overexpression (143).

Collectively, these results indicate that enhancing wild-type huntingtin level may be beneficial and that downstream targets of wild-type huntingtin activity, for example, BDNF and other RE1/NRSE regulated genes can be exploited in drug-screening strategies for the search of compounds that mimic normal huntingtin activity.

IV. MECHANISMS OF NEURODEGENERATION

The aim of this section is to describe the molecular pathways involved in HD and their relevance in pathogenesis. We focus on the pathways that have offered new targets for the development of therapeutics (see also Fig. 3 for a schematic representation of key cellular pathogenic mechanisms in HD).

A. Loss of BDNF

The importance of the BDNF for MSNs, the most affected neuronal population in HD, is well documented. A 1997 landmark discovery by Stanley J. Wiegand and colleagues (8) at Regeneron Pharmaceuticals, in New York, showed that the BDNF protein is produced in the cerebral cortex and anterogradely transported along the cortico-striatal tract to the MSNs. A large number of other experiments have confirmed that MSNs depend on cortically derived BDNF and that cortical BDNF is reduced to ~50% in HD (35, 667, 668). Thus it has been proposed that changes in its level or distribution in the cerebral cortex may contribute to striatal (and cortical) vulnerability in HD. Through loss and gain of function experiments, we now know that reducing or enhancing the production of BDNF in the cerebral cortex increases or reduces, respectively, neuronal loss in the striatum.

1. BDNF mRNA synthesis is reduced in HD

BDNF was first assessed in autoptic caudate and putamen from HD subjects showing that its levels were reduced in this brain region but preserved in the cerebral cortex and in the hippocampus (188). However, this study used a very limited number of samples, as well as qualitative western blot and immunohistochemistry. It was in 2001 that the link between BDNF and the HD mutation was first proposed by our group through studies performed in cellular and mouse models (669). Immortalized CNS cells bearing mutant huntingtin produced less BDNF than their wild-type counterparts (669). In the same study, we also demonstrated that the YAC72 transgenic mouse model of HD overexpressing full-length human mutant huntingtin exhibited a significant reduction in BDNF mRNA and protein levels in cerebral cortex and striatum. Changes in BDNF protein levels were consequent to a variation in the amount of transcribed BDNF mRNA in cerebral cortex in the presence of mutant huntingtin (669).

A systematic and quantitative assessment of BDNF levels in postmortem HD brain tissues revealed lower BDNF mRNA and protein levels in cortex, indicating reduced production of the neurotrophin in the brain of HD sufferers (671). Many laboratories have confirmed reduced BDNF levels in total brain or cortical samples from a large panel of HD animal models including R6/2, YAC, BAC-HD, N171-82Q and knock-in mice and HD rats (667, 668). Cortical BDNF mRNA levels undergo a significant reduction that starts from the early symptomatic stages and becomes more evident with disease progression in the R6/2 HD mouse model expressing an NH_2-terminal fragment of mutant huntingtin (668, 670).

Reduced BDNF gene transcription in HD was attributed to increased activity of the RE1/NRSE silencer within the BDNF promoter II due to increased binding of the repressor REST/NRSF to its consensus target site (666, 672). As described in section III*E3*B, huntingtin is part of a cytoplasmic complex that includes REST/NRSF and other proteins (HAP1 and RIPL) that allow REST/NRSF translocation into the nucleus. When huntingtin is mutated, REST/NRSF is released from the complex and accumulates in the nucleus, thus binding and activating the RE1/NRSE silencer and causing a reduction in the transcription of BDNF and of other RE1/NRSE neuronal genes. In HD cells, mouse, and human tissues, transcription from other BDNF promoters (BDNF promoter IV and VI) is also affected, suggesting that, in addition to reduced activity of BDNF promoter II (caused by the loss of wild-type huntingtin activity), other mechanisms are in operation that lead to reduced BDNF gene transcription that are more specifically linked to mutant huntingtin's gain of toxic function (668).

Mutant huntingtin may also affect BDNF transcription by altering the transcriptional activities of cAMP response element-binding protein (CREB), CREB binding protein (CBP), specificity protein 1 (Sp1), TBP associated factors 130 (TAF130), and other transcription factors that regulate the activity of the BDNF promoters (102). Moreover, mutant huntingtin can interact

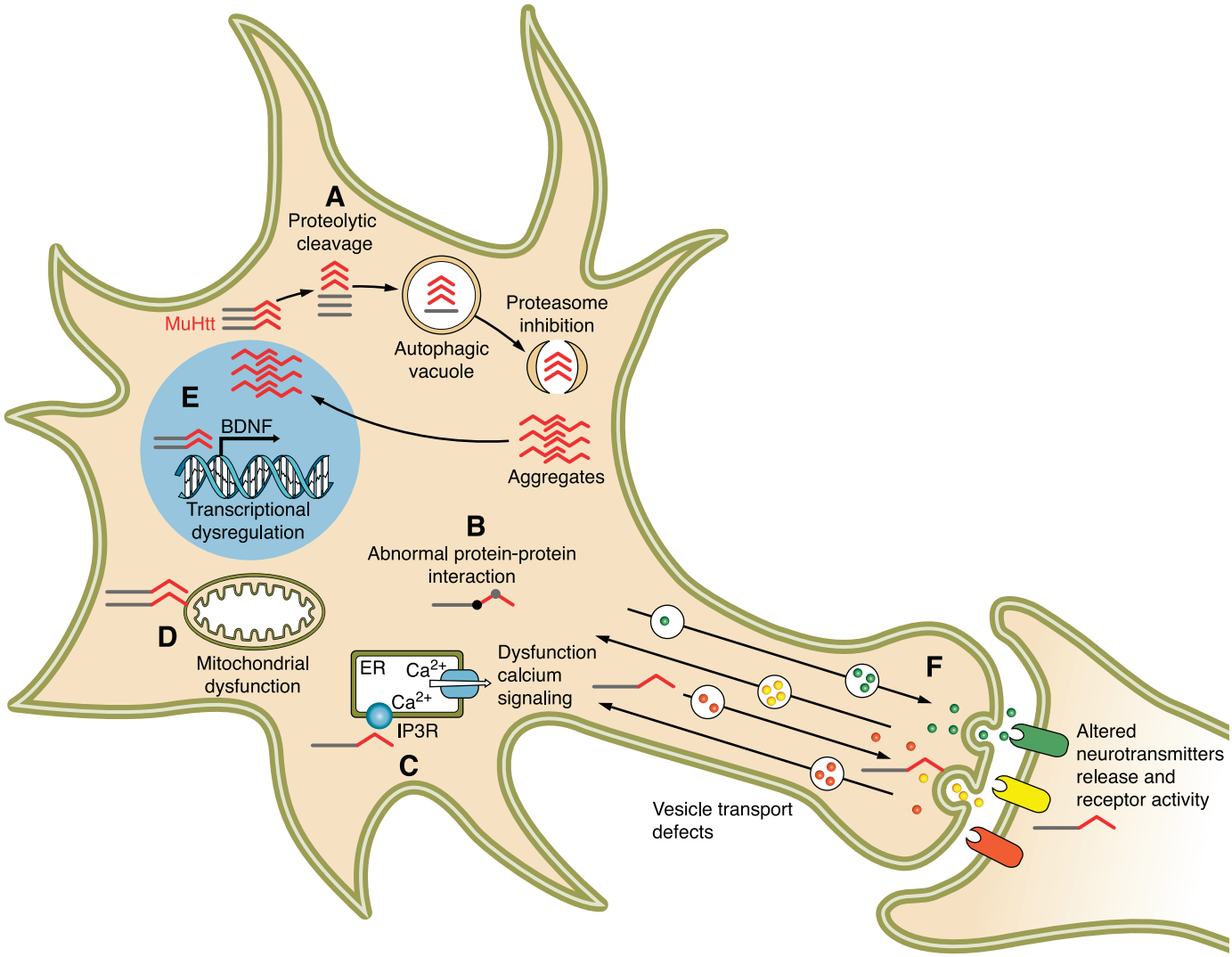


FIG. 3. Key cellular pathogenic mechanisms in Huntington's disease (HD). Multiple cellular pathways have been implicated in the pathogenesis of HD. These mechanisms could be exclusive or, more likely, have a high degree of cross-talk. *A*: the mutation in huntingtin causes a conformational change of the protein that leads to partial unfolding or abnormal folding of the protein, which can be corrected by molecular chaperones. Full-length mutant huntingtin is cleaved by proteases in the cytoplasm. In an attempt to eliminate the toxic huntingtin, fragments are ubiquitinated and targeted to the proteasome for degradation. However, the proteasome becomes less efficient in HD. Induction of the proteasome activity as well as of autophagy protects against the toxic insults of mutant huntingtin proteins by enhancing its clearance. *B*: NH_2-terminal fragments containing the polyQ strech accumulate in the cell cytoplasm and interact with several proteins causing impairment of calcium signaling and homeostasis (*C*) and mitochondrial dysfunction (*D*). *E*: NH_2-terminal mutant huntingtin fragments translocate to the nucleus where they impair gene transcription or form intranuclear inclusions. *F*: the mutation in huntingtin alters vesicular transport and recycling. muHtt, mutant huntingtin.

with both the glutamine-rich activation domain and the acetyltransferase domain of CBP, causing a reduction in histone acetylation, thus leading to a more compact chromatin structure that is less accessible to transcription factors, and therefore causing reduction in BDNF mRNA levels (550). Finally, it is also possible that mutant huntingtin alters the subcellular distribution of the different BDNF mRNA variants to regulate BDNF protein expression locally. This hypothesis is based on studies by Enrico Tongiorgi and colleagues at the University of Trieste (114), who revealed that the different BDNF mRNA isoforms can accumulate in distal dendrites or in the soma in response to stimuli triggering activation of NMDA receptor (NMDAR) and tropomyosin-related kinase B (TrkB) receptors.

2. BDNF vesicle transport is reduced in HD

In 2004, the group of Frederic Saudou (202) proposed that reduced BDNF levels in the HD striatum may depend also on reduced transport of BDNF vesicles along the cortico-striatal afferents. BDNF vesicular transport was shown to be significantly and specifically reduced in mutant huntingtin cells (202). The transport of mitochondria

was unaffected in the same homozygous mutant huntingtin knock-in cells, thus indicating that wild-type huntingtin specifically enhances BDNF vesicle transport in this cell system. However, these data conflict with other studies showing that mitochondria transport was specifically affected in cells depleted of endogenous huntingtin and in cells bearing mutant huntingtin (107, 420, 584). The different cell systems used may explain these opposite results and raise the question as to whether defects in BDNF vesicle transport represent a selective HD phenotype. To reinforce the selectivity of huntingtin involvement in the transport of BDNF vesicles, it was then demonstrated that the proteins involved in other neurodegenerative diseases do not affect BDNF transport (202).

To evaluate the possible in vivo alteration of BDNF transport in the brain, the composition of the microtubule transport machinery was evaluated by biochemical fractionation assays in brain homogenates from mutant huntingtin knock-in mice. Huntingtin was found to be part of the motor complex that drives anterograde and retrograde transport of BDNF vesicles along the microtubules. The results of experiments performed using HD cells and mice, as well as human post mortem brain tissues, suggest that this motor complex is altered in HD. In particular, increased binding of mutant huntingtin to HAP1 was found to reduce the association between HAP1/p150Glued dynactin and microtubules in heterozygous mutant huntingtin knock-in mice (202). This suggests that the mechanism controlling retrograde transport is altered in the presence of the polyglutamine expansion in huntingtin. In addition, as most striatal BDNF comes from anterograde (and not retrograde) transport from the cerebral cortex, Gauthier et al. (202) have investigated whether the association between kinesin and microtubules is also reduced and found this to be the case in in vitro experiments using homozygous mutant huntingtin knock-in cells. On the basis of the consideration that, in yeast two hybrid experiments, HAP1 may be pulled down with a human kinesin-like protein, it was suggested that the complex consisting of huntingtin/HAP1 and kinesin may be affected by the polyglutamine expansion, leading to impaired anterograde transport of BDNF vesicles (202).

Another critical factor implicated in the regulation of BDNF vesicle transport is the phosphorylation of huntingtin at serine-421 by Akt kinase. When phosphorylated at this position, huntingtin promotes anterograde transport (125). Conversely, when huntingtin is not phosphorylated, BDNF vesicles are more likely to undergo retrograde transport (125). Reduced phosphorylation of huntingtin at serine-421 is observed in cellular and animal HD models and in post mortem human tissues, and this is likely to impair the transport of the neurotrophin (125, 625).

A different study carried out in a knock-in mouse model of HD, which carries a 150 CAG triplet repeat expansion in the huntingtin gene [Hdh(CAG)150 knock-in], reported impaired movement of BDNF vesicles along microtubules in striatal and hippocampal primary neurons, but not in cortical neurons, the main source of striatal BDNF (252). Moreover, contrary to previous findings of Gauthier et al. (202), this study shows that the observed alteration of BDNF vesicles transport in HD is not attributable to a disruption of motor protein complexes (252). To test whether this discrepancy could be caused by differences in the HD mouse models used [Hdh-(CAG)150 vs. Hdh109Q/109Q used in the Gauthier's study], differences of age or methods, Her and Goldstein (252) performed sucrose gradient fractionation of brain extracts of 14-mo-old Hdh109Q/109Q mice using a 7.5–25% sucrose gradient as previously described. No gross changes in the pattern of the dynein and dynactin complexes and of kinesin or HAP1 between mutant and control mice were found. Although endogenous huntingtin is required for axonal transport of various cargoes, it is unclear whether mutant huntingtin disrupts all axonal transport. As suggested by Chang et al. (107) and Orr et al. (420), mutant huntingtin might form aggregates such that they bind to cargoes impairing their movement or physically blocking movement in axons. However, this is unlikely to occur in the Her and Goldstein experiments because no aggregates were observed in the presymptomatic primary neurons employed (252). The formation of huntingtin aggregates might precipitate motor proteins, thus reducing the soluble pool of motor proteins required for transport, as observed in both HD mouse brain and *Drosophila* embryos (230, 584). The mechanisms that underlie impaired BDNF transport in cortical neurons in HD require further investigation.

3. BDNF loss worsens the HD phenotype

In light of the evidence indicating reduced BDNF levels in HD, a number of studies involving genetically altered mice have evaluated the effects of the modification of the levels of this neurotrophin on disease onset and progression. In a first set of experiments, empty spiracles homolog *(Emx)*-BDNF^{KO} mice, which are genetically engineered to be deficient in BDNF production in cortical neurons with little BDNF reduction in the thalamus, midbrain, and hindbrain were produced. These mice gradually develop brain damage and a hindlimb clasping phenotype in a very similar pattern to HD mice (35). Cortical *Emx*-BDNF^{KO} mutants show significantly smaller striatal volumes due to reduced MSN soma size, thinner dendrites, and fewer dendritic spines than wild-type littermates. These data are in agreement with earlier studies demonstrating that BDNF stimulates the morphological differentiation of striatal neurons by increasing the length of their neurites, the number of branching points on the neurites, and the soma area (276). Another study has confirmed reduced BDNF support as one major pathway

causing striatal dysfunctions in human HD (553). The aim of this specific work was to identify the animal model that better recapitulates the striatal gene expression phenotype of human HD. This study included the most widely used genetic models of HD, i.e., the R6/2 line, three mechanistically motivated HD models of mitochondrial dysfunction [3-nitropropionic acid (3NP)-treated rats, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, and *PGC1-alpha* knock-out mice], and *Emx*-BDNF^{KO}. Remarkably, the authors found the *Emx*-BDNF^{KO} mutants to exhibit striatal gene expression abnormalities most similar to those described in human post mortem HD striatum (553).

In a second experimental paradigm to explore the relevance of BDNF depletion in HD pathogenesis, inactivation of one BDNF allele was achieved in a transgenic mouse line expressing human huntingtin exon 1 with an expanded CAG repeat (i.e., the R6/1 mouse) (92). These mice were reported to show a worsening of the HD phenotype as shown by anticipated age of onset and exacerbated behavioral deficits (92). These results provided further support to the idea that cortical BDNF depletion is a critical factor in the pathology of HD.

4. BDNF administration is neuroprotective in HD

During the early 1990s, studies aimed at testing a possible neuroprotective role of BDNF in HD started soon after the discovery that BDNF was a potent prosurvival and prodifferentiative factor for developing and mature neurons. The first experiments to assess the effect of BDNF augmentation in vivo in HD mice were performed in chemically induced models. BDNF delivery by protein infusion, intrastriatal injection of BDNF-expressing adenovirus, or grafting of BDNF-expressing cells conferred protection to striatal neurons exposed to excitotoxins (668). These early findings have been recently corroborated by a study in which the BDNF gene was delivered to the striatal neurons by use of adenoviral vectors. The authors found that transfer of low concentration of BDNF gene to striatal neurons using serotype adenoassociated viral vector (AAV) increased BDNF protein levels in the striatum and conferred protection to striatal neurons against excitotoxic insult, thus attenuating motor impairment (46, 303, 304).

The impact of BDNF delivery has also been evaluated in genetic models of HD. Experiments on cultured cells showed that BDNF conferred protection against death in neurons transfected with mutant huntingtin (505). Later, three independent studies tried to establish whether BDNF could be neuroprotective in vivo. In one study, BDNF was delivered via osmotic minipump into the striatum of mice overexpressing exon 1 of human mutant huntingtin (R6/1 mice). It was found that daily treatment of BDNF for 1 wk succeeded in increasing the expression of enkephalin, as well as in augmenting the number of enkephalin-expressing striatal neurons, the most severely affected cells in HD (92). The same study showed a slight improvement of the behavioral phenotype after BDNF administration (92). To better address the potential of BDNF increase in the brain of HD mice, in a separate study, the neurotrophin was constitutively overexpressed in R6/1 mice by means of the promoter of the α-subunit of CaMKII (207). Such overexpression in the striatum and cerebral cortex of HD mice substantially ameliorated motor dysfunction, reversed brain weight loss, restored TrkB signaling in the striatum, and reduced the formation of mutant huntingtin aggregates in neurons (207). Finally, in a third study, BDNF was delivered by means of adenoviral vectors in combination with noggin, a molecule that promotes neurogenesis and regulates striatal neuronal regeneration. In this case, the authors observed delayed motor impairment in the BDNF/Noggin-treated R6/2 transgenic mice (115). In particular, these mice exhibited a significant slowing in latency to fall and in rotarod impairment relative to untreated HD mice. Moreover, the BDNF/Noggin-treated mice survived an average of 16.8% longer than the respective controls. BDNF alone treated mice showed a slight improvement of motor impairment with respect to untreated mice but did not significantly survive longer than controls (115). These results suggest that the neurotrophic action of BDNF in combination with molecules that stimulate neurogenesis might confer considerable therapeutic potential for mitigating both neuropathological and motor function deficits in the brain of patients with HD.

5. BDNF polymorphisms in HD

One known polymorphism of the human BDNF gene is a valine-to-methionine substitution at position 66 (Val66Met BDNF) that is located in the 5′ pro-BDNF sequence encoding the precursor peptide (pro-BDNF), which is proteolytically cleaved to form the mature protein. This BDNF polymorphism does not affect mature BDNF protein function nor its rate of transcription, but it has recently been shown to dramatically alter the intracellular trafficking and packaging of pro-BDNF, and consequently the regulated secretion of the mature peptide (113, 171).

The BDNF Val66Met polymorphism is highly conserved across species and relatively common in the human population with a prevalence for heterozygotes of 20–30%, and a prevalence for the homozygote of ~4% (171, 241, 408, 519). Several genetic linkage and behavioral studies have shown that this polymorphism is associated with neuropsychiatric disorders, including Alzheimer's disease, Parkinson's disease, bipolar disorders, depression, obsessive compulsive disorder, and schizophrenia, as well as with normal personality traits (399, 407, 408, 531, 609).

In the case of HD, it was found that mutant huntingtin does not affect the transport of BDNF Val66Val nor of Val66Met BDNF from the endoplasmic reticulum to the Golgi apparatus. Instead, it specifically alters the post Golgi trafficking of BDNF vesicles. In particular, the post Golgi trafficking of Val66Val BDNF was significantly blocked in mutant huntingtin cells, whereas the transport of Val66Met BDNF was not affected. These data clearly indicate that the mutant protein affects solely the trafficking of Val66Val BDNF form, without causing a major retention of Val66Met BDNF in the Golgi apparatus (139).

Linkage studies reported a later age of onset in HD patients who were heterozygous for the Val66Met polymorphism compared with individuals who were homozygous for valine or methionine at this position, although this association was restricted to the group of patients with huntingtin CAG repeats between 42 and 49 (667). However, four subsequent independent studies did not confirm an effect of Val66Met and other BDNF polymorphisms, representing the entire variability of the BDNF gene, on the age of onset of HD (667). Collectively, these studies conclude that there is no convincing genetic link between BDNF polymorphisms and HD. As the Val66Met polymorphism influences BDNF transport from the Golgi region to the appropriate secretory granules, without affecting the transcriptional or biological activities of this molecule, we proposed that the lack of an association might indicate that the defect in BDNF transport has no impact on the age of disease onset, although it may still affect disease progression. However, this evidence does not exclude the possibility that a defect in BDNF transcriptional activity may affect age of onset and/or disease progression (668).

The evidence reviewed above points to a deficit in BDNF as a major contributor to HD pathogenesis, thus suggesting the possibility that delivering BDNF or increasing endogenous BDNF production may stop or delay the progression of the human disease. Ongoing strategies to increase BDNF levels in the brain will be described in section v*B*.

B. Excitoxicity and Corticostriatal Dysfunction

Excitotoxicity is the first identified pathogenic mechanism that results in the dysfunction of neuronal interaction and circuitries at the corticostriatal synapse. In the context of HD, this hypothesis stipulates excessive activation of glutamate receptors that may be due to increased glutamate release from cortical afferents and reduced uptake of glutamate by glia. Moreover, hypersensitivity of postsynaptic glutamate receptors on striatal projection neurons, likely in combination with pathological signaling downstream of glutamate receptor, may also contribute to pathogenesis. In this section we discuss the evidence in support of a role of excitotoxicity in HD, focusing on the more recent studies elucidating the molecular mechanisms underlying this dysfunction. We also describe the defects in other neurotransmitter systems that control the activity of the corticostriatal synapse and that contribute in rendering the striatal neurons more vulnerable to excitotoxic stimuli. Figure 4 shows neurotransmitter systems that are dysfunctional at the corticostriatal synapse and the reported underlying mechanisms.

1. NMDAR activity is altered in HD

The theory of excitoxicity as a pathogenic mechanism of HD has emerged when abnormalities of the glutamatergic neurotransmission were found in autoptic human brain tissues. Striatal neurons, the most affected neuronal population in HD, depend for their survival and activity on glutamate release from the cortical afferents. The first evidence supporting the excitotoxic model came from the discovery that glutamate receptors are lost in HD, and the evidence of a significant decrease of NMDA receptor (NMDAR) binding at pre- and early symptomatic stages in the human HD (4, 146, 167, 353, 654). Further data in rodent models showed that intrastriatal injections of glutamate agonists, particularly those acting on NMDA receptors, caused a pattern of striatal cell death similar to the one observed in human HD brain (42, 43, 86, 184, 239, 495, 515). Similarly, increased generation of the endogenous NMDAR agonist quinolinic acid, and its bioprecursor 3-hydroxykynurenin, may contribute, at least in part, to excitotoxicity (227, 228, 516).

With the production of transgenic mice, a unique opportunity became available to assess the influence of expanded polyQ tracts on the susceptibility to excitotoxicity of striatal neurons in vivo. Many reports documented increased glutamate release from cortical afferents in HD mouse models and increased susceptibility to excitotoxic insults while other work showed a complete resistance to excitotoxicity (176, 238, 401). This discrepancy seems to be related to the different transgenic models employed in the different studies and, particularly, to the size of the transgene they express. Mutant huntingtin-induced excitotoxicity has been confirmed in striatal neurons from mice expressing full-length human mutant huntingtin (YAC lines), but not in transgenic mice bearing an NH_2-terminal portion of the protein (R6/2 and N171–82Q lines) that rather show striatal resistance to excitotoxicity. The development of resistance to excitotoxicity in these models may represent an adaptive measure in response to mutant huntingtin expression to generate compensatory mechanisms that reduce some of the toxic effects of the polyQ expansion fragment. In this regard, it is interesting to note that the expression of polyQ-expanded exons 1–2 of huntingtin ("Short-Stop") or full-length huntingtin mutated to eliminate caspase-6 cleavage in the YAC128

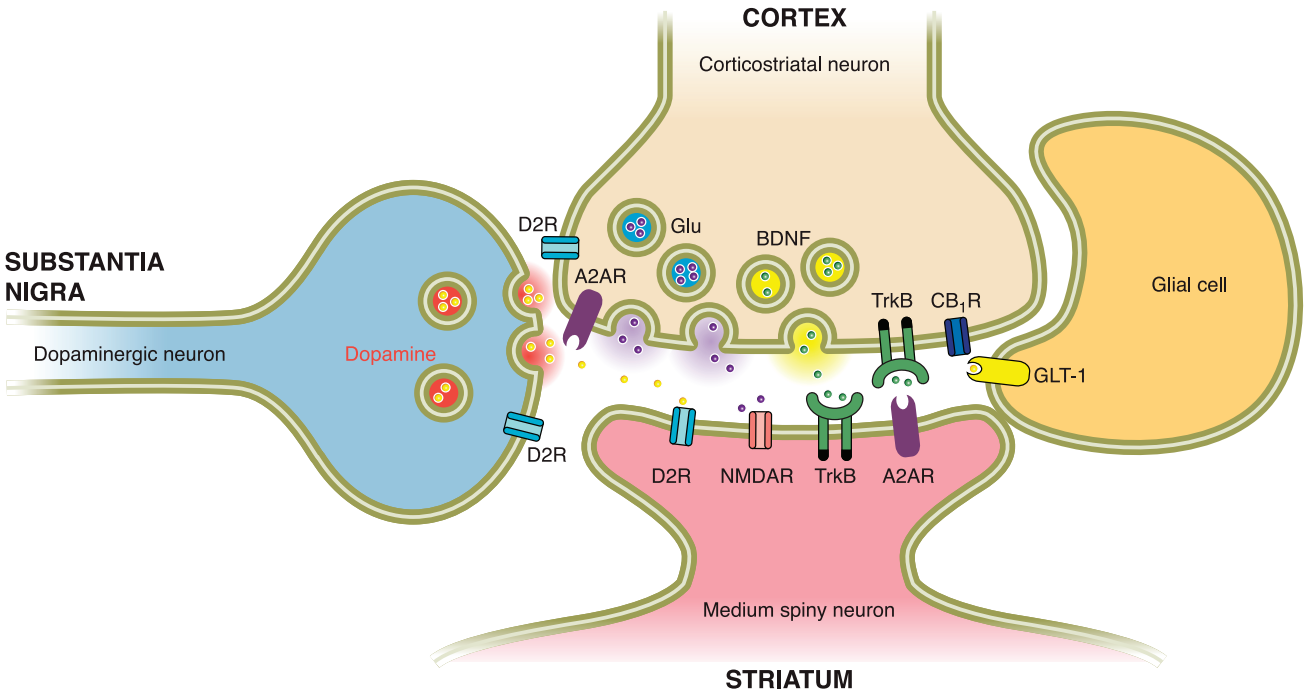


FIG. 4. Neurotransmitter systems and growth factors that are dysfunctional at the corticostriatal pathway. Neuronal death may depend on excitotoxicity that results from increased glutamate (Glu) release from cortical neurons and increased activity of the glutamate receptor (NMDAR). In addition to glutamate, other neurotransmitter systems that control the activity of the corticostriatal synapse can contribute to render striatal neurons more sensitive to excitotoxic stimuli. Adenosine A2 receptors (A2AR) and cannabinoid receptors (CB_1R) are particularly abundant on the corticostriatal terminals, where, when activated, they increase glutamate release. A crucial input to the striatum comes from the substantia nigra pars compacta, whose fibers represent the main striatal source of dopamine. Dopamine can directly regulate glutamate release from corticostriatal terminals by stimulating the D2 receptors (D2R) located on the cortical afferents. Glial cells may also play important roles through cell-cell interactions. For example, decreased glutamate uptake in glial cells by GLT-1 contributes to increased neuronal vulnerability and neuronal excitotoxicity in neurons. Reduced BDNF production and release from the cortical afferents contribute to neuronal death. Striatal BDNF is produced in the cortex where its transcription is downregulated in the presence of mutant huntingtin. In addition, mutant huntingtin reduces BDNF vesicle transport. Both mechanisms result in loss of BDNF trophic support to striatal neurons.

model confer resistance to excitotoxic stimuli in vitro and in vivo, and also rescue neuronal degeneration and HD-like symptoms (220, 533). It is therefore likely that certain NH_2-terminal fragments of mutant huntingtin are required for enhanced sensitivity to excitotoxicity and that this characteristic correlates well with the selective neuronal degeneration also observed in human HD.

Aside from altered glutamate release, impaired clearance of glutamate from the synaptic cleft may contribute to enhance excitotoxic neurodegeneration in HD. Increasing evidence implicates GLT1, the Na^+-dependent glial transporter of glutamate in HD. GLT1 is responsible for the removal of most extracellular glutamate, and there is mounting evidence that GLT1 actively participates in the regulation of synaptic transmission (587). In some HD mouse models (R6/2 and R6/1 lines), GLT1 is downregulated and appears responsible for decreased striatal glutamate uptake (44, 173, 345, 525). Decreased GLT1 mRNA and deficient glutamate uptake has also been reported in post mortem brain tissues taken from HD patients (21, 244). In a recent study, Miller et al. (391) have shown that increasing striatal GLT1 expression by pharmacological treatment attenuates the neurological signs of HD in R6/2 mice, suggesting that a dysregulation of striatal glutamate uptake by glial cells may play a key role in HD.

Electrophysiological recording confirmed the excitotoxic hypothesis. Enhanced NMDAR sensitivity to NMDA and increased NMDA evoked currents in striatal neurons from full-length mutant huntingtin mice, leading to impaired synaptic plasticity, have been described (176). Electrophysiological (single-unit recording and iontophoresis) and neurochemical (microdialysis and voltammetry) techniques have also highlighted altered information processing in the prefrontal cortex of HD mouse models and dysregulation of coordinated neuronal firing patterns in striatum (392, 620). These results suggest that the loss of connectivity between the cortex and striatum may participate in the excitotoxic process and contribute to the development of the HD phenotype (101).

2. Mechanisms of NMDAR alteration

Changes in NMDAR protein level or subunits have been proposed as possible mechanisms responsible for aberrant NMDAR activity in HD (6, 51, 100, 175, 282). Increased mRNA for NR1A subunit has been observed to

associate with increased NMDAR activity (175). In contrast, Lynn Raymond and colleagues at the University of British Columbia found that the level of NR2B subunit of NMDAR was reduced in the HD striatum, due to both reduced transcription of the NR2B gene and increased proteolysis of the subunits by calpain proteolytic enzymes (132, 658). NR2B subunits are particularly abundant in the striatum compared with other regions of the forebrain. Thus the presence of NR2B-type NMDAR complex with impaired functional characteristics may help to explain the preferential vulnerability of these cells in HD (132, 658). Gene expression studies in the R6/2 transgenic mouse model of HD also showed reduced expression of metabotropic glutamate receptor mGluR2 that could lead to glutamate-mediated overstimulation of the postsynaptic striatal neurons (103, 104, 357, 359).

In addition, the polyglutamine expansion was found to interfere with the ability of wild-type huntingtin to interact with PSD95, a scaffolding protein of the postsynapse, resulting in the sensitization of NMDA receptors (558). NMDAR function is also modulated by posttranslational modifications, such as phosphorylation. Mutant huntingtin-induced phosphorylation of the NR2B subunit contributes to promoting overactivation of NMDAR (539). NMDAR trafficking is also impaired in HD by destabilization of the clathrin-mediated endocytotic complex that involves NMDAR, huntingtin, and HIP-1 (175, 389).

3. Altered signaling pathways downstream of NMDAR

The overactivation of NMDARs has been mechanistically linked to intracellular calcium signaling, mitochondrial activity, and apoptotic pathways (Fig. 5). Mutant huntingtin binds the COOH terminus of the inositol 1,4,5-triphosphate receptor 1 ($InsP_3R1$) on the endoplasmic reticulum and renders the receptors more sensitive to IP_3 (569). The stimulation of glutamate receptors known for activating the IP_3 signaling pathway causes increased calcium release from $InsP_3R1$ in cells expressing mutant huntingtin (568). It was demonstrated that mutant huntingtin exerts an early compensatory attempt to prevent the calcium stress by reducing the transcription of components of the phosphatidylinositol cycle, and by decreasing basal calcium levels (346). This compensatory effect is, however, lost as disease progresses and intracellular calcium concentration is elevated in striatal neurons from symptomatic HD in basal condition and after exposure to NMDA (176, 568, 569). As mitochondrial calcium storage capacity is exceeded with time, mitochondria swelling and release of cytochrome *c*, apoptosis-inducing factor (AIF), calcium, and other proapoptotic factors into the cytoplasm occurs (657). This process is exacerbated by the direct action of mutant huntingtin on the mitochondrial transition pore that decreases the calcium threshold necessary to trigger the pore opening (116, 390).

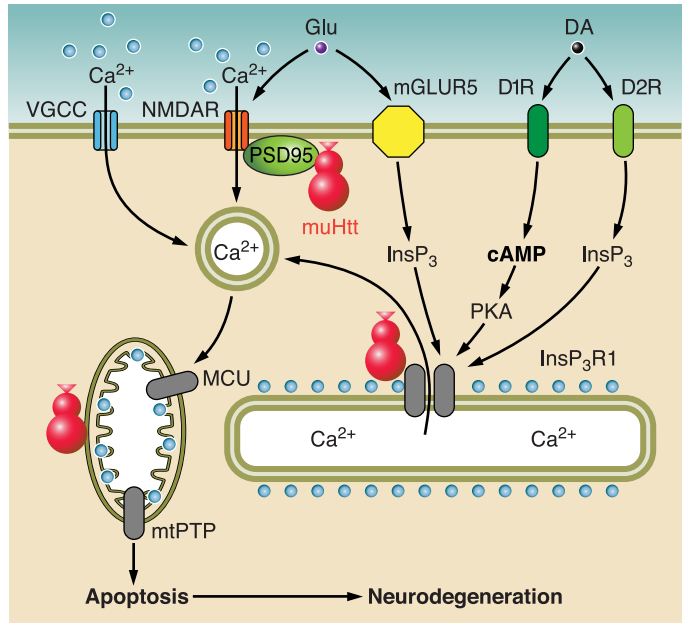


FIG. 5. Dysfunction of Ca^{2+} signaling in HD. Mutant huntingtin causes cytosolic and mitochondrial Ca^{2+} overload and apoptosis of HD MSN. Mutant huntingtin perturbs Ca^{2+} signaling by enhancing NMDAR function, possibly through decreased interaction with the PSD95-NR1A/NR2B complex. Moreover, mutant huntingtin binds strongly to $InsP_3$ R1, causing Ca^{2+} release through the $InsP_3$ R1. Dopamine (DA) released from midbrain dopaminergic neurons stimulates D1 and D2 receptors (D1R, D2R). D1R are coupled to activation of adenyl cyclase, increase in cAMP levels, and activation of PKA. PKA potentiates glutamate-induced Ca^{2+} signals by facilitating the activity of NMDAR and $InsP_3$ R1. D2R are coupled directly to $InsP_3$ production and activation of $InsP_3$ R1. Supranormal Ca^{2+} signals activate calpain, which cleave huntingtin and other substrates. Excessive cytosolic Ca^{2+} signals result also in mitochondrial Ca^{2+} uptake, which eventually triggers mtPTP opening and apoptosis. The mitochondrial Ca^{2+} handling is further destabilized by direct association of mutant huntingtin with mitochondria. muHtt, mutant huntingtin; MCU, mitochondrial calcium uniporter; mtPTP, mitochondrial permeability transition pore; VGCC, L-type voltage-gated calcium channel.

The evidence described above documents that cortical hyperexcitability is a major event in HD that is likely triggered by the increased sensitivity of NMDARs located on striatal neurons. The discovery of multiple mechanisms underlying this event provides new targets for therapeutic intervention and paves the way for treatments with NMDAR antagonists.

4. Defects in neurotransmitter systems that influence glutamate release

Beyond glutamate, other neurotransmitter systems controlling the activity of the corticostriatal synapse can help to render striatal neurons more sensitive to excitotoxic stimuli (Fig. 4). Adenosine (A) and A2 receptors, as well as cannabinoids (CB) receptors, which are particularly abundant on the corticostriatal terminals, can enhance glutamate release upon activation. In addition, a crucial input to the striatum comes from the substantia

nigra pars compacta, whose fibers represent the main striatal source of dopamine (DA). DA can directly regulate glutamate release from corticostriatal terminals by stimulating the D2 receptors located on cortical afferents (34) (Fig. 4).

Evidence of aberrant A2A receptor function has been found in a striatal cell model of HD, as well as in R6/2 mice that exhibit a transient, but significant, increase of A2A adenosine receptor density and A2A receptor-stimulated adenylyl cyclase activity in the striatum, possibly contributing to excitotoxicity (570, 605).

CBs also play a critical role at the cortico-striatal synapse by regulating corticostriatal glutamate release (361). Two subtypes of CB receptors have so far been identified: the CB1 receptor, which is predominantly distributed in the CNS and testis (205, 633), and the CB2 receptor, which is restricted to the periphery (199). Cannabinoids inhibit glutamatergic synaptic transmission by acting on presynaptic CB1 receptors that densely populate the striatum and cerebral cortex. More importantly, it has been demonstrated that the activation of these receptors increases the amount of BDNF released from the presynaptic terminals (370).

Downregulation of the dopamine transporter and D1 and D2 dopamine receptors occurs in HD brains (26, 103, 104, 211), implicating a role of the dopamine pathway in HD. It is known that degeneration of nigrostriatal projections (69, 182, 211, 559) of dopaminergic neurons in the substantia nigra (421, 653) and a reduction in the dopaminergic striatal neuronal population occurs in HD brains (272). Dopamine can directly regulate glutamate release from corticostriatal terminals by stimulating the D2 receptors located on a subpopulation of cortical afferents (34). Reduced levels of dopamine or dopamine receptors may contribute to alter glutamate release. An interesting study from Bezprozvanny's group at the University of Texas Southwestern Medical Center in Dallas (567) has further confirmed the impairment of the dopamine signaling pathway in HD. They discovered that glutamate and dopamine signaling pathways act synergistically to induce elevated calcium signals, causing apoptosis of YAC128 MSNs in vitro (Fig. 5). Consistent with these findings, persistent elevation of striatal dopamine level in vivo exacerbated the behavioral motor deficits and MSN neurodegeneration in YAC128 mice (567). Furthermore, the same group demonstrated that dopamine inhibitors protect HD MSNs from cell death both in vitro and in vivo in YAC128 mice. These results suggest that the dopamine signaling pathway plays an important role in HD pathogenesis and that antagonists of the dopamine pathway such as tetrabenazine or dopamine receptor blockers may have therapeutic potential in the treatment of HD.

C. Proteolysis

One of the landmark discoveries in HD was pioneered by Michael Hayden's laboratory in 1996 (218), when huntingtin was found to be subject to proteolytic cleavage by caspases and that the blocking of mutant huntingtin proteolysis was beneficial in treating HD mice. For the first time, a substrate involved in a neurological disease was shown to be cleaved by caspases (218). After the finding that cellular toxicity was increased in cells expressing huntingtin fragments, the discovery of huntingtin as a caspase substrate led to the development of a model connecting caspase cleavage of huntingtin to the amplification of additional caspases activity in a vicious cycle. This model, known as the toxic fragment hypothesis, postulates that proteolytic cleavage of huntingtin liberates toxic fragments containing the expanded polyglutamines and that their accumulation may lead to the activation of additional proteolytic caspases and the eventual demise of the cell. A more recent finding from Hayden's group indicates that a crucial proteolytic cleavage event in HD is mediated by caspase-6. In fact, the blockade of caspase-6-mediated huntingtin cleavage improves the disease phenotype in HD mice (220), and it was shown that pharmacological inhibition of caspases is beneficial to HD mice (111, 254, 418, 622). Further evidence for huntingtin proteolysis as a pathological mechanism operating in HD comes from studies by Marian DiFiglia and colleagues at the Massachusetts General Hospital who showed that both normal and mutant huntingtin are cleaved by calpain cysteine proteases (310).

1. Evidence of huntingtin cleavage

The identification of huntingtin as a substrate for caspase-3, in 1996, suggested that huntingtin proteolysis was involved in HD (218). In addition to caspases (218, 253, 630–632), calpains were found to also cleave huntingtin (59, 197, 198, 217, 308, 310). Huntingtin is also susceptible to aspartyl proteases that, like caspases and calpains, cleave the protein within the NH_2-terminal region (355). The cleavage events occur both in normal and mutant huntingtin, but the latter is more susceptible to proteolysis and generates NH_2-terminal fragments that are found in the cytoplasm and nucleus of neuronal and nonneuronal cells (137, 148, 246, 310, 355, 630).

In contrast to these findings, one study conducted on human post mortem material reported that mutant huntingtin is more resistant to cleavage than wild-type huntingtin (169). This study suggested that, in addition to proteolytic enzymes, other factors could be involved in the cleavage of huntingtin. Among them, posttranslational modifications were suggested as important regulators of huntingtin proteolysis. In fact, huntingtin phosphorylation at S434 by Cdk5 prevents the cleavage of the protein,

while phosphorylation at S421 reduces huntingtin cleavage by caspase-6 and the nuclear accumulation of caspase-6 fragments (356, 625, 626).

The evidence for caspase cleavage of huntingtin is progressively expanding. We now know that, in vitro, huntingtin is cleaved by caspase-3 and caspase-7 at amino acids 513 and 552, by caspase-6 at amino acid 586, and by caspase-2 at amino acid 552 (220, 253, 630, 632). These data are validated by multiple experiments showing that treatment of cells with caspase inhibitors or mutations in the identified cleavage sites block the cleavage of huntingtin (632). Caspase fragments of huntingtin were observed in vivo, in HD mice, and in post mortem brain tissue from HD patients (310, 630). Robert Friendlander's group at the Brigham and Women's Hospital, Harvard Medical School (418), produced evidence of caspase-1 activation in the brains of mice and humans suffering from the disease. The same authors found that the expression of a dominant-negative caspase-1 mutant extends survival and delays the appearance of neuronal inclusions, neurotransmitter receptor alterations, and onset of symptoms, indicating that caspase-1 is important in the pathogenesis of the disease. Similarly, intracerebroventricular administration of a caspase-1 inhibitor delays disease progression and mortality in R6/2 mice (418).

Tissue-specific proteolysis in striatum and cortex has been also reported in HD (384, 582). These observations were made by using specific antibodies, but must be interpreted with care, as a cross-reaction with other proteins is always possible. One way to overcome this potential problem is to sequence the fragments to confirm that they are derived from huntingtin; however, this has not yet been undertaken. The histological analysis of mouse and human HD brains revealed activation of caspase-2 in MSNs and cortical projection neurons compared with controls, suggesting that caspase-2 may be involved in the selective neuronal cell death associated with HD (253).

Huntingtin fragments are produced also by calpain and aspartic endopeptidases (59, 197, 198, 217, 308, 310, 355). Calpains are a family of calcium-dependent intracellular cysteine proteases activated by many apoptotic and necrotic stimuli, particularly those that alter calcium levels in the cell. In the context of HD, disturbances in calcium homeostasis, due to excitotoxicity and/or mitochondrial dysfunction, may result in activation of calpains. Calpain activation has been detected in human HD tissue, and the overall levels of both active and inactive calpains are increased in the brain of HD patients (197, 310). Calpains I, II, and m have all been implicated in the cleavage of huntingtin with a rate that is polyglutamine-length dependent (197, 217, 308, 310). Two specific calpain cleavage sites have been identified in huntingtin protein at residues 469 and 536, in the same region as the caspase cleavage sites (310).

The aspartic endopeptidase produces two fragments called cp-A and cp-B, with cp-A preferentially located in the nucleus (355). More recently, two novel fragments called cp-1 and cp-2 have been identified both in the cytoplasm and nucleus and are produced by caspase-independent cleavage of huntingtin (460). These fragments have been defined predominantly by cell model studies, and the enzymes that produce this cleavage have so far remained elusive. Further studies are required to better understand the role of these fragments in HD pathogenesis.

2. The relevance of caspase-6-mediated cleavage in HD

One recent study performed in Michael Hayden's lab indicates that cleavage of huntingtin by caspase-6 is particularly toxic and largely responsible for the HD behavioral and neuropathological phenotype observed in YAC128 mice (220). The authors have targeted two sites of mutant huntingtin in the YAC128 mouse model of HD where caspase-mediated fragmentation might occur, i.e., the site or part of the huntingtin protein that is cleaved in response to caspase-3 and the part that is cleaved in response to caspase-6 activation. Using striatal volume as a primary end point, this group found that caspase-3-resistant YAC128 mice developed the disease but the caspase-6-resistant YAC128 mice did not. In particular, they showed that inhibiting caspase-6 and not caspase-3 cleavage of mutant huntingtin protects against striatal atrophy and against neurotoxicity induced by multiple stressors. Moreover, it was shown that blocking the generation of the caspase-6 cleavage fragment is sufficient to prevent the motor dysfunction observed in the YAC model.

The studies described above strengthen the evidence for a role of mutant huntingtin proteolysis in HD and point at caspase-6-mediated cleavage as a crucial event in the disease process. Studies are underway to cross the YAC128 transgenic mouse with a caspase-6 knock-out mouse, as these will be critical for defining the role of caspase-6 in HD pathogenesis (589). As postulated by Hayden and colleagues, the activation of caspase-6 may be a primary event in the proteolytic process of mutant huntingtin that leads to the activation of additional proteolytic caspase activities (for example, to activation of caspase-2 and -3), which exacerbate neurodegeneration and contribute to the appearance of the disease phenotype. It is now therefore imperative to look for inhibitors of caspase-6 that may prevent or slow down the progression of the disease by preventing the production of toxic fragments.

D. Misfolding, Aggregation, and Clearance of Mutant Huntingtin

Historically, aggregates of mutant huntingtin have had a leading role in HD pathogenesis. The presence of

abnormal neuronal membrane ultrastructure in HD was first described by Roizin et al. (472) by electron microscope investigation of post mortem human brain tissues and later discovered in the brain of R6/2 mice in the form of aggregates (137). At that time, the puzzling evidence for the presence of aggregates in HD mice with little evidence that the same were present also in the human HD brain led DiFiglia et al. (148) to a careful reassessment of the presence of aggregates in autoptic HD brains.

The evidence collected clearly indicated that aggregates are present in neurons of all cortical layers and were more frequent in juvenile patients (present in 38–52% of total neurons) than in adult patients (present in 3–6% of total neurons). Aggregates were also observed in medium-sized neurons in the striatum but not in neurons of the globus pallidus or cerebellum. As expected, they were absent in the cortex and striatum of control subjects. The authors also reported that aggregates were positioned variably throughout the nucleus, adjacent to or distant from the nucleolus. Compared with the nucleolus, which fills 0.8–18% of the cross-sectional area of the nucleus, aggregates in ~30% of neurons covered 20–45% of nuclear cross-sectional area. This study revealed that one aggregate per cell was most common, but two or three per neuron were also seen in 5–7% of the labeled neurons (148). Mutant huntingtin aggregates are also ubiquitinated. This is in line with observations by Davies et al. (137) that showed that nascent nuclear inclusions contain huntingtin and ubiquitin immunoreactivity. Subsequently, aggregates have been described in a large set of HD transgenic mice overexpressing full-length or truncated forms of the mutant protein.

Huntingtin fragment length and amount, as well as the length of the glutamine repeat, are critical factors in determining the aggregation process (112, 235, 336, 341). The frequency of aggregates formation is higher in the presence of short NH_2-terminal huntingtin fragments (235). Biochemical analyses of nuclear and cytoplasmic inclusions showed that nuclear aggregates are composed mostly by the NH_2 terminus of mutant huntingtin fragments (130, 235, 371). On the other hand, extranuclear neuronal inclusions contain both full-length mutant and truncated huntingtin (130, 235, 371).

1. The process of aggregates formation

Important strides have been made in understanding the mutant huntingtin aggregation process. Two major aggregation pathways are in competition with each other and explain how the polyQ expansion can facilitate aggregation (Fig. 6). The first pathway, described almost 10 years ago, is mediated by aggregation of the polyQ stretch (37, 482, 624). PolyQ aggregation displays kinetics of nucleated-growth polymerization with a prolonged lag-phase required for forming an aggregation nucleus, followed by a fast extension phase during which additional polyglutamine monomers rapidly join the growing aggregate. The aggregates consist of β-sheet-rich fibrils aligned side-by-side to form ribbonlike structures and exhibit several defining features of amyloid, such as binding to thioflavin T, Congo red birefringence, and reactivity with a generic antiamyloid antibody.

The second pathway, recently elucidated by Ron Wetzel's group at the University of Pittsburgh (573), depends on the first 17 NH_2-terminal amino acids and involves intermediate structures. It is characterized by the formation of oligomers having the first 17 amino acids of the protein in its core and polyQ sequences exposed on the surface. As the polyQ increases, the structure decompacts and oligomers or protofibrils rearrange into amyloid-like structures capable of rapidly propagating via monomer addition (573). The same authors showed previously that the proline-rich flanking sequence (oligoPro) on the COOH-terminal side of the polyQ in exon 1 reduces aggregation kinetics and aggregate stability but does not fundamentally change the aggregation mechanism. Its effect is also directional; oligoPro added to the NH_2 terminus of polyQ has no impact on aggregation (55). These results indicate unprecedented complexity in how the primary sequence of huntingtin controls for polyQ aggregation (573).

2. Toxicity of aggregate

The toxicity of aggregate is based on the evidence that inclusion formation in cultured cells correlates with susceptibility to cell death (235, 258). Huntingtin aggregates were considered to be particularly toxic when specifically directed to the cell nucleus (652). Toxicity could arise from the recruitment of other polyQ-containing proteins that therefore may lose their physiological function. Many proteins in the cells contain a polyQ tract, in particular transcription factors and transcriptional regulators such as CBP. There is evidence that huntingtin aggregates accumulate in the nucleus and sequester transcriptional regulators (102, 413). Aggregates also affect other cellular processes such as axonal transport between the cell body and the synaptic terminal because of their skill to recruit soluble motor proteins (230, 336, 432).

Consistent with a toxic role of aggregates in the disease, aggregation increases with the progression of the disease in HD mice. Mutant huntingtin inclusions correlate with the disease progression in a conditional mouse model expressing exon 1 containing 94 CAG (HD94) (648). In the same study it is shown that the aggregates disappear when mutant huntingtin expression was turned off, leading to an amelioration of the behavioral and cognitive deficits (648). This study demonstrated for the first time that, despite the difficulties in biochemically dissolving or denaturing aggregates, the neurons are capable of

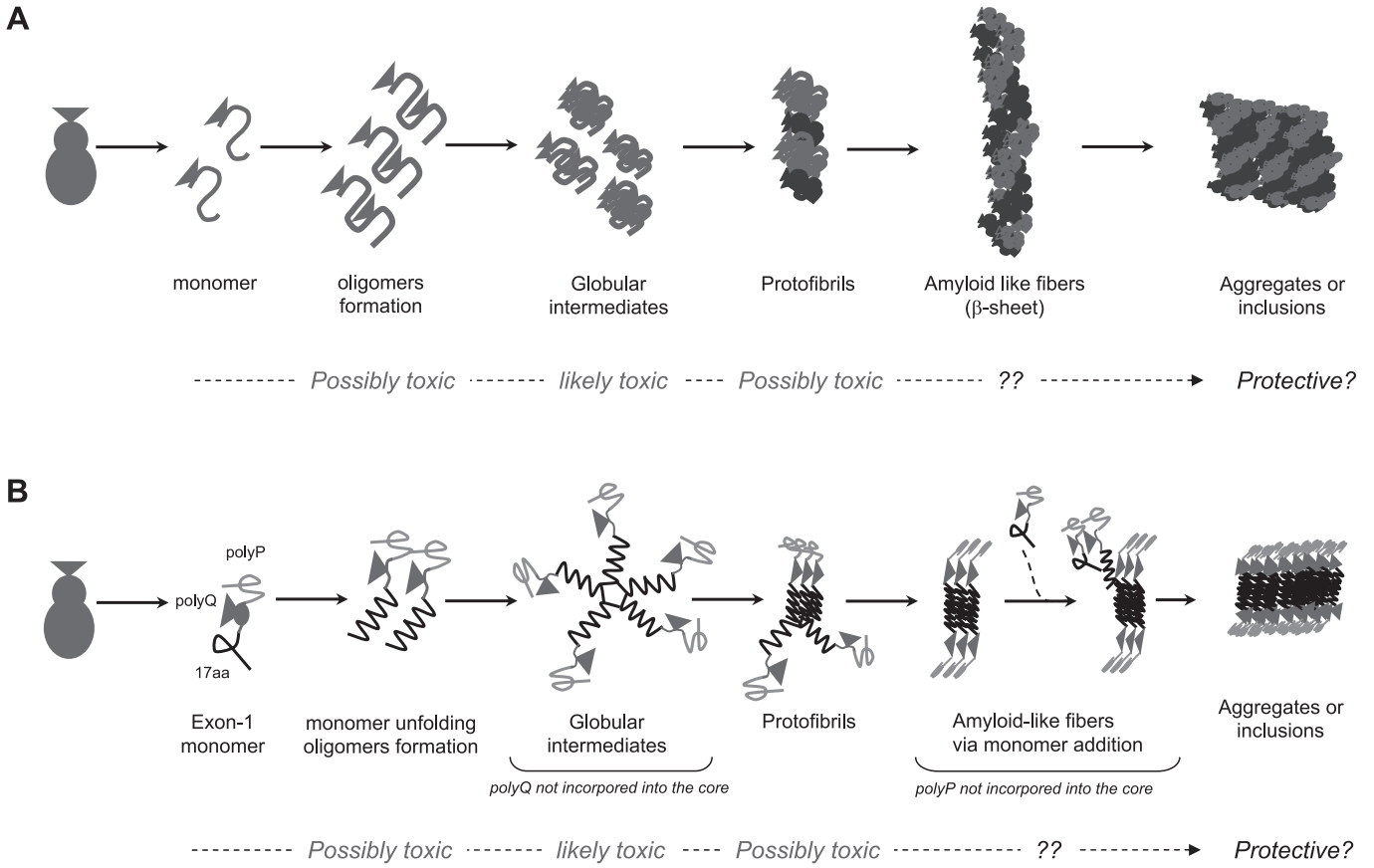


FIG. 6. The process of aggregate formation. Two major aggregation pathways are in competition with each other and explain how the polyQ expansion can facilitate aggregation. *A*: in the first pathway, mutant huntingtin undergoes covalent modifications (posttranslational modification or cleavage), determining the conversion of the protein to an abnormal conformation. The mutant protein forms oligomer intermediates that then give rise to globular intermediates from which protofibrils are generated. Protofibril intermediates associate to produce amyloid like structures, resulting in aggregates or inclusions. *B*: in the second pathway, oligomers having the first 17 amino acids of the protein in its core and polyQ sequences exposed on the surface are formed. As the polyQ increases, the structure decompacts and oligomers or protofibrils rearrange into amyloid-like structures capable of rapidly propagating via monomer addition and producing aggregates.

dispelling the nuclear and extranuclear accumulation of huntingtin. Therefore, therapeutic approaches aimed either at reducing aggregate formation or inactivating mutant huntingtin protein might be beneficial.

An increasing body of evidence implicates the ubiquitin-proteasome system (UPS) in the failure of cells to degrade mutant huntingtin, leading to aggregates formation. Huntingtin aggregates are labeled with antibodies to ubiquitin (48, 148, 619) and contain several proteasome subunits and chaperones (290, 494). This suggests that mutant huntingtin is targeted for degradation but cannot be properly cleared because the UPS is dysfunctional. Such impairment seems more pronounced in neurons than in glial cells, indicating that the lower neuronal UPS activity may account for the preferential accumulation of misfolded proteins in neurons, as well as for their preferential vulnerability (586).

Consistent with these results, administration of specific proteasome inhibitors in cellular and animal models of HD increases formation of huntingtin aggregates (48, 96, 266, 281, 312, 611). Instead, overexpression of heat shock proteins or chaperone proteins, which are components of the proteasome machinery, reduces huntingtin aggregation and increases life span in HD *Drosophila* and mice (96, 312, 590, 627, 646).

Proteasomal enzymes, paradoxically, seem to degrade polyQ flanking sequences, but not the polyglutamine tract itself (266, 608), resulting in the production of polyglutamine peptides that may be more prone to aggregation and toxicity, compared with the polyQ proteins containing the flanking sequences (608). New results suggest that among all cytoplasmic proteases, only one is able to digest polyQ fragments. It is the puromycin-sensitive aminopeptidase (PSA), a largely obscure enzyme that is abundant in the brain (56). PSA is upregulated in polyQ diseases and has drawn attention for its expression pattern in human brain areas resistant to tauopathy and for its ability to degrade tau and to protect against neurodegeneration in a fly model of tauopathy (294, 520). These findings suggest a role for PSA in huntingtin clearance

(56). It still remains to be determined whether PSA induction can retard the onset and progression of HD.

Several studies have focused on the identification of molecules that are able to interfere with aggregation of mutant huntingtin. One interesting strategy is based on intrabodies directed against the elongated polyQ tract or the NH_2-terminal region of huntingtin (see sect. VI*B*). High-throughput screens using in vitro and cell culture assays have been employed over the past few years to identify compounds that interfere with the aggregation process (248, 661). Compounds like Congo red, thioflavin S, gossypol, green tea, and trehalose have been found to prevent huntingtin aggregation (172, 249, 496, 565) (see sect. V*D*). However, it is not yet known how or at which stage of the aggregation process these compounds exert their inhibitory influence.

3. Neuroprotective role of aggregates

Several lines of evidence support the idea that mutant huntingtin inclusions are not pathogenic but rather an attempt of the cells to sequester toxic soluble fragments (321, 505). Evidence suggesting that aggregation is not sufficient to cause neurodegeneration comes also from HD mice expressing a CAG expanded huntingtin gene truncated after intron II, called Short-Stop mice (533). These mice show abundant aggregates but no behavioral dysfunction or neuronal loss (533). In a study by Steven Finkbeiner's group at the University of California (20), a robotic microscope was used to follow the fate over time of thousands of individual primary neurons expressing the exon 1 fragment of huntingtin fused to GFP. It was shown that neurons bearing aggregates survived significantly longer than those without aggregates. Similarly, other authors have demonstrated that aggregates per se are insufficient to determine cortical and striatal pathology in vivo (224, 226). Accordingly, compounds that promote the formation of mutant huntingtin aggregates have been suggested to rescue some of the toxic effects observed in the disease (67).

In conclusion, the role of aggregates (toxic or protective) may depend on the cell type and disease stage. Aggregates are present in the CNS but also in extra CNS territories apparently excluding any possible link between their distribution and the selective vulnerability observed in the disease (397, 504). A new view supports the hypothesis that small aggregates or even aberrantly folded monomeric forms of mutant huntingtin are toxic to cells and that inclusions may be protective by sequestering these diffuse toxic forms (20, 53, 462). Therefore, it is imperative to develop new approaches for assessing the earlier molecular aggregation. Understanding these steps will enable a better understanding as to how huntingtin aggregation affects cellular functioning and will influence the development of therapeutic compounds that could ameliorate this phenotype.

E. Autophagy

An independent set of evidence provided by the group of David Rubinsztein at the University of Cambridge, in the United Kingdom, argues that aggregates in HD are protective because they stimulate the autophagic process and clearance of mutant huntingtin (610). Autophagy is a bulk degradation process in which a portion of the cytosol and its content is enclosed by double-membrane structures called autophagosomes/autophagic vacuoles, which ultimately fuse with lysosomes for the degradation of the contents. Ravikumar et al. (462) in Rubinsztein's lab were the first to report that a negative regulator of the autophagic pathway, mTOR, is sequestered into huntingtin-polyQ aggregates in HD cell models, transgenic mice, and patients' brain. This ultimately leads to the induction of autophagy and clearance of mutant huntingtin fragments, which protects cells from death (462) (Fig. 7). These findings were in line with earlier studies showing increased numbers of autophagosome-like structures in the brain of HD patients (137, 300, 445, 456, 497). Furthermore, administration of chemical activators of autophagy or overexpression of genes implicated in autophagy enhance the clearance of mutant huntingtin, reduce aggregate formation, and improve the behavioral phenotype in HD mice, *Drosophila*, and *C. elegans* (285, 461, 462). In contrast, when the autophagy-lysosomal pathway is inhibited, soluble mutant huntingtin levels, aggregate formation, and toxicity increase (461). Recently, it has also been reported that autophagy in HD cells can be induced by acetylation of mutant huntingtin at lysine residue 444 (K444) (284). Increased acetylation at K444 facilitates trafficking of mutant huntingtin into autophagosomes, significantly improves clearance of the mutant protein by macroautophagy, and reverses the toxic effects of mutant huntingtin. Mutant huntingtin that is rendered resistant to acetylation dramatically accumulates and leads to neurodegeneration in the mouse brain. Acetylation is therefore proposed as a mechanism for the removal of accumulated proteins in HD and for actively targeting proteins for degradation by autophagy (284).

For the degradation by lysosomal acidic hydrolases, the fusion of autophagosomes with lysosomes is necessary (420). Without this fusion event, autophagosomes accumulate, thus increasing the toxic species within the cell. A recent study led by Andrea Ballabio at the Telethon Institute of Genetics and Medicine in Italy (498) has shown that stimulation of lysosomal activity by overexpression of transcription factor EB (TFEB), a master regulator gene of most lysosomal genes, in an inducible cell model of HD increases the degradation of the mutant

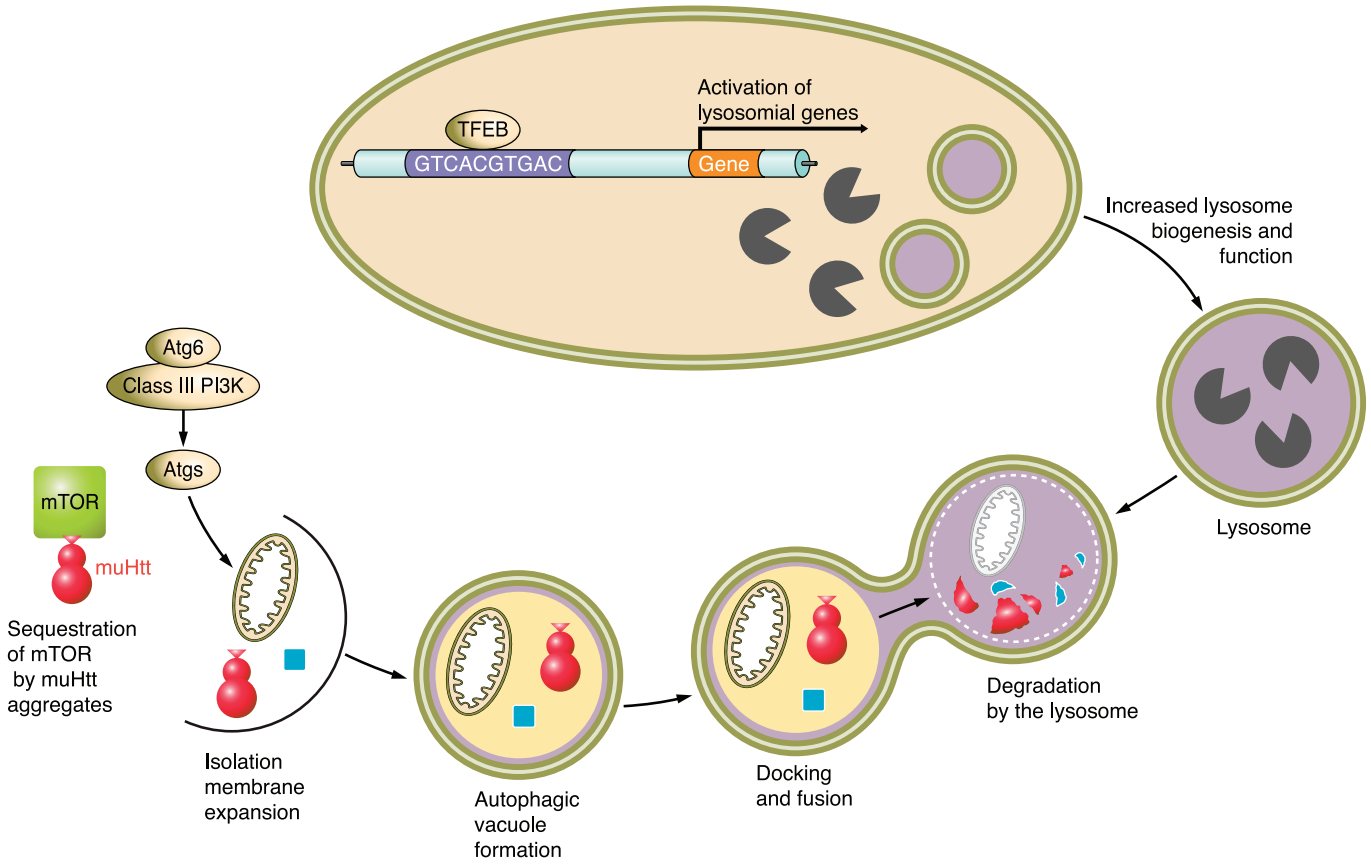


FIG. 7. The autophagic process. A signal, such as starvation under physiological condition, induces the formation of double-membrane structures, known as autophagic vacuoles or autophagosomes, that sequester portions of cytoplasm along with proteins or damaged cell organelles to be degraded. The autophagosome ultimately fuses with the lysosome to form an autophagolysosome, where its content is degraded by acidic proteases. Increased lysosome biogenesis and function is increased by TFEB, a master transcription factor responsible of activation of lysosomial genes. In HD, mutant huntingtin aggregates sequester mTOR, a negative regulator of the autophagic pathway, activating the autophagic process to degrade mutant protein. The overexpression of TFEB in a cellular model of HD also increases the clearance of mutant huntingtin in a cellular model of HD. Atgs, autophagy specific genes.

protein. Although preliminary, these results suggest that the genetic program that controls lysosomal biogenesis and function may represent a novel potential therapeutic target on which to intervene to enhance cellular clearing of mutant huntingtin in HD.

Very recent findings suggest that huntingtin clearance by the lysosome depends on LAMP-2A, the integral membrane receptor protein that can directly import proteins across the lysosomal membrane (579). Since autophagy by lysosomes decreases with age due to a gradual decrease of LAMP-2A levels (133), a decline of LAMP-2A levels with age may enhance mutant huntingtin accumulation and HD pathogenesis. Increasing level of functional LAMP-2A early in the disease process could delay HD onset and serve as a therapeutic strategy (579).

F. Mitochondrial Dysfunctions

Mutant huntingtin binds directly to mitochondria (116, 420, 427), thereby altering their metabolic activity and motility within the cells (420, 584). Increased mtDNA mutations and deletions that can affect mitochondrial respiration have been detected in neurons of the cerebral cortex of HD patients (95, 267). In this section we describe how energy metabolism is affected in HD, and we focus on the reports indicating how mitochondrial calcium handling is impaired in the disease (427). Finally, we describe more recent data suggesting how a primary nuclear action of mutant huntingtin may indirectly influence mitochondrial function via effects on the transcription of genes involved in the functioning and biogenesis of this organelle.

1. *Energy metabolism defects*

The first evidence of a mitochondrial defect in HD came from very early studies indicating ultrastructural abnormalities in mitochondria isolated from HD cortical autoptic tissue (201, 215). In the following years, several imaging studies implicated bioenergetic defects in HD pathogenesis. With the use of ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS), the basal ganglia and thalamus of

symptomatic HD patients were shown to be depleted in *N*-acetylaspartate, a molecule abundant in brain cells, whose production is linked to and often regarded to reflect mitochondrial metabolic function (283, 396). In the cerebral cortex and basal ganglia of HD patients, increased production of lactate was also observed (283, 314), suggestive of an elevated glycolytic rate. Furthermore, positron emission tomography (PET) showed altered glucose metabolism from the early stages of the disease (81, 178, 322–324). A number of studies have examined whether alterations in mitochondrial respiration contribute to the observed bioenergetic defects. Biochemical studies of brain and peripheral tissues from HD patients, as well as studies on HD cells and animal models, revealed decreased activity of several enzymes involved in oxidative phosphorylation such as complex I, II, III, and IV (16, 81, 82, 91, 110, 119, 214, 223, 283, 314, 351, 366, 451, 492, 507, 510, 561, 584).

2. Oxidative stress

Mitochondria are both a target and an important source of reactive oxygen species (ROS). Although data on post mortem tissues show contrasting results on the presence of oxidative stress products (3, 83), further analyses in animal models of HD, obtained by systemic injection of 3-nitropropionic acid causing mitochondrial complex II inhibition, suggest that oxidative stress may play an important role in the pathogenesis of HD (90). Kovtun et al. (316) also showed that oxidative damage can produce somatic expansion of the CAG triplet repeat tract in postmitotic neurons, resulting in a longer and more toxic polyglutamine expansion in huntingtin, with possible consequences on disease onset and progression (316).

3. Defects in mitochondrial calcium handling

Timothy Greenamyre's team at that time at the Emory University School of Medicine (427) demonstrated that mitochondria from the whole brain of presymptomatic YAC72 mice (expressing full-length human mutant huntingtin) exhibit reduced calcium uptake capacity. The observed defect in calcium capacity was evident in 3- to 4-mo-old mice, several months before any behavioral or pathological changes appear in these animals (427). Similar results have been obtained on mitochondria isolated from HD patient lymphoblasts. Mitochondria from lymphoblasts of adult-onset HD patients were less resistant to calcium challenge, and the mitochondrial membrane potential decreased at an earlier time point compared with mitochondria isolated from lymphoblasts of normal individuals. The mitochondria isolated from lymphoblasts of juvenile-onset HD patients were even more sensitive to calcium (427). It was suggested that mitochondrial calcium handling defects may be partly due to a direct deleterious effect of mutant huntingtin on the outer mitochondrial membrane, since incubation of normal mitochondria with the mutant protein reproduced the calcium-handling defect seen in human HD mitochondria (116, 426, 427). As might be expected, the mitochondrial defect is not restricted to the CNS. Reduced mitochondrial calcium capacity has also been reported in mitochondria from skeletal muscle of end-stage R6/2 mice, concomitant with muscle degeneration (214). Analyses of mitochondria isolated from several mouse models indicate that the sensitivity to calcium handling may be tissue specific (85). Mitochondria in striatum appear to be more sensitive to calcium than those in cortex (85). In particular, in contrast to previous findings (427), the evidence that calcium loading capacity is increased in isolated mitochondria from transgenic and knock-in HD mice (85) raised for the first time the problem that isolation of mitochondria from their physiological cellular context, as well as differences in the methodological approaches, cell or tissue types, and/or ages of the used animals, may seriously affect measurement of calcium handling. This discrepancy can be solved by evaluating the mitochondrial respiratory function in situ in intact cells and mice. When in situ mitochondria have been challenged with high calcium, by transiently activating NMDA receptors, primary striatal neurons from YAC128 transgenic mice failed to reestablish calcium homeostasis in higher proportion compared with neurons from wild-type littermates (417). These results confirm the presence of altered mitochondrial calcium homeostasis and stress the importance of experimental conditions when assessing HD mitochondrial function in cells. Recent advancements in imaging and genetically encoded sensor technologies now allow for the visualization of mitochondrial Ca^{2+} transients in live mice (452). The application of these techniques (GFP two-photon microscopy or bioluminescence imaging of cameleon or aequorin-GFP calcium sensors) to mouse models of HD will help in elucidating defects in mitochondrial calcium handling and evaluating their impact on pathogenesis.

4. Mutant huntingtin and transcription factors involved in mitochondrial function

There are a number of transcription factors known to regulate genes responsible for mitochondrial function and oxidative stress (102). Mutant huntingtin was found to bind p53 and increase p53 levels and transcriptional activity, leading to the upregulation of two proapoptotic downstream targets, Bcl2-associated X protein (BAX) and p53-upregulated modulator of apoptosis (PUMA), as well as to mitochondrial membrane depolarization (33). In an elegant study, the group of Dimitri Krainc and colleagues (134) showed that mutant huntingtin also represses transcription of PGC-1α, a gene encoding for a transcriptional coactivator that regulates expression of genes involved in

mitochondrial biogenesis and respiration. The expression of these genes is severely impaired in the disease. In line with these findings, PGC-1α knock-out mice exhibit mitochondrial defects accompanied by hyperkinetic movement disorder and striatal degeneration (134).

Despite the extensive information on mitochondrial dysfunction in HD patients and models, and data indicating that mitochondrial dynamics are affected by mutant huntingtin expression, the contribution of mitochondria to the disease etiology is still unclear. The observation that 3-nitropropionic acid and malonate mitochondrial toxins, which selectively inhibit succinate dehydrogenase and complex II, respectively, induce a clinical and pathological phenotype that closely resembles HD is consistent with the thesis of dysfunctional mitochondria in HD (79). Overexpression of complex II subunits restores complex II activity and blocks mitochondrial dysfunction in a cellular model of HD (50). Similarly, overexpression of the free radical scavenger Cu/Zn SOD1 reverses mutant huntingtin-induced oxidative stress (219). Consistently, treatment of HD animals and patients with antioxidants such as creatine (11, 180) and coenzyme Q (179, 514) has been beneficial, although not curative, in preliminary clinical trials.

Despite this evidence, other data refute the widely held view of a direct mutant huntingtin effect on the mitochondrion (332). It was found that pathways by which cellular energy is lost are clearly different in 3-NP-treated neural cells versus the same cells bearing the expanded polyglutamine tract of the HD protein, suggesting that mitochondrial defects may be a consequence of neurodegeneration (332). However, the majority of the cellular events compromised during disease progression are highly energy-dependent processes. Hence, impairments of these functions could stem from or be amplified by mutant huntingtin-induced mitochondrial and energetic defects.

G. Transcriptional Dysregulation

The first evidence indicating the presence of transcriptional dysregulation in HD came from in situ hybridization studies on post mortem human HD brains showing that certain mRNA species encoding for signaling neuropeptides and neurotransmitter receptors were specifically decreased in striatal neurons (21, 26, 27, 412). Consistent with these early findings, gene expression studies performed by Jang-Ho Cha and colleagues (103, 104) at the Massachusetts General Hospital on the R6/2 mouse model confirmed alteration in the messenger RNA levels of the dopamine receptors D1 and D2. Subsequently, a large number of studies have provided evidence for transcriptional abnormalities in HD. These discoveries include demonstration of consistent changes in steady-state mRNA levels, direct interactions between huntingtin and proteins of the transcriptional machinery, and inhibition of enzymes involved in chromatin remodeling.

Investigations based on DNA microarray technology showed a large number of gene expression changes in cellular and mouse models of HD. They indicate also that gene dysregulation occurs before the onset of symptoms, suggesting that transcriptional dysregulation is an important causative factor in the disease (102). A large set of data also indicates the absence of a single transcriptional regulator having a primary role in HD and rather demonstrates the involvement of different transcription factors and DNA target sequences and some critical pathways in HD, such as the GC-box/Sp1-mediated, the CRE/CREB regulation systems, and the REST/NRSF regulon (102, 287)(Fig. 8).

1. Microarray studies

In 2000, over 50 scientists from 19 universities joined forces and started the Hereditary Disease Array Group (HDAG) with the support of the Hereditary Disease Foundation. The group's goal was to create an expression database that they hoped would reveal the biological pathways affected by the mutant HD gene. To speed research and get the most possible comparable results, the HDAG scientists, coordinated by Jim Olson at the Fred Hutchinson Cancer Research Center in Seattle, standardized their experimental methods using a single, robust microarray platform (Affymetrix GeneChip microarrays) to conduct whole-genome analysis on a variety of different disease models. Collectively, these assays, published in Human Molecular Genetics in 2002, allowed the identification of groups of genes altered at different stages of the disease (106, 192, 358, 360, 529). The mRNAs in question were found to be associated with transcriptional processes, neurotransmitter receptors, synaptic transmission, cytoskeletal and structural proteins, intracellular signaling, and calcium homeostasis, all phenomena that have been implicated in HD. Decreases in mRNA levels were more common than increases, and the set of genes that were decreased at symptomatic stages was larger than the one affected before symptoms onset, suggesting that transcriptional dysregulation is an early and progressive event in HD. The initial studies on HD transgenic mice have also shown fewer transcriptional changes in mice expressing the longer or full-length transgenes, compared with those expressing the shorter NH_2-terminal fragments, indicating that the cleavage products, rather than full-length mutant huntingtin, more robustly affect gene transcription. In the same studies, it was also suggested that many of the changes that occur in HD models also occur in models of other degenerative brain disorders.

In a recent study, the group of Luthi-Carter at the École Polytechnique Fédérale de Lausanne has confirmed that HD mice expressing a short NH_2-terminal fragment of mutant huntingtin exhibit the most significant effects on gene expression (325), but in contrast to previous find-

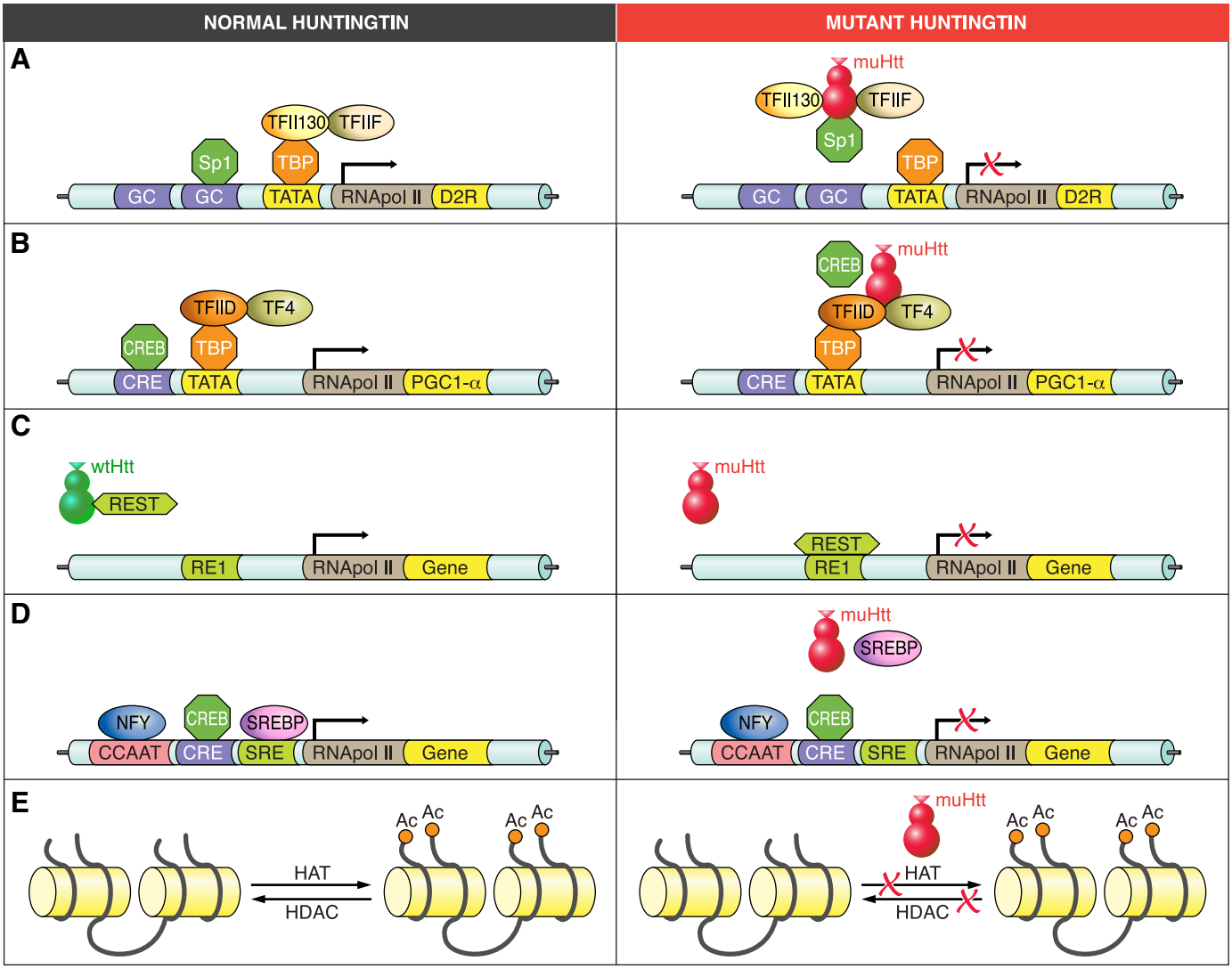


FIG. 8. Transcription factors, DNA target sequences, and chromatin structure in HD. *A*: expanded polyQ in huntingtin represses transcription of Sp1-dependent promoters (i.e., D2R gene) by abnormally interacting with specific transcription cofactors such as Sp1 itself, TFIIF, and TFII130. *B*: the transcription factor cAMP-responsive element (CRE)-binding protein (CREB) binds to DNA elements that contain a CRE sequence, as in the promoter of the PGC1-α gene, a master regulator of genes involved in mitochondrial function and energy metabolism. Mutant huntingtin interferes with CREB and TFIID, leading to reduced activation of PGC1-α gene, reduced PGC1-α protein levels, and consequently, downregulation of its mitochondrial target genes. *C*: the transcription factor REST/NRSF binds to RE1/NRSE elements in neuronal gene promoters such as in the BDNF gene. Wild-type huntingtin sustains the production of BDNF by interacting with REST/NRSF in the cytoplasm, thereby reducing its availability in the nucleus to bind to RE1/NRSE sites. Under these conditions, transcription of BDNF and of other RE1/NRSE regulated neuronal genes is promoted. Mutant huntingtin fails to interact with REST/NRSF in the cytoplasm, which leads to increased levels of REST/NRSF in the nucleus. Under these conditions, REST/NRSF binds avidly to the RE1/NRSE sites, suppressing the transcription of BDNF and of other RE1/NRSE regulated neuronal genes. *D*: SREBP binds to SRE to regulate the transcription of genes involved in the cholesterol biosynthesis pathway. Under physiological conditions, SREBP is transported from the endoplasmic reticulum to the Golgi region, where it is cleaved to obtain a fragment that enters the nucleus and activates cholesterogenic genes. In the presence of mutant huntingtin, this mechanism is impaired, which leads to the reduced expression of SREBP-dependent genes and decreases the biological effects of cholesterol biosynthesis. *E*: levels of histone acetylation at specific lysine residues are determined by concurrent reactions of acetylation (Ac) and deacetylation, which are mediated by histone acetylases (HATs) and histone deacetylases (HDACs). Histone acetylation is vital for establishing the conformational structure of DNA-chromatin complexes suitable for transcriptional gene expression. Mutant huntingtin leads to disruptions in HAT and HDAC balance, leading to general transcriptional repression. wtHtt, wild-type huntingtin; muHtt, mutant huntingtin.

ings, gene expression profile of full-length huntingtin transgene models had no discernable differences with fragment models (325). Most of the decreases in mRNAs measured in the striatum of R6/2 mice likely reflect neuronal, as opposed to glial, expression changes, as neuronal populations obtained through laser capture microdissection (LCM) recapitulate the findings found for striatal homogenates (491, 673).

In 2006, Luthi-Carter with several other collaborators was also the first to produce a gene expression profile of human HD cases (261), showing that gene expression changes are more pronounced in the caudate and motor

cortex and that many genes among those most downregulated had also been identified in expression profiling studies in HD transgenic mice (358–360). Gene expression profile studies revealed that transcriptional abnormalities occurred also in the skeletal muscles (358, 552) and in a more accessible tissue such as blood, although with contrasting results with respect to an earlier study by Dimitri Krainc's group at the Massachusetts General Hospital (72, 486). Importantly, expression profile data in the forebrain of the BDNF conditional knock-out mice largely overlapped with the transcriptional changes observed in the human HD caudate, pointing at cortical BDNF loss as one of the major causes of striatal dysfunction in HD (553).

2. *From microarray studies to a new pathogenic mechanism implicating cholesterol dysfunction in HD*

As a member of the HDAG, we analyzed gene expression profiles in striatal cell clones engineered to express a 548-amino acid NH_2-terminal portion of mutant or normal huntingtin under the control of an inducible promoter. The final goal was to identify the early transcriptional events taking place as soon as the expression of the mutant protein was turned on. We reported that expression of mutant huntingtin reduces the transcription of many genes involved in the cholesterol biosynthesis pathway (529). Subsequent studies in vivo validated these results with the observation of reduced expression of some key cholesterogenic genes in brain tissues from HD mice and human samples (592). We also identified a potential underlying molecular mechanism that involves the sterol responsive element binding protein (SREBP), a transcription factor that controls the expression of many genes involved in cholesterol and lipid metabolism. The HD cells showed reduced SREBP nuclear translocation and reduced activity of a SRE-reporter gene in the presence of mutant huntingtin. These results imply that less SREBP reaches the transcriptionally active sites in the nucleus causing reduced expression of SRE-regulated genes; reduced SREBP translocation was also confirmed in HD mouse brain (594) (Fig. 8). Interestingly, these data have been linked to early reduction in the levels of cholesterol precursors and cholesterol production in the brain of HD mice (R6/2 line and YAC models) (591, 593).

In brain, cholesterol is an essential component of cell membranes and is involved in a number of biological functions such as membrane trafficking, signal transduction, myelin formation, and synaptogenesis. Moreover, brain cholesterol does not come from the periphery but is synthesized locally, particularly during early development in parallel to myelin production (145). During brain maturation, and before astrocytic differentiation, neurons fulfill their own cholesterol requirements through endogenous neosynthesis. In postnatal life, however, this ability is strongly reduced, and neurons have to rely on the delivery of cholesterol from astrocytes (376, 446). Given the essential role of brain cholesterol as well as of its precursors, during development and in adulthood, impairment of the cholesterol biosynthetic pathway in HD may contribute to the pathogenesis at multiple levels (592).

3. *Transcription factors and DNA target sequences involved in HD*

Unexpected evidence from Robert Tjian and colleagues (659) at the University of California in Berkeley and from Jang Ho Cha and co-workers (52) indicated that huntingtin itself binds to the DNA and impairs the transcriptional machinery by directly repressing transcription of genes (Fig. 8). TFIID, TFIIF, and TAFII130, which are components of the core transcriptional machinery, are direct targets of mutant huntingtin (162, 523, 659). Huntingtin's effect on the core transcriptional machinery may also involve RNA polymerases, since RNA polymerase II large subunit can be recruited to polyglutamine inclusions (360). These observations suggested that mutant huntingtin can disrupt the transcriptional complex, but they do not explain the regional specificity that is seen in gene expression profiles (141, 261).

Other reports indicated that soluble mutant huntingtin interferes with the activity of transcription factors (102, 509, 556) or may function to disrupt coactivator complexes on susceptible gene promoters (659). Nuclear receptor corepressor (N-Cor)(76), mammalian SIN3 homolog A, transcription regulator (mSin3a), p53 (551), TATA-binding protein (TBP) (270), CBP (414, 551), the coactivator CA150 (265) transcriptional corepressor COOH-terminal binding protein (CtBP) (301), and Sp1 (162, 340) were found to selectively bind the expanded polyQ. Consistently, the activity of well-known transcriptional systems as the GC-box/Sp1-mediated and the CRE/CREB regulation system were found severely reduced in HD (102).

One of the best-understood transcriptional abnormalities in HD is the one involving aberrant neuronal gene repression by the transcription factor REST/NRSF (666, 672)(Fig. 8). REST/NRSF represses a large cohort of neuronal-specific genes through recruitment of a DNA regulatory motif, the RE1/NRSE (419). As previously described in section III*E*, the transcription of RE1/NRSE regulates neuronal specific genes, among which is the BDNF gene, that is significantly decreased in the disease and is one of the contributors to HD pathogenesis (666, 672). By using a microarray-based survey of gene expression in a large cohort of HD patients and matched controls (261), REST/NRSF target genes were found to be enriched among the genes whose expression is downregulated in the HD caudate (287). These findings strongly support the model of increased REST/NRSF repression of its target genes in HD brain. As specific nonneuronal

REST/NRSF-regulated genes have been identified in the human genome (84, 288), we have recently exploited the possibility that the binding of REST/NRSF to its target RE1/NRSE sites may also be altered in the peripheral tissues of HD patients. Our data show that REST/NRSF occupancy is increased in lymphocytes from HD subjects, thus mirroring a pathological event occurring in the nervous system (373).

Transcriptional dysregulation in HD was also linked to energy defects (see also sect. IV*F*). Particularly, it was found that mutant huntingtin inhibits expression of PGC-1α, a master regulator of mitochondrial biogenesis and function, by interfering with CREB/TAF4 at the PGC-1α promoter (134).

4. Mutant huntingtin and chromatin structure

Regulation of gene expression is accomplished through the action of transcription factors and enzymes that modify chromatin structure. Histones are the main target of these modifications that include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation.

Acetylation and deacetylation of histones play a critical role in gene expression through the interplay of histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs). In a simplified way, HAT activity leads to an increase in gene transcription through the opening of chromatin architecture by adding acetyl groups. Conversely, HDACs remove acetyl groups leading to gene repression through chromatin condensation. Inhibition of HDAC activity can result in a general hyperacetylation of histones, which is followed by the transcriptional activation of certain genes through relaxation of the DNA conformation.

Reduction in CBP-mediated HAT activity, when mutant huntingtin is expressed, was first described by Joan Steffan and Leslie Thompson and colleagues at the University of California Irvine in 2001 (550) (Fig. 8). They found the expanded polyQ to directly bind the acetyltransferase domain of CBP and p300/CBP associated factor (P/CAF), blocking their acetyltransferase activity (550). This causes a condensed chromatin state and reduced gene transcription. In particular, hypoacetylation of histone H3 in HD associates with downregulated genes (490). These results indicate that reduced acetyltransferase activity might be an important component of polyglutamine pathogenesis and pave the way for HDAC inhibitors as part of important therapeutic strategies to pursue the restoration of gene transcription in HD (186, 260, 490, 550)(also see sect. V*F*).

5. Posttranscriptional dysregulation

Two independent and recent studies have shown that the microRNA (miRNA) system is perturbed in HD (289, 422). MicroRNAs are essential gene regulators in the nervous system, both during development and patterning, as well as in mature neurons (315). In particular, reciprocal actions of REST and a microRNA were found to promote neuronal identity (126). The first miRNA abnormality in HD concerns the reduction of the levels miRNA genes targeted by REST (289). Among these miRNAs mir-7, mir-124, mir-129, mir-132, mir-137, and mir-184 are found (289, 422). Particularly, the neural-specific mir-124a that is highly and specifically expressed in the CNS is decreased in human and mouse HD cortex (289, 422). Therefore, the level of mRNA targets of mir124a should be increased in the HD brain. To test this hypothesis, Johnson et al. (287) have consulted the human HD microarray expression data from human HD caudate in conjunction with a published set of 174 experimentally determined mRNA targets of mir-124a. Interestingly, it was found that mir-124a target genes are significantly enriched among those that are upregulated in HD (287).

Mutant huntingtin also indirectly regulates the transcription of microRNA genes by destabilizing the interaction of Ago2 with P-bodies, which are both key components of the microRNA silencing pathway (506). These findings suggest that microRNA processing, as a whole, is impaired in HD.

The study of gene transcription has generated novel targets and pathways that were not previously suspected to contribute to HD pathogenesis, such as BDNF, PGC-1α, and the cholesterol biosynthesis pathway. New DNA targets (the RE1/NRSE and SRE, the CRE, and SP1-binding sites) found affected by mutant huntingtin may be exploited as tools for drug screening strategies. The critical tests will consist of determining if by correcting these abnormal phenotypes pathogenesis in HD mouse models can be ameliorated. Many of these strategies are under investigation (667, 668). In this regard, recent experience with HDAC inhibitors has been very instructive (491). In transgenic HD mice (i.e., R6/2 and N171–82Q mice), motor deficits were in fact improved when treated with HDAC inhibitors. Phenylbutyrate, an HDAC inhibitor, is now being tested in HD patients (see sect. V*F*).

H. Summary and Conclusions

For most of the abnormalities described above, their relevance to HD pathogenesis is still uncertain. For example, we know the importance of BDNF for brain neurons, in particular for the neurons affected in HD, but the exact implication of REST/NRSF dysfunction in HD pathogenesis has still to be elucidated through experiments aimed at inhibiting the activity of this repressor complex in vivo. Moreover, some of the abnormalities described above could be primary events, but many may represent secondary events that, if so, would be less interesting as targets for therapeutic intervention. For example, the emerging cholesterol biosynthesis dysfunc-

tion is observed in several HD animal models early, before or concomitant with the first symptoms, although we do not know yet if this phenotype is one of the detrimental events in HD. We also know that cell-autonomous mechanisms contribute to the toxicity elicited by mutant huntingtin in affected neurons. One of the most recent examples is the Rhes protein that is suggested to cooperate with mutant huntingtin to cause selective loss of striatal neurons, although more detailed studies are needed to validate its role in HD. However, it is also clear that non-cell autonomous mechanisms contribute to the disease. For example, one of the fundamental consequences of HD is a breakdown in the information processes between cortex and striatum.

Impairment of the cortico-striatal synapse due to excitotoxity as well as to loss of the trophic BDNF support from the cortex may predominantly contribute to the preferential loss of striatal neurons in the disease. These dysfunctional cell-cell interaction mechanisms may, in turn, intoxicate individual neurons, thus forming a positive-feedback loop that will ultimately culminate in neuronal cell death. Moreover, other cells, microglia, and astrocytes may also have a role in pathogenesis, and there is now the need for a more thorough understanding of the leitmotif of all these dysfunctions and of their interconnections to select those pathways that are more interesting from a therapeutic standpoint.

V. THERAPEUTIC STRATEGIES AGAINST PATHOGENIC MECHANISMS

The majority of therapeutics currently used in HD are designed to ameliorate the primary symptomatology of the HD condition itself, i.e., psychiatric agents for the control of behavioral symptoms, motor sedatives, cognitive enhancers, and neuroprotective agents (237). These drugs have limited benefits, however, and do not address the inexorable disease progression.

As discussed previously, neuronal dysfunction and cell death in HD are due to a combination of interrelated pathogenic processes. Affected targets have been used to set up specific assays based on high-throughput screening of licensed pharmaceuticals to identify and possibly develop novel compounds that might be useful for the treatment of HD. Many of these chemicals are being tested in cell culture and animal models of the disease (Table 2). Here we will discuss the HD therapeutics currently under development focusing on their benefits and limitations.

TABLE 2. *Therapies in the pipeline targeting specific molecular mechanisms*

Pipeline	Basic Research	Preclinical	Phase I	Phase II	Phase III	Available
Targeting excitotoxicity						
Riluzole and memantine	==========	==========	==========	==========	==========	==========
Tetrabenazine	==========	==========	==========	==========	==========	==========
Strategies to increase BDNF						
Recombinant BDNF	=======					
BDNF gene therapy	=======					
BDNF-releasing cell grafts	=======					
BDNF mimetics	=======					
Diet and environment enrichment	==========	==========				
BDNF inducers	=======					
Targeting caspases and huntingtin proteolysis						
Minocycline	==========	==========	==========	==========		
Caspase 6 inhibitors	=======					
Targeting huntingtin aggregation and clearance						
Trehalose	==========	==========				
C2-8	==========	==========				
Drugs stimulating huntingtin clearance	=======					
Targeting mitochondrial dysfunction						
Creatine	==========	==========	==========	==========		
Coenzyme Q10	==========	==========	==========	==========		
Eicosapentaenoic acid (EPA)	==========	==========	==========	==========		
Cysteamine	==========	==========				
Targeting transcriptional dysregulation						
Sodium phenylbutyrate	==========	==========	==========			
HDACi 4b	==========	==========				
Targeting mutant huntingtin						
RNA interference	==========	==========				
Artificial peptides and intrabodies	==========	==========				
Targeting cell loss						
Fetal cells	==========	==========	==========			
Embryonic/neural stem cells	=======					

A. Drugs Against Excitotoxicity

As described in section IV*B*, excitotoxicity is considered one of the major causes of cell death in HD. Excitotoxicity relies on increased glutamate release and increased NMDAR activity, ultimately resulting in impaired calcium signaling and cell death.

1. Riluzole and memantine

One of the first attempts to counteract excitotoxicity was through the blockade of the excessive glutamate release from corticostriatal terminals. Riluzole, an inhibitor of glutamate neurotransmission in the CNS, was among the first compounds found to be protective in chemical models of HD and in HD transgenic mice (232, 375, 423, 511). This promising finding led to a pilot open-label human trial. In this study, the enrolled HD patients showed reduced chorea, but subsequent studies demonstrated that the beneficial effect was only transient (476, 521). The finding that riluzole upregulates the levels of neuroprotective factors, including BDNF and glial-derived neurotrophic factor (GDNF), has led to extensive reevaluation of this drug in a 3-yr-long clinical trial involving a total of ~400 early-symptomatic European patients (394). Unfortunately, the tested dose of riluzole used in this study had no beneficial symptomatic or neuroprotective effects in HD, in contrast to previous results (327). Riluzole neither slowed down progression nor improved symptoms (327).

The drug memantine, acting on the glutamatergic system by blocking NMDAR, may hold more promise than riluzole. Memantine reduces striatal cell death in chemical models of HD (333). An open label study has suggested that memantine might slow down progression, and anecdotal reports have indicated that it may lead to cognitive improvement (94). However, further investigations on a larger cohort of patients will be required to confirm the neuroprotective role of this drug in HD.

2. Lamotrigine, remacemide, ifenprodil, and calcium signaling blockers

Lamotrigine, a glutamate antagonist, and remacemide, a noncompetitive inhibitor of the NMDA receptors, have also been tested in clinical trials, but despite the promising results obtained in HD mice (179, 514), no significant effects on disease progression were reported (307, 317, 576). Moreover, each of these drugs was associated with significant side effects, precluding their use in the clinic.

Lynn Raymond and colleagues (658) found that specific NMDA receptor subtypes (those comprised of NR1A/NR2B subunits) are partly responsible for the selective striatal neuronal vulnerability in HD (658). These data will hopefully provide the impetus for the development of novel compounds that use multiple approaches to modulate the activity of NR2B subunit-containing NMDA receptors. To date, ifenprodil, a NR2B-specific antagonist, has been shown to reduce excitotoxic cell death in medium spiny neurons from HD transgenic and wild-type mice after exposure to NMDA, thus bringing to fruition the promise of a therapeutic efficacy utilizing this approach (658). Bezprozvanny and Hayden (54), who mechanistically linked excitotoxicity to abnormal calcium signaling in HD, suggested that calcium signaling blockers, in particular $InsP_3R1$ inhibitors, might be also beneficial for disease treatment. Further investigations in animal models are required before proposing a clinical use of $InsP_3R1$ inhibitors.

3. Dopamine pathway inhibitors

Dopamine is released in the striatum from nigrostriatal terminals and is neurotoxic after direct injection into the striatum (47, 189, 245). Initial studies provided evidence that a "hyperactive" dopaminergic system could contribute to choreic symptoms and is implicated in neurotoxicity in HD (280). Other studies supported this hypothesis (49, 108, 135, 428, 447). The crossing of a dopamine transporter knockout mouse, which exhibits elevation of extracellular dopamine levels in the striatum and locomotor hyperactivity, with a knock-in mice carrying 92 CAG repeats exacerbates locomotor abnormalities and accelerates the formation of aggregates of mutant huntingtin in the striatal projection neurons (135). Therefore, drugs that decrease dopaminergic neurotransmission could be beneficial in HD.

Tetrabenazine (TBZ), a dopamine pathway inhibitor, alleviates the motor deficits and reduces striatal cell loss in HD mice, confirming that the dopamine signaling pathway plays an important role in HD pathogenesis (567). A randomized controlled trial provided encouraging evidence in favor of the use of TBZ for the control of choreic movements in HD (193, 577). Although side effects have been identified such as depression, akathisia, parkinsonism, and sedation, TBZ has been recently approved by the Food and Drug Administration (United States) for the treatment of chorea associated with HD.

B. Strategies to Increase BDNF in HD

We described in section IV*A* that reduced levels of BDNF are contributing to the HD phenotype. These findings have generated considerable excitement about the possibility of establishing a "BDNF therapy" for neurodegenerative diseases (Fig. 9). The proposition of BDNF administration as a possible therapeutic strategy for patients with HD is based on solid preclinical data in animals, although a number of issues such as dosage and delivery method remain to be established.

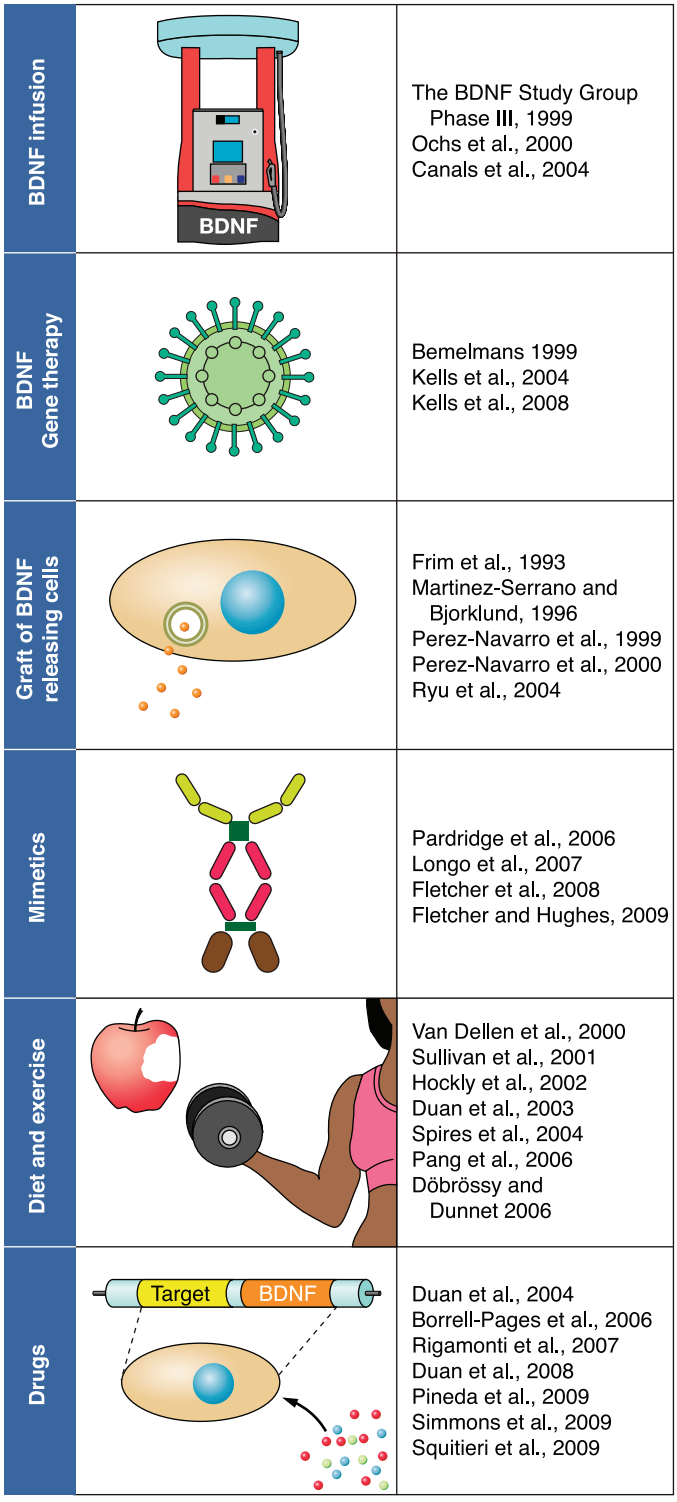


FIG. 9. Experimental therapeutic strategies for restoring BDNF function in HD.

When designing therapeutic strategies based on BDNF administration, one important consideration is the level of BDNF receptor expression in the neurodegenerating brain. A reduction in the number and activity of TrkB receptors has been described in the striatum in mouse models of HD and patients with this condition (210, 671). However, it is surprising and encouraging to note that the administration of exogenous BDNF in mice with reduced TrkB levels and activity can improve the neuropathological phenotype (92). Overall, it is likely that residual TrkB molecules in affected individuals are still capable of efficiently transducing BDNF-dependent cell signaling, thereby justifying the effort to develop strategies aimed at increasing BDNF levels in the brain.

1. Recombinant BDNF

The first clinical trial that explicitly investigated the role of BDNF in neurodegenerative diseases was performed in patients with amyotrophic lateral sclerosis (ALS) (416, 574). Methionyl human BDNF, infused subcutaneously or intrathecally, was well tolerated but failed to demonstrate a statistically significant effect of BDNF on the survival of patients with ALS. It is possible that the promising results obtained in animal models of the disease have not translated well into clinical trials, owing to the poor pharmacokinetics associated with the intact protein (667). In particular, BDNF has a short in vivo half-life and a low blood-brain barrier penetrability and undergoes only limited diffusion. The delivery of BDNF into the brain by minipump allows monitoring of the delivery dose. However, there is a serious drawback associated with this administration route due to the generation of a steep concentration gradient, originating from the infusion point, which could lead to alteration of the infused tissue and the development of adverse effects, such as edema (209). The aforementioned problems, and the limited neuroprotective effects observed in ALS, led to the cessation of trials with BDNF. On the basis of the evidence described above, other approaches to efficiently deliver optimum doses of BDNF to the brain have been considered.

2. Gene therapy

BDNF can be delivered locally to neurons through gene therapy. Constant and local production would offer many advantages over other delivery systems, such as minipump, that need to be refilled repeatedly over time. Moreover, gene transfer would prevent the problem of protein instability that may be encountered by long-term storage inside pumps. Durable expression of BDNF (or other neurotrophins such as GDNF) from adenoviral, adeno-associated viral, or lentiviral vectors has been successfully tested and developed over the past decades in animal models of HD (668). Nevertheless, there are still a number of problems to be overcome if this approach is to be used in the clinic. The first challenge is to regulate the amount of BDNF produced locally, as an excess of BDNF could have a deleterious effect on neuronal circuits, learning, and memory (15). The second problem is that trans-

duction is often associated with inflammation, which is usually accompanied by vector toxicity, and together these effects prohibit long-term therapy on safety grounds. Another major problem is the risk of accidental insertional mutagenesis by viral vectors and subsequent tumor formation (234). To overcome these problems, a large effort has been undertaken to produce new adeno-associated viral vectors, which lack both pathogenicity and immunogenicity. Moreover, alternative methods utilizing integration-deficient lentiviral vectors and nontoxic viral systems are under scrutiny. These approaches would allow the transduction of BDNF in a cell-specific and inducible manner (57, 650).

3. BDNF-releasing cell grafts

Another possible strategy to increase BDNF levels in the brain is to perform an intrastriatal graft of cells engineered to stably express BDNF. The feasibility of this approach was tested years ago in animal models of HD, initially with immortalized rat fibroblasts genetically engineered to secrete BDNF, and later with bona fide neural stem cells taken from human fetal brain (194, 372, 440, 441, 489, 668). The rats that were injected intrastriatally with the donor cells from rats or humans demonstrated improved motor performance and showed reduced striatal neuronal damage compared with control animals (668). Since this approach may still have some problems (xenogenic cells are at risk of being rejected and immortalized cells can cause tumor growth), researchers envisage to encapsulating cells with new materials under development (585). These materials would serve as biological shields, preventing immune rejection and eliminating the need for immunosuppression (493). Finally, there is considerable interest in the development of stable, nontumorigenic human neural stem cell lines that release BDNF (129, 483).

4. BDNF mimetics

As many of the issues surrounding BDNF efficacy and safety result from the need to deliver the neurotrophin close to the target site, investigators have considered the interesting possibility of using small molecules directed specifically to the BDNF receptors. These BDNF mimetics, which are currently under investigation in mouse models of Alzheimer's disease and HD, have been designed in accordance with the three-dimensional structure of BDNF, in particular, loops 1, 2, and 4, which are required for binding of BDNF to TrkB receptors (190). The synthetic molecules are also modified in such a way as to penetrate the blood-brain barrier more efficiently than BDNF (354, 429). The use of BDNF mimetics applied locally or systemically is the most promising strategy, because it avoids the adverse effects associated with invasive methods of delivery or uncontrolled dosing, while improving upon the diffusion properties of BDNF.

5. Diet and environmental enrichment

An interesting approach to achieve higher BDNF levels in the brain is the use of dietary restriction regimens, intermittent fasting, and environmental enrichment (409). Environmental enrichment in mice involves providing animals with regularly changed, complex tools that promote physical exercise as well as sensory and cognitive stimulation. Sensory stimulation includes visual, auditory, and olfactory stimulus, while cognitive stimulation relates to spatial maps and object recognition.

Preclinical studies show that an intermittent fasting dietary restriction regimen, in which HD N171-82Q mice were deprived of food for a 24-h period every other day, normalizes brain BDNF levels and delays the onset of motor dysfunction (160). Similarly, environmental enrichment has been shown to delay disease progression in R6/1 and R6/2 animal models and to partially rescue BDNF deficits in the brain, improving behavioral and cognitive deficits (152, 259, 425, 541, 596). Notably, remotivation therapy improves functioning in HD patients, indicating that stimulation may also benefit human HD (557).

6. Drugs or small molecules

Current experiments are aimed at developing techniques or isolating compounds that will increase endogenous BDNF levels. Such a strategy would circumvent the problems related to invasive methods of BDNF delivery in humans, including achieving the correct dosage and maintaining stability of the neurotrophin.

The noncompetitive inhibitors of ionotropic glutamate NMDA receptors, memantine and riluzole, increase BDNF expression and are beneficial for treating HD symptoms (544, 668). Antidepressants, such as paroxetine and sertraline (selective serotonin reuptake inhibitors), also increase endogenous BDNF levels and prolong survival of neurons, improve motor performance, and ameliorate brain atrophy in N171–82Q mice (159, 161). It is possible that the administration of antidepressants at very early stages of the disease may contribute to improvements in the clinical status through elevation of BDNF levels. In a very recent paper, Gary Lynch's group at the University of California Irvine (527) showed that upregulation of endogenous BDNF levels with an ampakine, a positive modulator of AMPA-type glutamate receptors, rescues neuronal plasticity and reduces learning problems in mutant huntingtin knock-in mice. Ampakines are well tolerated in clinical trials and have shown efficacy in this study after brief exposures, suggesting that they may be useful for chronic treatment of the cognitive difficulties in the early stages of HD.

Since the mechanisms of reduced BDNF gene transcription and protein transport have been well elucidated, a valid option could be to increase BDNF transport and levels by pharmacologically targeting specific mecha-

nisms that are responsible for BDNF dysfunction. For example, cystamine can increase the transport and release of BDNF from brain neurons (74). BDNF vesicle transport depends on S421 phosphorylation (125). Pineda et al. (448) found that pharmacological inhibition of calcineurin, the bona fide huntingtin S421 phosphatase, restored the BDNF transport defects observed in HD. The effective compound is FK506 that is approved by the Food and Drug Administration. Moreover, our laboratory has developed a cell-based reporter assay to monitor RE1/NRSE activity in cultured brain cells with the final aim to identify compounds that specifically upregulate BDNF expression in HD. Large-scale screenings are ongoing (468). Compounds identified in high-throughput screenings as blockers of the RE1/NRSE silencing activity could alleviate REST/NRSF-dependent repression and, hence, ameliorate the global transcription repression in the disease (468). Both strategies, which act specifically on a defined molecular dysfunction, could be more effective than drugs that increase BDNF levels but do not specifically target a disease mechanism.

C. Targeting Caspase Activities and Huntingtin Proteolysis

A therapeutical approach aimed at targeting caspase activity relies on data showing that the mutation of caspase cleavage sites prevents neurodegeneration and improves disease phenotype in HD mice (220).

1. Caspase inhibitors

The production of toxic fragments through the cleavage of huntingtin by caspases is a key event in the development of HD. Minocycline, a second-generation tetracycline, was found to inhibit caspase 1 and 3 in R6/2 mice, improving the disease phenotype (111, 254, 418, 622). Although the replication of these results in mouse models has been difficult, probably because a different experimental paradigm has been followed, the preliminary effective animal studies have inspired clinical trials with minocycline (534). A two-year open label study of minocycline was recently carried out, and patients were evaluated at baseline and after 6 and 24 mo of treatment. After the first 6 mo, minocycline did not have major, obvious toxicity and did not worsen HD features (70). Unlike the expected natural course of HD and after an improvement in the first 6 mo, patients exhibited stabilization in general motor and neuropsychological functions at end point (24 mo). Moreover, after the first 6 mo, a significant amelioration of psychiatric symptoms was noticed (71). These outcomes supported the idea that minocycline could exhibit neuroprotective features in HD. A multi-center, randomized, double-blind study of minocycline in HD individuals is currently being conducted by the Huntington Study Group to assess long-term safety and to gather more information on the possible efficacy of this drug (www.huntington-study-group.com).

Although the neuroprotective activity of caspase inhibitors in the human pathology still has to be proven, several efforts are ongoing in this direction. Multifunctional caspase inhibitors have been created, and by using high-throughput screenings, another set of neuroprotective compounds (R1–R4 compounds) that also appear to act through a selective caspase inhibitory mechanism have been generated by Varma et al. (606). As previously described, mice expressing mutant huntingtin, and resistant to cleavage by caspase-6 but not by caspase-3, maintain normal neuronal function and do not develop striatal neurodegeneration. These caspase-6-resistant mutant huntingtin mice are protected against neurotoxicity induced by multiple stressors, including NMDA, quinolinic acid, and staurosporine. Recently, a series of isatin sulfonamide acceptors having a high nanomolar potency for inhibiting caspase-6 and increased selectivity for caspase-6 versus caspase-3 inhibition have been described, providing novel molecules for the experimental treatment of HD (118).

D. Targeting Aggregation

The precise role of huntingtin aggregates in HD is unclear. Large inclusions of mutant huntingtin at first seemed toxic, as their presence appeared to correlate with pathology. More recent studies suggest that inclusion formation extends survival of cells expressing mutant huntingtin. Further investigations are required to elucidate the pathways leading to aggregate formation, to identify the different types of aggregates that are formed during disease progression, and to discriminate which ones are protective or toxic. Being that these processes are not yet fully understood, the drug discovery efforts have so far focused on blocking the formation of aggregates and on promoting their formation.

1. Screens for antiaggregation compounds

Numerous compounds acting as aggregation inhibitors have been isolated in models of HD. A significant example is represented by Congo red, which was found to decrease neuronal aggregates and to promote phenotypic improvement in a mouse model of HD (496). Congo red specifically inhibits polyglutamine oligomerization by disrupting preformed oligomers, prevents ATP depletion and caspase activation, preserves normal cellular protein synthesis and degradation functions, and promotes the clearance of expanded polyglutamine repeats in vivo and in vitro (496). However, Jennifer Morton and colleagues at University of Cambridge in the UK (645) were not able to replicate these outcomes. In their study, Congo red treatment failed to ameliorate either motor or cognitive defi-

cits in the same mice, thus questioning the use of Congo red derivatives as a therapy for HD (645).

In 2004, Nobuyuki Nukina and collaborators at the Riken Brain Science Institute in Japan (565) used an in vitro aggregate formation assay with myoglobin containing an expanded polyglutamine tract as target molecule and discovered that the disaccharide trehalose prevented nuclear inclusion formation, improved motor dysfunction, and prolonged survival in R6/2 mice, without any deleterious side effects (565).

A few years before, the group of Erich Wanker at the Max Planck Institute in Berlin proposed the first in vitro automated screen for antiaggregation compounds (248). The assay was based on the finding that polyQ-containing protein aggregates are resistant to SDS and were selectively retained on a cellulose acetate filter, unlike SDS-soluble proteins under the same conditions. The aggregates retained on the filter membrane were detected and quantified by immunoblot analysis using specific antibodies. Through this assay, several benzothiazole derivatives were found to inhibit huntingtin fibrillogenesis in a dose-dependent manner (248). Riluzole is one of these derivatives that has entered the clinical trials.

For the isolation of aggregates inhibitors, high-throughput screening based on computational structure-based drug design has also been used (117, 661). Examples of antiaggregation compounds isolated by this approach are represented by C2–8 and epigallocatechin-3-gallate. The small compound C2–8, an inhibitor of polyQ aggregation identified by a yeast-based high-throughput screening assay, reduces neurodegeneration in vivo in a *Drosophila* model of HD (661). Further studies showed that C2–8, at nontoxic doses, penetrates the blood-brain barrier and is present in the brain at a high concentration. When administered to R6/2 mice, C2–8 improved motor performance and reduced both neuronal atrophy and the size of huntingtin aggregates (117). Epigallocatechin-3-gallate (EGCG), the most abundant catechin in tea, was discovered by screening a library of natural compounds and was found to be a potent inhibitor of mutant huntingtin exon 1 protein aggregation, acting similarly to other polyphenols (172).

Recently, a qualitative and quantitative biochemical aggregate detection system has been developed by the group of Paolo Paganetti at Novartis Institutes for BioMedical Research in Basel (629). The system, named AGERA (agarose gel electrophoresis for resolving aggregates), enables a sensitive and quantitative assessment of the loaded aggregates and could be useful in tracking changes within disease-specific aggregates pools, specifically visualizing subtle differences caused by experimental drugs.

Despite promising data in cell-based assays and nonmammalian model organisms as *Drosophila* (see for example a recent paper from Doumanis et al., Ref. 155), few convincing results in mice have been described. One potential problem is that in HD mice, compounds designed to prevent formation of large aggregates may not stop the initial pathological misfolding of protein monomers, which will retain their capacity for pathogenesis, either as single molecules or as toxic oligomers. For these reasons, pharmacological stimulation of cellular degradation pathways that preferentially target misfolded disease proteins should be also investigated.

2. Drugs stimulating huntingtin clearance

Emerging evidence implicates autophagy as a protective mechanism in polyglutamine diseases. The induction of autophagy by the mTOR inhibitor rapamycin resulted in a significant reduction in mutant huntingtin aggregates, improved neuronal survival in HD *Drosophila*, and motor performance and striatal neuropathology in HD mice (462). Unfortunately, the side effects of rapamycin, especially immunosuppression, preclude its use in patients, spurring interest in finding novel drugs that selectively enhance autophagy. Sarkar and colleagues (500, 501) showed that combining inhibition of mTOR and inositol monophosphatase (IMPase) by rapamycin and lithium, respectively, resulted in additive clearance of mutant huntingtin in vitro, providing a basis for preclinical trials in HD models. At the same line, trehalose has been found to induce autophagy of mutant huntingtin and protect cells against subsequent pro-apoptotic insults via the mitochondrial pathway (499). It is therefore possible that trehalose acts by a double mechanism of action, i.e., by reducing huntingtin aggregates and by promoting the clearance of huntingtin toxic fragments. These data suggest that a combinatorial strategy with rapamycin or other drugs promoting autophagy may be relevant to the treatment of HD and related diseases.

The list of new drugs that upregulate autophagy and enhance the clearance of mutant huntingtin is growing (502). A subsequent screen of small molecules capable of modulating autophagy independently from mTOR was performed. Many small autophagy enhancer molecules were shown to reduce mutant huntingtin aggregation in vitro and in nonmammalian models (499, 640). While the precise therapeutic mechanism remains elusive, it is possible that these compounds act downstream of mTOR to induce autophagy (191). A crucial step will be to assess the safety of these compounds in vivo as well as their ability to induce autophagy in HD mice.

E. Drugs Against Mitochondrial Dysfunction

Mutant huntingtin impairs mitochondrial energy production and cellular respiration, leading to a reduction of the intracellular level of ATP, thus promoting apoptosis, oxidative stress, and susceptibility to excitotoxicity. Con-

sequently, drugs that enhance mitochondrial function or antioxidants may represent a potential neuroprotective strategy in HD. However, to date, the majority of preclinical mouse trials designed to test the effects of different neuroprotective agents demonstrated limited success, probably because of an underestimation of the optimal therapeutic dose (237).

1. Creatine and coenzyme Q_{10}

Oxidative damage plays a role in HD; thus therapeutic strategies that reduce ROS may slow the neurodegenerative process.

Creatine stimulates mitochondrial respiration and has antioxidant properties. Several preclinical studies showed that this drug is neuroprotective in chemical models of HD and in R6/2 mice (11, 180). Human clinical trials showed that administration of creatine to HD patients for 2 yr prevented weight loss and, in some patients, caused an improvement in the scores for neurological testing (560). Administration of creatine in HD patients reduced serum levels of 8-hydroxy-2-deoxyguanosine (8-OH-2′-dG), an indicator of oxidative injury to DNA, suggesting that creatine may effectively counteract oxidative stress in HD patients (255). While it is clear that this drug has a therapeutic indication in mice, the challenge will be to determine which dose might be of effective value in patients, as the pharmacokinetics in mice and humans differ. The relative clinical studies are ongoing.

Coenzyme Q_{10}, a lipid-soluble benzoquinone playing a vital role in ATP production, can also stimulate mitochondrial activity. Although coenzyme Q_{10} was shown to be protective in R6/2 and in N171–82Q mice (179, 514), the first human clinical trial showed no significant benefits for HD patients (576). The Huntington Disease Study Group has recently conducted open-label studies to assess and gather information on the safety and tolerability of higher doses of coenzyme Q_{10} in HD patients. The low doses of coenzyme Q_{10} administered in the first clinical trial could account for the lack of efficacy. A smaller, short-term study, called Pre-2CARE (Pilot Safety and Tolerability Study of coenzyme Q_{10} in Huntington's Disease and in Normal Subjects), has revealed that the optimal dose of coenzyme Q_{10} was felt to be 2,400 mg/day (unpublished data from Huntington Study Group, 12th Annual Meeting in St. Louis, MO, 2004). A larger study, called 2CARE (coenzyme Q_{10} in Huntington's disease), is currently ongoing for reevaluation of coenzyme Q_{10} as a neuroprotective drug in HD (http://www.huntington-study-group.org).

2. Eicosapentaenoic acid

Therapies targeting alternative aspects of mitochondrial function include eicosapentaenoic acid (EPA). This *n*-3 fatty acid seems to act as a mitochondrial proliferator while promoting mitochondrial fitness. Significant improvements in multiple motor and behavioral abnormalities have been found in different HD animal models after treatment with purified EPA, known as ethyl-EPA (123, 601). A subsequent 6-mo clinical trial using ethyl-EPA in advanced patients (stage III) demonstrated significant improvement of the orofacial component of the Unified Huntington's Disease Rating Scale (UHDRS) and delay of progressive cerebral atrophy as assessed by MRI (454). More recently, a 1-yr multicenter, double-blind, placebo-controlled trial, involving 135 patients with HD (stages I and II), showed no significant benefits in any functional scale in the entire study population. However, some beneficial effect on motor function was found in those patients who followed the entire protocol without any violations (455). The Huntington Study Group is now conducting another 1-yr multicenter double-blind, randomized, placebo-controlled trial in more than 300 HD subjects with mild and moderate HD to determine definitely the effect of ethyl-EPA on motor signs and symptoms in HD. Preliminary results show that ethyl-EPA is not beneficial in HD patients during the 6 mo of the placebo-controlled evaluation (575).

3. Cystamine and MPTP blockers

Dysfunction in mitochondrial activity has been prevented by small molecules that inhibit oxidative damage, such as cystamine (74, 367). Prosurvival effects have also been observed when MPTP blockers have been administered to HD cells. Recently, a number of biologically active Food and Drug Administration approved heterocyclic, tricyclic, and phenothiazine-derived compounds have been identified as putative MPTP blockers. Future studies with animal models of HD or human clinical trials will test the utility of these compounds as potential therapeutics for HD.

F. Targeting Gene Transcription

Transcriptional dysregulation is an early event in HD pathology and is considered to significantly contribute to the molecular pathogenesis of the disease. Dysfunction at the transcriptional level in HD mainly involves the down-regulation of gene expression with the greatest number and magnitude of differentially expressed mRNAs detected in the caudate nucleus, followed by the motor cortex and then the cerebellum (102, 261). This has led to the initiation of new lines of research aimed at testing the ability of a number of compounds to restore gene transcription in HD models.

1. HDAC inhibitors

All the findings highlighting transcriptional dysregulation in HD support the notion that treatment with HDAC

inhibitors may ameliorate mRNA abnormalities by a direct effect on histone acetylation. Therefore, the administration of HDAC inhibitors became a good strategy to render the HD chromatin more relaxed and prone to gene transcription.

Earlier evidence from a *Drosophila* model of HD has shown a decrease in degeneration of rhabdomeres, the subcellular light-gathering structures of the photoreceptor neurons in the fly compound eye, and extended survival after exposure to HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA) or sodium butyrate, for 6 days after eclosion (550). Such pioneering findings indicated that these drugs may improve the HD phenotype, leading to subsequent preclinical studies in mice that have confirmed the neuroprotective effects of HDAC inhibitors (SAHA, sodium butyrate, and phenylbutyrate) (36, 186, 200, 260, 273, 578).

The first study using an HDAC inhibitor (i.e., SAHA) as a therapeutic agent in a mouse model of HD was performed in 2003 in Gillian Bates laboratory at the King's College (260). This study demonstrated that SAHA crosses the blood-brain barrier, increases histone acetylation in the brain, and reduces the motor impairment in R6/2 mice (260). Another positive example comes from a novel pimelic diphenylamide HDAC inhibitor, named HDAC inhibitor (HDACi) 4b, that was found to efficiently prevent motor deficits and neurodegenerative processes with a low toxicity profile in vitro (578). Moreover, HDACi 4b treatment partly ameliorates gene expression abnormalities detected by microarray analysis in these mice (578).

Several studies have shown that some of the reported ameliorative effects of HDACi can be mediated also by effects that go beyond the rescue of mRNA abnormalities (299). HDAC6, for example, was shown to be implicated in the autophagic degradation of mutant huntingtin aggregates. The use of tubacin, a selective HDAC6 inhibitor, leads to a rescue of the mutant huntingtin aggregation (277). Other studies highlighted anti-inflammatory and antiapoptotic properties of the HDAC inhibitors (212). One report shows that the class III HDAC SIRT1 can also be a potential modulator of the lipid metabolism and glucose homeostasis (343). At present, it still remains unknown how much of the reported ameliorative effects of HDACi in HD animal models rely on epigenetic modifications and how much can be ascribed to nonhistone effects. It is clearly important to further assess both contributions, when considering HDACi as a therapeutic intervention (89).

These promising results have led to the enrollment of HD patients in clinical trials as HDAC inhibitors (specifically sodium phenylbutyrate) are safe and well-tolerated in HD patients (264). However, these compounds may cause growth arrest and induction of apoptosis, leading to chromosomal instability and other off-target effects (89). It is therefore important to improve our understanding of the molecular basis of the potential selectivity of HDAC inhibitors in altering gene transcription and to identify the biological functions of the different HDACs to generate specific and effective HDAC inhibitors. There is a major effort being made by the pharmaceutical industry in this direction, which is also working to develop selective HDAC inhibitors able to easily penetrate the blood-brain barrier.

Sirtuins, human histone deacetylases belonging to class III of HDAC enzymes, have been a recent focus of therapeutic development for neurodegenerative diseases (64). Sirtuins are NAD-dependent deacetylases, which uniquely distinguish them from other HDACs. Interest in sirtuins was precipitated by experiments demonstrating the regulatory effects of sirtuins on aging and longevity in many biological models, and further by the discovery of neuroprotective effects of the sirtuin ligand resveratrol (40, 612). Interestingly, activation, rather than inhibition of sirtuins, with the small molecule resveratrol was found neuroprotective in HD worms (73, 431). Preclinical trials with resveratrol and development of potent and selective activators of human sirtuins as well as further validation experiments are needed to assess the therapeutic potential of these enzyme targets in HD (298).

2. *Compounds interacting with DNA*

In addition to the compounds that directly interact with HDACs, compounds interacting with DNA could have a potential therapeutic value in HD, by influencing transcriptional activity. For example, mithramycin and chromomycin, two anthracycline antibiotics that inhibit neuronal apoptosis, bind DNA and modulate epigenetic histone modifications that influence transcription (105). Preclinical trials with these compounds have already been performed in HD (187, 487, 546). In the first study, pharmacological treatment of R6/2 mice with mithramycin extended survival by 29.1%, higher than any single agent reported to date. Behavioral and survival data were obtained from ~120 R6/2 and 120 wild-type mice, which represents a very large cohort of animals compared with other studies in which 12–20 animals per group are usually analyzed. The use of a large enough number of animals was important to achieve statistical significance in the analyses. Increased survival was accompanied by improved motor performance and markedly delayed neuropathological sequelae (187). Furthermore, treatment with anthracycline compounds was able to rebalance epigenetic histone modification in R6/2 and N171–82Q HD mouse lines, providing the rationale for the design of clinical trials in HD patients (487, 545).

3. *Compounds targeting transcription factors or the activity of DNA regulatory elements*

A separate route into drug discovery for HD is emerging from studies looking at those disease mechanisms

triggered by gain or loss-of-function defects caused by mutation in huntingtin. Examples are represented by the findings that expression of Sp1-dependent genes and SRE-dependent genes (involved in cholesterol biogenesis) is reduced in HD models. Therefore, Sp1 and SRE DNA target sites can be considered as read outs for the search of compounds that ameliorate the activity of these specific pathways (102). Another strategy takes inspiration from the finding that diminished wild-type huntingtin function causes hyperactivation of the RE1/NRSE silencer of gene expression with subsequent reduction in the transcription of the BDNF gene as well as of many other neuronal genes (666, 672). The demonstration that this occurs because of a pathological accumulation of REST/NRSF repressor complex at the level of the RE1/NRSE DNA target site of many neuronal genes has led us to the development of a cell-based reporter assay for monitoring RE1/NRSE silencing activity after administration of a number of chemicals to *DiaNRSE*^{Luc8} cells (468). With the use of this strategy, three closely related structural analogs that upregulate RE1/NRSE reporter expression at low nanomolar concentrations have been identified. The capability of these compounds to inhibit the activity of endogenous RE1/NRSE loci was confirmed by the evidence of an increase in BDNF mRNA levels and for two additional neuronal genes known to contain a RE1/NRSE sequence in their promoter, namely, synapsin I and M4 muscarinic receptor (468).

Since loss of BDNF in mice causes defects in gene transcription with a pattern that is very similar to the one observed in the human HD brain, the increase in striatal BDNF should in turn increase the transcription of other genes in this brain area (553). In this scenario, the RE1/NRSE found in BDNF promoter II and in many other neuronal genes and the other BDNF promoters (IV and VI) that are dysfunctional in the disease emerge as primary targets for the development of therapeutics.

G. Summary and Conclusions

Several promising targets and assays for drug screening have been identified. Currently, there are several potential therapeutic agents (memantine, tetrabenazine, minocycline, treaholose, C2–8, creatine, coenzyme Q_{10}, ethyl-EPA, cysteamine, HDAC inhibitors, mitramicycin) mostly acting on the above-mentioned downstream targets that have shown improvement of motor and/or cognitive dysfunction mostly in the R6/2 and N171–82Q mouse lines.

Up to now, seven compounds have been systematically tested in HD patients at different stages of the disease. Currently, these compounds are in phase II (creatine, coenzyme Q_{10}) and in phase I (minocycline, cysteamine, memantine, ethyl-EPA). Only one is now available in several countries (tetrabenazine) (see Table 2). Unfortunately, as described above, only a few drugs, all belonging to old-generation drugs, have been tested in HD patients with some benefits. One possible explanation can be found in the discrepancies existing between mouse and human trials. These discrepancies highlight the difficulty in predicting the efficacy of new drugs in humans based on animal models of HD. All of the existing transgenic HD mouse models share features with the human pathology, but not unexpectedely, none of them individually seems to recapitulate the entire spectrum of phenotypes of human HD. It is therefore now a consensus in the field that a single agent with potential therapeutic effects needs a systematic evaluation in more than one animal model, possibly a short model (expressing the NH_2-terminal portion of mutant huntingtin) and a full-length mouse model, either knock-in or YAC or BAC. Only afterwards, the selected compounds can be proposed for use in clinical trials. However, this rule should be applied with some flexibility, as compounds with only moderate efficacy in all mouse models may still be effective when combined with other treatments in humans.

Given the multiple pathogenic mechanisms involved in HD, it is expected that a compound targeting one pathological mechanism may not be effective alone. Combinations of therapeutic agents that target different pathogenic mechanisms should have greater efficacy. Evidence of the effectiveness of drug combinations has already arisen from studies performed in mutant flies and mice where compounds acting on several cellular mechanisms (transcriptional dysregulation, aggregation, energy dysfunction) contributed to improve the HD phenotype in an additive and synergistic manner (1, 179, 488, 514, 548). The main obstacles remaining include our inability to discriminate between primary and secondary disease mechanisms and our ability to target and select only those pathogenic mechanisms that effectively influence disease onset and progression. Moreover, these mechanisms should also be the earliest dysfunctions to occur in HD cells.

VI. TARGETING MUTANT HUNTINGTIN

Recently, a new attractive option for treating HD has emerged, based on the possibility to block the production of the mutant protein. One strategy employs targeting mutant huntingtin mRNA by the use of RNA interference, while other strategies aim to block the protein product using small synthetic peptides or antibodies that recognize mutant huntingtin.

A. Targeting Mutant Huntingtin RNA: Antisense Oligonucleotide and RNA Interference

In 1997, Haque and Isacson at the Harvard Medical School/McLean Hospital in Belmont, Massachusetts

(240), were the first to treat mice with repeated intrastrial infusion of 18-mer fluorescein-labeled phosphorothiorated antisense oligodeoxynucleotide (ODN), targeted against the start site of the first exon of the IT15 gene. Although fluorescein-labeled ODN appeared to penetrate several cells and did not cause any apparent toxicity to the neurons, the average reduction of the huntingtin levels did not differ significantly between the striatal tissues of antisense ODN-treated animals and the animals treated with a sense ODN or with vehicle only (240). This pioneeristic work suggested that an improvement of the methods for molecular modifications of the IT15 gene was needed for therapeutical initiatives. Three years later, putative antisense short oligonucleotide (ASO) candidates were screened for downregulation of the huntingtin gene in a cell model of HD. This study provided the first evidence that expression of the HD gene could be downregulated using ASO oligomers directed to the methionine initiation codon of huntingtin (66). In a separate attempt, a phosphorothioated antisense oligonucleotide was delivered to NT2 cells, leading to the downregulation of mutant huntingtin and reduction of aggregates formation (405). As a consequence, the development of the ASO technology has steadily progressed, and efforts in the last 10 years have focused on extensive medicinal chemistry research to find backbone modifications of ASO able to increase resistance to nuclease, maintain or improve affinity and specificity to the target RNA, and reduce off-targets and immune effects (53). More recently, modified ASOs (named peptide nucleic acid, PNA) have been produced that enable the selective recognition of the mutant allele and the determination of the selective inhibition of mutant protein expression in human fibroblasts (269). These findings have opened to the fascinating possibility to reduce in vivo the expression of huntingtin by means of ASO. In the same years, researchers put much effort also on reducing mutant huntingtin expression via RNA interference approaches.

In 2005, Beverly Davidson and colleagues at the University of Iowa (243) demonstrated that brain delivery of adeno-associated virus (AAV1)-short hairpin RNA (shRNA) directed against human mutant huntingtin mRNA reduces mutant huntingtin mRNA and protein level in the striatum of N171–82Q mice (243). The shRNA was designed to reduce the expression of the mutant human huntingtin transgene, without affecting normal huntingtin expression thanks to sequence differences between mouse and human genes. Notably, silencing the exogenously expressed pathogenic gene prevented behavioral and neuropathological symptoms in HD mice, with particular improvements in stride length measurements and rotaroad performance, and also reduction in mutant huntingtin inclusions (243). In a parallel study, Ron Mandel and colleagues at the University of Florida used AAV5 to deliver two different human huntingtin-specific shRNA to R6/1 mice striata (140). One shRNA (si-Hunt1) reduced mutant huntingtin mRNA expression and inclusions and improved hindlimb clasping, an indicator of neurological impairment in mice. Moreover, striatal proenkephalin, dopamine- and DARPP-32 mRNA transcripts, which are reduced in the mouse and human HD brain, were partially normalized in treated mice (471). Together, these studies demonstrated that RNA interference could be used as a potential HD therapy.

An alternative strategy to knockdown mutant huntingtin expression is the use of small-interference RNA (siRNA), which seems more effective and safe than shRNAs (5, 222, 537, 538) when introduced in mice and non-human primates (153, 247, 572). Delivery of lipid-encapsulated siRNA in a single intraventricular infusion to 2-day-old R6/2 mice led to a significant reduction of mutant huntingtin transcripts up to 7 days after treatment, but silencing was almost completely lost 1 wk later. Despite this relatively short period of mutant huntingtin suppression, long-lasting behavioral improvements were reported, including reduced hindlimb clasping and improved open-field behavior up to 14 wk of age, significantly better rotarod retention times, diminished striatal inclusion at 8 wk, and a 14% life span increase (623).

In a later study, chemically modified and cholesterol-conjugated siRNAs (cc-siRNA) were used to improve in vivo siRNA stability and cellular uptake, without using potentially deleterious liposome encapsulation. In this case, adult wild-type mice were injected with AAV1/8 vectors carrying expanded human huntingtin cDNA encoding for 365 amino acids apart from the polyglutamine repeat. Coinjected siRNA targeting human mutant huntingtin mRNA silenced the human gene by 65%, reduced both monomer levels and huntingtin protein aggregation in striata 3 days and 2 wk after treatment, respectively, and improved clasping and motor deficits after 1 wk (149).

Although these findings are extremely relevant in terms of HD therapeutics, in HD patients the type of mutation (>36 CAG repeats) and the small size (~21 nucleotides) of the iRNAs, either shRNA or siRNA, preclude the design of small RNAs specifically directed to the mutation since they do not discriminate between the normal and the mutant allele. In the first HD RNA interference studies, performed in mice bearing the two normal huntingtin alleles, there was no evidence of an effect of normal huntingtin knock-down, either because it was not measured or because its levels were unaffected by human huntingtin-targeted inhibitory RNAs. However, there are concerns about off target effects that would especially involve unwanted reduction in the availability of wild-type huntingtin, whose beneficial activity for brain neurons is now well established. Three recent studies have attempted to address whether HD-affected neurons would benefit from a non-allele-specific RNA interference strategy that results in simultaneous knockdown of mutant and wild-type alleles. In the first study, huntingtin-

target miRNAs were delivered to the wild-type brain. Four months later wild-type huntingtin mRNA and protein levels were reduced by 70% and 83%, respectively, and animals showed no overt histopathological changes (378). Therafter, it was found that co-knockdown of mutant and wild-type huntingtin could improve HD-related histopathology. In this study, mutant huntingtin was expressed in rat or mouse striata by using lentiviral vectors. Coexpression of huntingtin-target shRNA in affected neurons prevented DARPP-32 loss and reduced nuclear inclusions up to 9 mo after injections (158). However, the level of remaining normal huntingtin associated with these histopathological improvements was unclear. Specifically, huntingtin mRNA was reduced by 86% at 3 wk following injections but only by 50–60% at 16 wk, while no huntingtin knock-down data were reported for the 9-mo time point (158). In a parallel study from Davidson's group, it was shown that cosuppression of mutant human huntingtin and mouse endogenous huntingtin in the same neurons could improve HD-associated motor deficits 11 wk after injections in N171-82Q HD mouse striata (75). Together, these three studies support the feasibility of nonallele specific gene silencing strategies to treat HD. However, the latter two studies reported alterations in molecular pathways associated with huntingtin loss of function. These results indicate that further investigation of the implications of RNA interference-mediated normal huntingtin reduction is necessary.

Altogether, one might consider that RNA interference-based clinical strategies able to silence only the mutant allele are desirable to avoid potential side effects due to a partial reduction of normal huntingtin and loss of its beneficial activites in the brain.

One challenge for HD scientists now is to identify and target heterozygous single nucleotide polymorphisms (SNPs) selectively associated to the disease-causing allele. An increasing number of disease-linked polymorphisms are being identified and characterized (19, 352, 471, 517, 595, 662). It has been recently demonstrated that the majority of HD patients may be treatable by individualized allele-specific RNA interference that selectively target the mutant huntingtin mRNA (352).

Although the potential therapeutic applications of siRNA-based strategies are promising, many technical issues remain unsolved, including the stability of the silencing, as well as the delivery modes, timing, and cellular targets (19). HD is characterized by the loss of striatal MSNs, which an effective HD treatment should prevent. Since cell-autonomous mechanisms likely play a role in HD pathogenesis, medium spiny neurons are certainly targets of RNAi therapy. However, recent evidence suggests that non-cell-autonomous mechanisms (alteration of the cortico-striatal circuitry) are also operating in HD, indicating that a broader treatment regimen to additional brain regions should be considered.

Finally, recent studies have highlighted the potential side effects of siRNAs, such as changes in expression of off-target genes (140, 278, 508), induction of cellular immunoreactive responses (532), or competition of exogenous RNA interference with endogenous, intracellular mRNA silencing systems (335). Advances in the understanding of RNA interference biology will contribute to solve such technical problems to develop siRNAs that act with greater efficiency and specificity. Beverly Davidson's group (378) has recently shown that the toxicity of shRNAs could be alleviated by moving the inhibitory RNA sequences into an artificial miRNA scaffold, highlighting the fact that miRNA-based approaches might be more suitable for achieving RNA interference-mediated silencing of mutant huntingtin in the brain.

B. Targeting the Mutant Protein: Artificial Peptides and Intrabodies

Another intriguing approach to reduce mutant huntingtin toxicity is to interfere with mutant huntingtin at the posttranslational level. Kazantsev et al. (297) demonstrated that pathogenesis can be reduced by targeting the polyQ domain with an artificial polypeptide. These experiments raised the possibility that small molecules targeting the polyQ might be beneficial to HD.

A similar mechanism of action is exerted by intrabodies that have previously been developed as therapeutics against cancer and HIV. The intrabodies are recombinant antibodies (Abs) or Ab fragments that function and target antigens intracellularly. Intrabodies maintain the key characteristics of traditional Abs, which are diversity, high specificity, and high affinity to the target site. However, they are smaller than whole Abs and have been genetically engineered to cross the blood-brain barrier and to function in the intracellular environment (387). The group of Ann Messer at the University of Albany in the state of New York has pioneered this field in HD producing intrabodies that target the huntingtin exon 1 translation product and successfully reduce mutant huntingtin, cell death, and aggregation in vitro (124, 305, 331, 393), in organotypic slices from HD mice (402), and in vivo in HD flies, leading to a reduction in the number of aggregates and prolonged life span (380, 644). The in vivo delivery of the intrabody scFV-C4, using AAV2/1, resulted in a significant reduction in the size and number of huntingtin aggregates when intrastriatally injected into R6/1 transgenic mice. Similarly, AAV delivery of scFv-EM48 in the striatum of N171–82Q mice reduced neuropil aggregate formation and could alleviate motor deficits across the 8-wk duration of the experiment (621). More recent findings from the group of Paul Patterson at the California, Institute of Technology, California, have shown that the AAV2/1-mediated delivery of intrabody Happ1, which

recognizes the proline-rich domain of huntingtin, ameliorates motor, cognitive, and neuropathological symptoms in multiple mouse models of HD (R6/2, N171-82Q, YAC128, and BACHD)(540).

Altogether, these results build the basis for novel therapeutics. However, the use of intrabodies as therapeutics against neurodegenerative diseases is still in the very early stages. Future directions include more informed and focused antigen choice, enhanced cytoplasmic stability and solubility, more robust delivery methods, and extension of their use in combinatorial therapies.

VII. TARGETING CELL LOSS: CELL REPLACEMENT APPROACHES

Cell transplantation for HD has developed over the last decade to clinical application in pilot trials in France, United States, and United Kingdom (165, 166). Although the evidence for efficacy is still limited and several parameters have yet to be completely defined, studies in HD animal models and pilots in HD patients underline the potential for cell replacement strategies in HD.

In general the development of cell-based therapies for HD has been heavily dependent on the animal models available. The first experiments aiming at intrastriatal transplantation of freshly dissociated striatal progenitors from the fetal brain into the lesioned animal's brain started in 1988 and made use mostly of the excitotoxic lesion models (163, 233, 403, 530). Only a few studies of cell transplantation in HD transgenic mice have been carried out (164, 286, 597). This was mainly due to the fact that the available genetic models of HD did not produce the expected neuronal cell loss and, therefore, reduced the rationale for an approach aimed at assessing the value of cell replacement strategies in these mice. Given the more recent success in modeling HD in mice using YAC and BAC constructs, which gave evidence of neurodegeneration, it is likely that new transplantation trials will be performed, especially with the new stem cell lines that have become available.

On the basis of the experimental and preclinical studies conducted in excitotoxin-lesioned animals transplanted with fetal striatal tissues, the first pilot clinical trials of neural transplantation in patients commenced in 1990 (165). So far, the most promising study that uses objective assessment protocols has been performed in Creteil, France, by Marc Peschanski, Anne-Catherine Bachoud-Levi, and colleagues (32). In 2000 they reported that fetal striatal neural allografts in five patients with HD were well tolerated, and 2 years after transplantation, functional, motor, and cognitive improvements were seen in three of five patients (32). The five patients were then followed annually for up to 6 yr after grafting. This long-term follow-up study demonstrated that neuronal transplantation in HD provided a period of several years of improvement and stability, but not a permanent cure for the disease (31). In fact, clinical improvement plateaued after 2 yr and then faded off 4–6 yr after surgery. In particular, dystonia deteriorated consistently, whereas chorea did not. Cognitive performance remained stable on nontimed tests, whereas progression of motor disability was shown by deterioration on timed tests. Two patients who had no benefit from grafting at 2 yr continued to decline in the same way as nongrafted patients (31).

The findings of these and other pilot studies in HD patients performed so far indicate that cell replacement might be useful in HD, but the approach is still in its early stages of development. Moreover, it was suggested that also a placebo effect should be considered since these pilot studies were not blinded (166). However, there is general agreement that such an approach may be valuable and should be pursued while waiting for stem cell lines that are safer and validated for their use in clinical trials through rigorous and systematic testing of the most prominent candidate cells in appropriate animal models. Accordingly, European groups led by Peschanski and Bachoud-Levi are conducting a phase II clinical trial with 60 patients (named Multicentric Intracerebral Grafting in Huntington's Disease, MIG-HD), and results are expected by 2011.

The improvement of the surgical procedure in the selection of patients, and in the choice of the best source of cells to be used for transplantation, are now crucial limiting factors. Beneficial effects in patients have been observed when human fetal cells were grafted, but their availability is limited and their application is cumbersome. Alternative sources are envisaged that include embryonic stem cells, neural stem cells, and iPS cells, to generate a "bankable" and renewable source of functional and transplantable neurons.

Human embryonic stem cells (hESCs) should be taken into account for their capacity to self-renew while maintaining their pluripotency, which leads them to give rise to a wide variety of mature cell types. These cells should be able to produce the brain neurons of the specific subtype that undergo degeneration in HD. However, these cells need to be better characterized for their differentiation properties as well as for possible posttransplantation side effects, such as overgrowth. Further studies are required to optimize conditions to obtain human neural stem cells that fully accomplish the requisites for cell therapy. One major recent advancement is that of Perrier and Peschanski's group (24) who designed the so-far best available in vitro differentiation protocol for converting hESC into postmitotic neurons expressing striatal markers and demonstrated the therapeutic potential of such derivatives in vivo following xenotransplantation into adult rats.

Preclinical trials in HD mice are a fundamental prerequisite before considering cell replacement approaches with hESCs in HD patients. However, one should also consider large-animal models such as monkeys or minipigs which would allow more appropriate testing of the engraftment capacity of the donor cells in species that are immunologically more similar to the human condition.

It is also possible that the search for disease mechanisms will generate results useful for future cell transplantation strategies. As shown in section IV*A*, cortical BDNF production is severely diminished in HD. The risk that stem cell lines fully suitable for generating striatal neurons may be less efficacious once transplanted because of the lack of BDNF support from cortex should not be underestimated. Similarly, it will be important to verify that the disease mechanism itself will not be propagated to the healthy donor neurons. However, analyses on post mortem brain tissue from Parkinson disease (PD) patients that had received a graft 16 years before revealed that the pathology was transferred to the donor cells (313, 339), although one other study reported the opposite (385). The good news, yet, may be that the patients and the neurons survived for that long even in those conditions. Cicchetti et al. (121) performed a similar immunohistochemistry and electron microscopy study on post mortem tissue from two HD patients that were transplanted a decade before and did not find genetic markers of HD in the grafted cells. However, this study, which included three subjects, indicated reduced graft survival and long-term diseaselike neuronal degeneration in the area of the implant (121). Bachoud-Lévi, the principal investigator of MIG-HD study, noted that the cells from striatal anlagen transplanted in the striatum of the three HD subjects included in the study can connect to the human host brain, similar to results reported in animals (30). Recent observations suggest that in addition to therapies based on cell replacement, strategies that combine recruitment of endogenous stem cells and growth factor delivery should be considered as therapeutically useful. In fact, a work from Cho et al. (115) (described also in sect. IV*A4*) shows that BDNF/Noggin-stimulated neurogenesis may represent a means of both replacing neurons lost to striatal neurodegeneration and conferring therapeutic benefit in HD mice (115).

VIII. BIOMARKERS IN HUNTINGTON'S DISEASE

The current standard method for the assessment of the clinical stages of HD patients is the UHDRS (526). The UHDRS is a primary measure of on-going clinical trials for detecting the onset of symptoms in asymptomatic at-risk individuals and to follow disease progression in the patients. However, this method is not able to distinguish between pure symptomatic benefit and modification of the underlying disease process. In addition, it is prone to subjectivity since it is based on clinical evaluation of motor, cognitive, behavioral, and functional capacity. Moreover, the disease progresses slowly, and HD patients present variable clinical phenotypes making this scale of limited sensitivity (434).

Biological markers, or biomarkers, are defined as "cellular, biochemical or molecular characteristics that can be objectively measured and evaluated as indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). The identification of such markers is now considered a top priority in the HD field, since many disease-modifying compounds have been identified in several HD models, and clinical trials aimed at validating such treatments are close to initiation (41). There is, therefore, an urgent need for reproducible, sensitive, and specific outcome measures that can be used to track disease progression in patients and that may also reflect disease-related changes in presymptomatic HD gene carriers (250, 437). The ideal biomarker needs to be easily measurable, objective, reliable, and repeatable in different cohorts from world population. In addition, for evaluation of therapies, it needs to change linearly with disease progression and to correlate with established clinico-pathological parameters of the disease. It is unlikely to find one biomarker with all these characteristics. Rather, each marker might have its own application for specific disease stages, and therefore, more than one biomarker should be used for early diagnosis (i.e., at the time of phenoconversion) and others for testing the progression of the disease. Overall, a combination of clinical, neuroimaging, and biochemical biomarkers will be necessary to enhance the accuracy, specificity, and sensitivity in tracking disease onset and progression.

Several candidate HD biomarkers have emerged during the last years. They are under investigation for their potential application in the early diagnosis and evaluation of therapies. A promising candidate is 8-OH-2′-dG, an indicator of oxidative injury to DNA, which levels were markedly elevated in HD patients and reduced by creatine treatment (255; Table 2). Here we summarize the several strategies and technologies, including neuroimaging, proteomic, metabolomic, and genomic approaches, which are being undertaken in the search for biomarkers in HD (Fig. 10). We also focus on clues and problems that are emerging from these studies.

A. Imaging Studies

New advances in modern neuroimaging techniques are providing additional information about structural, physiological, and functional changes occurring in HD

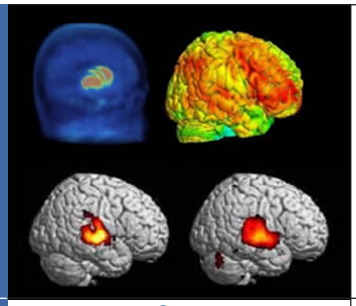
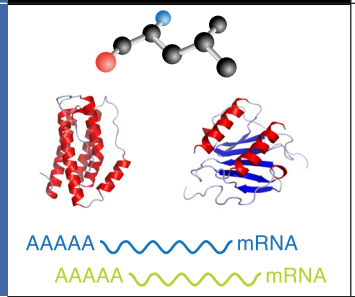
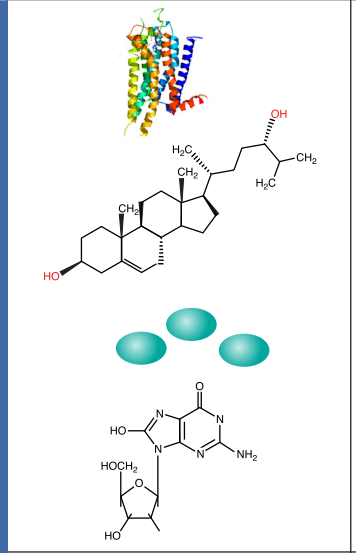
Imaging studies		MRI	Rosas et al., 2002; 2005; 2008
		fMRI and PET	Paulsen et al., 2004; Zimbelman et al., 2007; Wolf et al., 2007; 2008; 2009
		DTI	Rosas et al., 2004; Weaver et al., 2009; Douaud et al., 2009
Metabolic, proteomic, transcriptomic approaches		BCAA	Underwood et al., 2006; Mochel et al., 2007
		IL-6, IL-8, TNF-a	Dalrymple et al., 2007
		Subset of transcripts	Borovecki et al., 2005; Runne et al., 2007
Hypothesis -driven		A2AR; ECS	Varani et al., 2007; Battista et al., 2007
		24OHC	Leoni et al., 2008
		BDNF	Borrell-Pages et al., 2006; Ciammola et al., 2007; Conforti et al., 2008
		8-OH-2'-dG	Hersch et al., 2006

FIG. 10. Biomarkers for HD that are currently under investigation. Imaging studies, metabolic, proteomic, and transcriptomic approaches, as well as hypothesis driven studies are presented.

and their potential correlation with behavioral cognitive and motor symptoms. These methods offer noninvasive, reproducible, and objective analyses, which may be used to develop valid, reliable, and reproducible surrogate markers of both disease onset and progression.

Magnetic resonance imaging (MRI), functional MRI (fMRI), and positron emission tomography (PET) studies have improved the understanding of striatal atrophy and cortical dysfunction, which appear to be characteristic abnormalities in HD, while diffusion tensor imaging (DTI) has recently yielded significant insights into brain microstructure in both clinical and preclinical HD. By using in vivo neuroimaging, abnormalities in brain structure and function have been detected in HD patients many years before the symptomatic onset. Altogether we now have a powerful set of complementary techniques to investigate dynamic and long-term aspects of brain structure, function, and connectivity to monitor disease progression before and after therapeutic intervention.

1. *Structural MRI*

MRI studies in HD patients showed that changes occur in caudate volume over time and that this change correlates with the age of onset and the CAG repeat length. These studies also suggested that striatal volume loss may be a surrogate marker of HD useful in clinical trials (28, 68). The potential of such a measurement has recently been supported by the PREDICT-HD study led by Jane Paulsen at the University of Iowa (435, 436). PREDICT-HD is a multicenter, longitudinal, prospective study which aims to establish predictors of HD diagnosis and to refine

measures that track disease progression for conducting preventive clinical trials. The PREDICT-HD study showed that MRI reveals a linear decline in striatal volume that begins ~20 yr before disease onset (58). These results indicate that MRI of striatal volume can be used in longitudinal studies to address the benefits of therapeutics on attenuation or slowing of brain atrophy, as well as in monitoring disease progression.

Results of structural MRI studies revealed also that widespread cerebral changes and white matter abnormalities are present in the HD brain (68). Notably, cortical thinning has been found before clinical onset, is progressive with measurable changes occurring in as little as a year, and is well correlated with performance on several cognitive tests (477). Rosas et al. (474) also detected atrophy in frontal, temporal, parietal, and cortical areas in preclinical HD. In a subsequent study, the same group found a correlation between cortical thinning and disease stage (479). To test cortical thinning's potential as a biomarker of HD, this measure has been used in a small creatine phase II study in which high doses of creatine were found to inhibit cortical thinning in HD patients (255). A 3-yr study enrolling more than 600 subjects is now ongoing to validate this result. However, further work is needed to determine the precise relationship between clinical symptoms and cortical thinning before considering this measure as an important surrogate marker of disease onset and/or progression.

2. Functional MRI and PET

The long length of time required for treatment to affect volume measurements is a significant disadvantage of using structural MRI alone as an outcome measure, and it needs, as a complement, a method for the short-term assessment of brain function and microstructure. Currently, fMRI provides a reliable and sensitive imaging tool to assess brain function over time during rest or, more commonly, in association with a cognitive task or a stimulus that engages relevant brain areas and networks. Blood Oxygen Level Dependent (BOLD) fMRI is the most common functional imaging method in HD that measures brain activity indirectly, relying on local alterations in blood flow associated with neuronal activity. Current fMRI research indicates that functional changes occur very early in HD, at least around the same time as striatal and cortical atrophy can be detected. fMRI was found to be sensitive to changes in striatal function long before the emergence of motor symptoms in pre-HD, indicating that fMRI can potentially be used to identify early neural degeneration before the onset of clinical symptoms. Moreover, neuronal dysfunction in premanifesting HD carriers was found to occur more than 12 yr before the estimated onset of manifest HD, suggesting that fMRI may be more sensitive to disease onset than other neuroimaging techniques (665). These findings have been corroborated by Bernard Landwehrmeyer and his group at the University of Ulm who, by fMRI analyses, have identified abnormalities in cortex and striatum in response to specific cognitive or motor tasks in preclinical HD (641–643).

Considering that neurophysiological dysfunctions are observed in HD mutation carriers prior to symptoms onset, longitudinal fMRI studies may provide a sensitive and reliable symptom-independent tool to monitor symptomatic onset, disease progression, and the dynamic effects of therapeutic intervention. However, it still remains a challenge to determine a temporal profile of activity changes during the preclinical period and during conversion to clinical HD. For this reason, longitudinal studies are warranted.

PET has also been applied in several studies where changes in brain metabolism in both HD carriers and patients and also changes that seem to be early in the pathogenic processes of HD, such as microglial activation, have been detected (434).

3. DTI

Diffusion imaging has recently been used to investigate microstructural changes in clinical and preclinical HD (68). These studies demonstrated that diffusion is consistently increased in the basal ganglia in HD. Diffusivity is also increased in the whole brain of HD patients but not in mutation carriers (473). More recently, a study from Elizabeth Aylward at the University of Washington School of Medicine in Seattle has provided the first longitudinal DTI evidence of white matter degeneration in HD, supporting the notion that this may result from axonal injury or withdrawal (628). Moreover, Douaud et al. (154) reported the ability of DTI to uncover in vivo evidence for selective subcortical degeneration in HD.

After therapeutic intervention, microstructural changes measured by using DTI would likely be discernible prior to those changes detactable by structural MRI, since microstructural recovery would be expected to precede macrostructural change. These results suggest that DTI could be used in longitudinal studies to evaluate therapeutic intervention in HD.

B. Metabolomic, Proteomic, and Transcriptomic Approaches

Apart from specific changes in the CNS, systemic abnormalities have been identified in HD models, and these observations are supported by the fact that huntingtin is expressed in most of the tissues. On this basis, different strategies are being used to identify biomarkers in blood, a desirable target for biomarker identification because of its accessibility.

1. Metabolomic approaches

In a non-hypothesis-driven search for disease biomarkers, several analytical approaches aimed at applying metabolic profiling to serum samples have been attempted. Altered levels of several metabolites have been identified in HD animal models and patients (588). For instance, similar changes in metabolite profiles in blood have been found between the N171-N82Q transgenic mouse line and human HD (588). In particular, markers of fatty acid breakdown (including glycerol and malonate) were shown to progressively change with the mutant condition in both animals and patients compared with respective controls, presymptomatic mice, and asymptomatic gene carriers.

Interestingly, lower levels of the branched chain amino acid (BCAA) have been found in HD samples by two independent studies (395, 588). BCAA is involved in mitochondrial intermediary metabolism, and its reduction may indicate a systemic attempt to compensate for an early energy deficit in HD. Reduced levels of BCAA are also associated with weight loss and might be considered a promising early biomarker of HD progression (395). However, in both studies, the low number of human HD samples analyzed (10–15 presymptomatic, 10–20 HD early-symptomatic, and 20 controls), the lack of late-symptomatic subjects, and the lack of longitudinal studies do not allow any conclusion to be drawn about the potential of these metabolites, including BCAA, as potential biomarkers in HD.

2. Proteomic profiling

In a study coordinated by Sarah Tabrizi at the University College London, proteomic profiling was applied to human plasma at various stages of HD, including premanifest gene carriers (136). Proteins involved in immune activation and neuroinflammatory processes, which are known to be activated in HD (330, 438, 563), have been tracked and some change with disease progression (136). Among them, the most promising ones were clusterin and interleukin-6 (IL-6). The overall study was conducted in 74 controls, 42 premanifest, 58 early, 66 moderate, and 10 advanced HD subjects. After the first identification of proteins differently regulated in HD patients and controls, subsequent experiments were performed in a smaller subset of subjects to validate the results (136). IL-6 was further analyzed in 194 plasma samples from HD mutation carriers ranging from premanifest to moderate HD and from control subjects. This study demonstrated that IL-6 levels were increased in HD gene carriers with a mean of 16 yr before the predicted onset of clinical symptoms and across disease stages, representing the earliest plasma abnormality identified to date in the human pathology (63).

3. Transcriptomic profiling

Based on known transcriptional disruptions occurring in brain and peripheral HD tissues, Krainc's group was the first to apply transcriptomic approaches to search for HD-associated changes in gene expression in blood samples from HD patients and control subjects. A subset of 12 mRNAs has been found to be upregulated in blood samples from symptomatic HD patients with respect to controls. Krainc and colleagues (72) reported that presymptomatic subjects showed similar gene expression profiles to those of controls, whereas late presymptomatic subjects showed altered expression that resembled that of symptomatic HD patients. A recent study headed by Luthi-Carter, however, did not confirm these data and did not find any differential expression between manifest HD and control samples in any of the 12 previously reported marker RNAs (486). In addition, among all the RNAs tested, overall changes in gene expression were weak. The same authors stated that the small differences observed, although significant and trending to track with HD status, offer little potential to provide sensitive disease monitoring (486). These two studies (72, 486) emphasize the need for further validation of candidate biomarkers before their use in clinical trials. In addition, interindividual variability in blood samples has to be taken in account, and optimal technical conditions are required when working with human blood (639). More recently, we have described a novel strategy for mRNA normalization in quantitative real-time PCR that is based on expressed Alu repeat (EAR) amplification as a measure for the mRNA fraction (374). This strategy should help in biomarker validation studies based on mRNA detection in human blood.

C. Biomarkers Built on Hypothesis-Driven Experiments

Although a good biomarker does not necessarily depend on its connection to a central disease mechanism, it is attractive to consider hypothesis-driven experiments and molecular and cellular pathways previously implicated in the disease, to identify candidate biomarkers that directly measure in blood a disease process occurring in the brain. In this case, the changes observed in blood may be due to CNS processes, or a disease mechanism may produce parallel changes in both central and peripheral tissues, causing plasma levels of a protein to mirror those in the CNS. A number of promising (but also negative) results have been obtained in the last years and some examples are described below.

1. A2A receptor and the endocannabinoids system

Changes in A2A receptor expression and signaling were reported in HD (450, 570, 605), and abnormalities in

the endocannabinoid system (ECS) have been found in several neurodegenerative diseases (361). The evidence that alterations in A2A receptor binding activity has been reported also in lymphocytes from HD patients and that such cells possess a complete and functional ECS (311, 362, 603) has opened up the possibility that the aberrant A2A receptor and/or ECS phenotypes might represent novel potential HD biomarkers. Subsequent studies conducted on have confirmed abnormalities of A2A receptor binding activity and of fatty acid amide hydrolase (FAAH) activity in large cohorts of HD patients, with respect to control subjects (39, 604). However, these studies failed to demonstrate significant differences across patients with different disease stages and with HD-positive premanifesting subjects, which presented abnormal values similar to those found in HD patients and a small group of spinocerebellar ataxia type 1 and 2 (SCA1 and SCA2) patients. Therefore, both A2A receptor activity and FAAH activity in blood samples are not suitable as markers of disease progression, although they may be of potential interest in the search for therapeutics (361, 450).

2. Neuroendocrine system

Increasing evidence points also towards the important contribution of the hypothalamus and endocrine system to HD (443). As many neuroendocrine factors are secreted into the cerebrospinal fluid, blood, and urine, their levels in such samples may reflect the disease state in the CNS (62).

3. 24-Hydroxycholesterol

A reduction in the activity of the cholesterol biosynthesis pathway in the brain of different HD animal models has been demonstrated (591, 593, 594). This defect has been mechanistically linked to altered trancription of SRE-regulated genes in the cholesterol metabolic pathway (594). To maintain homeostasis, brain cholesterol is converted into 24-hydroxycholesterol (24OHC), a more polar sterol that is capable of crossing the blood-brain barrier and entering the circulation (60). Therefore, the circulating levels of 24OHC might be an important index of cholesterol dysfunction occurring in HD brains. Our recent studies in YAC128 mice have shown a significant reduction of brain and plasma levels of 24OHC in YAC128 mice compared with littermate and YAC18 mice, prompting further investigation in blood samples of HD patients (591). When assayed in 96 HD patients, 33 premanifest, and 62 control individuals obtained from two different cohorts of populations from Italy and Great Britain, plasma levels of 24OHC were significantly lower in HD patients than controls from both countries (334). Intriguingly, premanifest HD subjects showed similar levels of 24OHC with respect to controls, but the subjects that were closer to developing motor signs of disease had lower 24OHC levels than those far from onset. However, this analysis was limited to 10 presymptomatic subjects close to and 23 presymtomatic subjects far from disease onset. A new study that includes a larger number of presymptomatic HD subjects and leaded by Stefano Di Donato at the Besta Neurological Institute in Milano is now under completion. In the above study, a correlation with the degree of caudate atrophy measured by morphometric MRI had been found in preclinical HD and stage I HD patients (334). Although no differences had been found across disease stages, 24OHC levels might be useful, combined with MRI or other biomarkers, to follow disease progression in early HD.

4. BDNF

BDNF is highly concentrated in the nervous system but is also found in the blood of human and other mammals, where its function is poorly understood (195, 457). The BDNF in blood derives from synthesis in mononuclear blood cells and endothelial cells, and from platelets release as well as, although to a very minor extent, from the passage through the blood-brain barrier (195). Although it is still unclear how BDNF expression and metabolism in human peripheral tissues are regulated, changes in serum BDNF levels in rats during development correlate with those in the brain (293). On the basis of these findings and the extensive data showing that BDNF is reduced in HD brain, it was proposed that peripheral BDNF could be used to measure the state of the disease and/or the effectiveness of a given treatment. Studies in rodents in which BDNF mRNA and protein have been evaluated both in basal conditions and after pharmacological treatment are promising (74, 127). In particular, the level of BDNF mRNA can be monitored systematically, and it correlates with disease progression in R6/2 mice and HD rats. Blood BDNF mRNA is also sensitive to pharmacological treatments as, for example, the acute and chronic treatment of R6/2 mice with CEP-1347, a mixed lineage kinase (MLK) inhibitor with neuroprotective and neurotrophic effects in mice, leads to increased total BDNF mRNA in blood and brain compared with untreated R6/2 mice (14, 127).

This means that it is possible to test compounds in HD mice, predict their ability to stimulate the production of BDNF in the brain, and possibly evaluate their therapeutic efficacy in the animals by a blood test. Although attempts at revealing BDNF protein levels in human HD blood have been performed (120), in our experience the detection of BDNF protein in human blood samples (total blood for RNA; plasma or serum for the protein) remains extremely complex and variable. Its detection in HD patients requires standardization in the collection and the processing methodology of a large number of samples (unpublished data).

IX. CONCLUSIONS

In the 17 years since the discovery of the HD gene, a large number of basic research studies has highlighted that multiple molecular and biochemical pathways ultimately lead to a complex disease phenotype. Today, the current status of HD research looks promising. The development of strategies to counteract HD at its primary source, including RNAi and intrabodies, is now well underway. Multiple downstream pathways and molecules are emerging as suitable therapeutic targets. Modulating the cellular stress response, correcting the BDNF deficiency associated with HD, and targeting specific proteins are some of the many encouraging candidate pathways that may yield effective treatments. Furthermore, systematic, unbiased approaches to identify HD modifiers promise to further enrich the opportunities for therapeutic intervention.

Despite significant achievements in our understanding of the pathogenesis of HD, more efforts are needed to clearly identify the abnormalities/pathways/targets that are most critical for neurodegeneration and to distinguish them from the ones that are secondary responses or simply related epiphenomena. Those abnormalities associated with normal huntingtin function and possibly influenced by the CAG expansion seem to have greater appeal. Some of the activites altered by the mutation might specifically occur in adulthood and in acute phases of the disease or be present since developmental stages, leading to subtle phenotypes. Overall, this classification is important to discriminate key targets specific to HD from those for more generic neurodegeneration, and to exclude the nonessential ones. Revealing disease trigger processes will provide potential routes to HD-specific therapeutics and allow prioritizing the growing portfolio of therapeutic candidates.

The identification of the timing of appearance of the different dysfunctions (early and late events) and of the mechanisms that explain the selective neuronal vulnerability, in combination with more precise guidelines to translate the results of animal studies into the clinic will provide new hopes for a cure. In addition, new biomarkers to follow HD progression and markers to monitor drugs' engagement in a given molecular mechanism will hopefully constitute new tools for moving faster into the clinic.

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As for the colleagues working on Huntington's Disease, our heart, mind, and hands are moved by the patients, by their stories of courage, dignity, and fear, and by those of their families. Of these stories, we wish to remember Andrea, who has sadly passed away on February 3, 2010, at the age of 35. On that day, his mother told us of her desperation but also of the importance that neither we nor the other colleagues perceive her loss as a defeat as "it is only through the work of dedicated scientists that there will be a day in which no more lifes will be given to this disease." In the darkest day of her life, her deep sense of hope and trust has turned into a new shining star for us all.

With this manuscript, we honor Andrea and his mom and all the families and patients who suffer from this disease.

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