Are there multiple pathways in the pathogenesis of Huntington’s disease?

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Studies of huntingtin localization in human post-mortem brain offer insights and a framework for basic experiments in the pathogenesis of Huntington's disease. In neurons of cortex and striatum, we identified changes in the cytoplasmic localization of huntingtin including a marked perinuclear accumulation of huntingtin and formation of multivesicular bodies, changes conceivably pointing to an altered handling of huntingtin in neurons. In Huntington's disease, huntingtin also accumulates in aberrant subcellular compartments such as nuclear and neuritic aggregates co-localized with ubiquitin. The site of protein aggregation is polyglutamine-dependent, both in juvenile-onset patients having more aggregates in the nucleus and in adult-onset patients presenting more neuritic aggregates. Studies in vitro reveal that the genesis of these aggregates and cell death are tied to cleavage of mutant huntingtin. However, we found that the aggregation of mutant huntingtin can be dissociated from the extent of cell death. Thus properties of mutant huntingtin more subtle than its aggregation, such as its proteolysis and protein interactions that affect vesicle trafficking and nuclear transport, might suffice to cause neurodegeneration in the striatum and cortex. We propose that mutant huntingtin engages multiple pathogenic pathways leading to neuronal death.

Keywords: Huntington's disease; neurodegeneration; basal ganglia; polyglutamine; trinucleotide repeats; protein aggregation

1. INTRODUCTION

(a) Historical background

In 1872, George Huntington graphically described the triad of chorea, dementia and autosomal dominant inheritance that marks the disorder now called Huntington's disease (Huntington 1872). He crafted his description from observations made by his father and grandfather, both physicians on Long Island, New York. Alzheimer is credited with showing that small neurons in the striatum disappeared in this illness. Widespread neurodegeneration is appreciated elsewhere in the brain, especially in layers 3, 5 and 6 of the cortex (Vonsattel & DiFiglia 1990). First and foremost, the pathogenic process in Huntington's disease afflicts the medium spiny neuron in the striatum (Aronin et al. 1983; Graveland et al. 1985). Its genetic cause remained obscure until 1993, when a multi-site collaboration reported that patients with Huntington's disease have an increase in the number of CAG repeats in a gene IT-15 (interesting transcript number 15), recently named huntingtin (Huntington's Disease Collaborative Research Group 1993). Unaffected subjects had fewer than 35 CAG repeats in tandem, whereas affected individuals had 37 or more of these repeats.

Huntington's disease is a prototype of neurological diseases with lengthened CAG repeats that express proteins with extended polyglutamine sequences; so far, genes with lengthened CAG repeats underlie eight neurological diseases (Bates & Lehrach 1994). It is now clear that the neuropathology of this kind of mutation depends not only on the length of the polyglutamine sequence but also on the parent protein (the context) in which the polyglutamine resides. Recent findings underscore this idea. The introduction of an increased CAG repeat into an otherwise innocuous gene, hypoxanthine phosphoribosyltransferase (HPRT), results in a neurological diathesis in transgenic mice (Ordway et al. 1997). We regard this result as fundamental to understanding trinucleotide repeat diseases, because it demonstrates an inherent toxicity of extended polyglutamine sequences. However, the neuropathology was widespread, whereas in each of the trinucleotide-repeat diseases specific brain regions are predominantly affected. Therefore we believe that the polyglutamine sequences confer on the parent protein altered properties culminating in cell-selective disease.

(b) Thesis

Our current knowledge of the pathogenesis of Huntington's disease is based on its neuropathology in the juvenile and adult-onset forms as well as insights from transgenic models of Huntington's disease and cultured striatal neurons transfected with mutant huntingtin. Changes in huntingtin localization occur in the Huntington's disease brain and most frequently involve a diffuse nuclear and focal perinuclear accumulation of the protein (Sapp et al. 1997). Additionally, in the largest group of Huntington's disease patients, those with adult onset (90-95% of patients) (Snell et al. 1993), aggregation of the N-terminal
region of mutant huntingtin in neurites predominate. In the remaining 5–10% of Huntington’s disease patients, those with juvenile onset, nuclear inclusions are abundant in the cortex and striatum. The transgenic models of Huntington’s disease reveal that expanded polyglutamine repeats serve to target the mutant huntingtin to the nucleus and that small mutant huntingtin fragments are delivered more effectively than large fragments or full-length huntingtin (Davies et al. 1997; Laforet et al. 1998; Reddy et al. 1998). These findings demonstrate, in the human, that cleavage of mutant huntingtin precedes its aggregation and neuropathological consequences. However, even abundant nuclear inclusions do not inevitably cause striatal neurodegeneration (Davies et al. 1997; Saudou et al. 1998). We propose that mutant huntingtin contributes to a number of nuclear and cytoplasmic events that lead to neuronal cell death; the handling of mutant huntingtin in juvenile-onset Huntington’s disease probably differs from that in adult onset, resulting in two presentations of the disease.

2. NEURONAL CHANGES IN HUNTINGTON’S DISEASE

(a) Localization of huntingtin and its implication for function in mammalian brain

We organized our research plan to determine the localization of wild-type huntingtin and the expression of the mutant protein. The protein coded by the Huntington’s disease gene, huntingtin (ca. 350 kDa) appears early in the development of the mammalian brain, by day 15 of gestation (Bhide et al. 1996). The amount of huntingtin increases in the rodent newborn to achieve adult levels by three weeks. In the adult rodent, wild-type huntingtin resides in the cytoplasm (DiFiglia et al. 1995; Sharp et al. 1995; Trotter et al. 1995). Ultrastructural and biochemical studies in brain point to an association of huntingtin with tubular cisternae and vesicles of the Golgi complex (figure 1a), synaptic vesicles (figure 1b) (see also DiFiglia et al. 1995) and microtubules (Gutenkunst et al. 1995; Tukamoto et al. 1997), in addition to huntingtin in soluble fractions (DiFiglia et al. 1995; Sharp et al. 1995). Huntingtin co-distributes with clathrin in differentiating neurons (figure 2d) (Kim et al. 1999a) and decorates clathrin-coated pits and mature clathrin-coated vesicles (figure 2a, b) (Nélier et al. 1998); and uncoated vesicles (figure 2e). Co-immunolocalization of huntingtin with synaptofisin in synaptosomes is consistent with the transport of huntingtin to presynaptic sites (DiFiglia et al. 1995). In axoplasmic flow studies in peripheral nerve, huntingtin is anterogradely and retrogradely transported (Block-Galarza et al. 1997). On the basis of these results, we speculate that wild-type huntingtin participates in vesicle transport.

Identifying partners for huntingtin has been used to elucidate functions of wild-type huntingtin, as well as possible changes in function of the mutated huntingtin. Huntingtin interacts biochemically with numerous, predominately cytoplasmic proteins, including huntingtin associated protein 1 (HAP-1) (Li et al. 1995), huntingtin interacting protein (HIP-1) (Kalchman et al. 1997; Wanker et al. 1997), a ubiquitin-conjugating enzyme (Kalchman et al. 1996), calmodulin (Bao et al. 1996), apopain or caspase-3 (Goldberg et al. 1996), α-adapin (Faber et al. 1998; Gusella & MacDonald 1998), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Burke et al. 1996) and cystathionine β-synthase (Boutell et al. 1998). HAP-1 attaches to dynactin (Engelender et al. 1997; S. H. Li et al. 1998), which contributes to the movement of particles along microtubules; HIP-1 is structurally similar to Slu2P, also implicated in cytoskeletal function in yeast; and adapin binds to clathrin-coated vesicles. There is also evidence that huntingtin interacts with WW and SH3 protein domains (Faber et al. 1998; Sittler et al. 1998). Collectively, these studies form the framework that huntingtin might participate, directly or indirectly, in vesicle transport.

(b) Expression of mutant huntingtin in Huntington’s disease

Three general mechanisms have been used to explain the pathogenesis of diseases with autosomal dominant
inheritance: (i) the mutated gene might not be transcribed, resulting in a decrease in active protein to levels insufficient to perform important cellular functions; (ii) a protein might be translated, but be unable to perform its activities and block the activities of the wild-type protein; (iii) the protein might change its function or non-specifically render the cell less hardy.

Current information provides evidence that the mutant huntingtin is expressed and maintains its normal activity (which is yet to be defined). We found that mutant huntingtin is expressed throughout the brain in patients with Huntington's disease (Aronin et al. 1995). A mechanism for cell death in Huntington's disease needs to consider that unaffected neurons also express the mutant protein. The absence of normal protein does not produce a disease phenotype. Crucially, patients homozygous for Huntington's disease have a phenotype similar to the hemizygous patients (Wexler et al. 1987). Collectively, these findings support the idea that the vulnerable neurons should handle the mutant protein differently from protected cells.

(c) **Nuclear inclusions and dystrophic neurites**

We examined the morphology and ultrastructure of neurons in the striatum and cortex in Huntington's disease. Our idea was that study of the human condition would best guide our future experiments. Our findings revealed a complex of subcellular changes, involving cytoplasmic and nuclear structures (DiFiglia et al. 1997; Sapp et al. 1997), possibly portending a series of molecular aberrations leading to apoptotic neuronal death in Huntington's disease (Portera-Cailliau et al. 1995; Butterworth et al. 1998). Nuclear changes were readily apparent, but we shall first describe findings in the cytoplasm.

Changes in the cytoplasm pervade the neuropathology in Huntington's disease. With an antiserum directed against an internal epitope of huntingtin (DiFiglia et al. 1995), we identified large, immunoreactive granules in the somatodendritic cytoplasm; under electron microscopy, these granules resembled multivesicular bodies, organelles dually involved in retrograde transport and protein degradation (Sapp et al. 1997; LaVail & LaVail 1974; Hollenbeck 1993) (figure 3). Huntingtin heavily concentrated in the perinuclear cytoplasm accumulated in part over endosomal–lysosomal organelles and tubulo-vascular membranes. Some of the full-length protein or large fragments of huntingtin recognized by the antibody might have been destined for the nucleus but was unable to penetrate its borders because it was irreversibly bound to subcompartments of the endoplasmic reticulum.

Another aspect of huntingtin's cytoplasmic localization in the brain with Huntington's disease was revealed by an N-terminally directed antiserum; huntingtin immunoreactivity resided in dystrophic neurites. The dense accumulations of huntingtin overlapped with ubiquitin (DiFiglia et al. 1997; Sapp et al. 1999) and were embedded in neurofilament-labelled processes (figure 4 a,b). Jackson et al. (1995) reported dystrophic neurites containing ubiquitin in Huntington's disease. Consisting largely of degenerating axons, the dystrophic neurites with huntingtin marked the striatal and cortical landscape in the adult brain in patients with Huntington's disease and were much more prevalent in adult-onset Huntington's disease than in the juvenile-onset disease (figure 5). A substantial portion of the dystrophic neurites comprised cortical–striatal projections that degenerate early in the disease (Sapp et al. 1999) (figure 4c).
Wild-type huntingtin is absent from, or rare in, the nucleus in neurons in control brains, on the basis of most studies (DiFiglia et al. 1995; Sharp et al. 1995; Troettier et al. 1995), although there is a report of nuclear localization with appropriate biochemical correlates (De Rooij et al. 1996). In post-mortem human striatum in Huntington’s disease, we found moderately intense, diffuse nuclear localization of huntingtin detected with the use of antisera against an internal epitope of the protein (Sapp et al. 1997). The N-terminal antiserum unmasked huntingtin in dense intranuclear inclusions in brains with juvenile-onset Huntington’s disease (figure 6), and to a smaller extent in adult-onset Huntington’s disease (DiFiglia et al. 1997). The nuclear inclusions were dispersed in neurons of cortex and striatum (figure 6), regions of most active disease, but not in globus pallidus or cerebellum (figure 6e,f), which are largely unaffected. Like the dystrophic neurites, the nuclear inclusions contained ubiquitin (figure 6a). Ultrastructural review revealed granular and filamentous configurations (figure 6g). Contemporaneously, nuclear inclusions were identified in other CAG trinucleotide repeat diseases (Becher et al. 1998; Holmberg et al. 1998; M. Li et al. 1998; Merry et al. 1998; Paulson et al. 1997).

The formation of nuclear inclusions was polyglutamine-dependent and was more frequent with larger
expansions; 30–50% of neurons in the cortex in juvenile-onset Huntington’s disease contained nuclear inclusions, compared with ca. 3–6% of cortical neurons in the adult-onset disease (DiFiglia et al. 1997; Vonsattel & DiFiglia 1998) (figure 5). This finding was not related to the neuropathological grade of striatal degeneration (DiFiglia et al. 1997). In the adult-onset patients with low-grade neuropathology (grades 1 and 2), nuclear inclusions were absent from, or rare in, the striatum when dystrophic neurites had formed (Sapp et al. 1999).

How does huntingtin enter the nucleus? The large size of huntingtin would predict its nuclear entry either by active transport or by its being processed to form smaller fragments. Measurement of huntingtin immunoreactivity in isolated nuclei demonstrated that much of the huntingtin in the nuclei consisted of N-terminal fragments of lower molecular mass, in the 20–40 kDa range (DiFiglia et al. 1997) (it should be noted that approximating the molecular mass of proteins containing lengthy polyglutamine sequences is risky). Proteins of this approximate size could enter the nucleus passively (Alpert et al. 1994). Smaller, cleaved products of mutant huntingtin were shown to enter the nucleus preferentially (Martindale et al. 1998; Hackam et al. 1998). Detlof and co-workers showed that highly extended polyglutamine sequences can promote the nuclear entry of an otherwise cytoplasmic protein (Ordway et al. 1997). We found full-length huntingtin in nuclear extracts of brain with Huntington’s disease. Larger proteins have access to the nucleus, but enter passively at a low rate, or enter by facilitated transport or active import. We contend that over a time frame of years to decades, with the lengthened polyglutamine sequences as facilitator, small and large huntingtin fragments and full-length protein have an opportunity to gain access to the nucleus.

3. IS NUCLEAR AGGREGATION OF HUNTINGTIN NECESSARY OR SUFFICIENT TO TRIGGER CELL DEATH IN STRIATAL NEURONS?

(a) Lessons from transgenic models of Huntington’s disease

Several laboratories have generated transgenic mice expressing either an N-terminal fragment or a full-length mutant huntingtin (Mangiarini et al. 1996; Reddy et al. 1998). Multiple pathways in the pathogenesis of Huntington’s disease

![Figure 5. Frequency of nuclear inclusions and dystrophic neurites in patients with Huntington’s disease with juvenile onset (J11, J12, J13) and adult onset (A12, A17, A20, A21). Nuclear inclusions are more frequent in juveniles, and dystrophic neurites are more abundant in adults. (Taken from DiFiglia et al. (1997).)](http://rstb.royalsocietypublishing.org/)

![Figure 6. Nuclear inclusions formed by N-terminal mutant huntingtin in the brain with Huntington’s disease.](http://rstb.royalsocietypublishing.org/)
1998; Cha et al., this issue; Davies et al., this issue; Reddy et al., this issue). Differences between the transgenic mice include the length of the polyglutamine expansion, the size of the huntingtin, the promoter and the background strain. The initial transgenic mice with Huntington's disease (as previously described) used a human huntingtin gene promoter to express mutant huntingtin with an expanded polyglutamine repeat (115–150 repeats) and a small huntingtin N-terminal fragment (exon 1, 69 residues) in a C57BL/6 mouse strain. This transgenic strain was very instructive. Despite extensive nuclear inclusions, striatal cell loss was absent or minimal. The animals succumbed within three months to general inanition, although recent evidence implicates diabetes mellitus (with hypoinsulinaemia) as a cause (Hurlbert et al. 1998).

Detloff and co-workers created transgenic mice expressing HPRT with a stretch of 146 glutamine residues. HPRT is ordinarily localized in the cytoplasm, but these animals showed abundant nuclear inclusions throughout the brain. No cell death was observed. The animals were short-lived and exhibited abundant seizures. These studies indicate that small proteins with expanded polyglutamine sequences are targeted to neuronal nuclei and accumulate in sufficient abundance to form large aggregates, detected as inclusions. Transgenic animals harbouring expanded polyglutamine stretches in the context of small proteins manifest phenotypic changes consistent with generalized brain dysfunction. However, the presence of inclusions does not predict cell-specific or region-specific neurodegeneration under these conditions.

Tagle and co-workers (Reddy et al. 1998, this issue) and our own laboratory (Laforet et al. 1998) made transgenic models of Huntington's disease expressing a substantially larger mutant huntingtin than in the initial transgenic mice with Huntington's disease. Our transgenic mice expressed a 46 or 100 CAG repeat (one base change at position 28 CAG) in the first 3221 bases of huntingtin complementary DNA (cDNA). The host strain was SJL/B6 F1 hybrid. Many animals exhibited motor deficits, lost ca. 20% of striatal cells and lived beyond one year (Laforet et al. 1998; G. A. Laforet, M. DiFiglia and N. Aromin, unpublished data). Nuclear inclusions were apparent but not abundant in the striatum of normal, affected mice in lineages with 46 or 100 glutamine repeats. Tagle and co-workers generated transgenic mice with 16, 48 and 89 glutamine repeats in a full-length huntingtin in FVB/N murine strain. Mutant huntingtin expression varied, from nearly equivalent to native protein to about fivefold greater. Nuclear inclusions were detected, but not abundantly, and the animals had a predictable 20% cell loss in the striatum and cortex (Reddy et al. 1998, this issue).

These studies in vivo reveal that the size of the huntingtin protein substantially influences the longevity of the transgenic mice with Huntington's disease and also the presence of striatal cell loss. Neither the background strain of the mice nor the abundance of nuclear inclusions accounts for neurodegeneration.

(b) Revelations in the short-term: studies in neurons and other cells in culture

Experiments in cell culture have provided important clues to the role of inclusions of huntingtin in the pathogenesis of cell death. We studied effects of mutant huntingtin expression in cultured hybrid striatal, medium-sized neurons, generated from clonal mouse foetal striatal-neuroblastoma fusions (Kim et al. 1999a). These cells maintain many fundamental characteristics of striatal spiny neurons, which are the initial targets of neurodegeneration in Huntington's disease (Wainwright et al. 1995). Constructs containing 18, 46 or 100 CAG repeats in either a full-length huntingtin cDNA or a large fragment of huntingtin cDNA (1–321) bases were transfected into the clonal striatal neurons. The cDNAs were intact or modified as N-terminal FLAG–huntingtin fusion proteins or huntingtin–green fluorescent protein (GFP) fusion proteins; huntingtin and FLAG were detected by immunohistochemistry and GFP was viewed by fluorescence (Kim et al. 1999a). Full-length and 3 kb mutant huntingtin cDNAs (irrespective of molecular tags) produced nuclear inclusions. Transfection with the 100 CAG repeat huntingtin cDNA resulted in a greater number of inclusions than with the 46 repeat cDNA; the 18 repeat produced none. Therefore the clonal striatal neurons recapitulated the polyglutamine-dependent formation of inclusions seen in the patient brain in Huntington's disease.

We have found that transfection of partial or full-length mutant huntingtin cDNAs into clonal striatal cells generated N-terminal fragments; the sizes varied between 70 and 100 kDa (Kim et al. 1999a). Because huntingtin can be a target of caspase cleavage (Goldberg et al. 1996), we introduced caspase inhibitors into the cultures of transfected cells. With the caveats that caspase inhibitors lack true specificity and probably affect separate components of apoptotic cascades, we considered the inhibitors primarily as tools to block huntingtin degradation.

The caspase inhibitor Z-DEV-D-FMK (Enzyme Systems) prevented inclusion formation after transfection with mutant huntingtin cDNAs. No abatement in cell death was observed (Kim et al. 1999a). An implication of this result is that huntingtin nuclear inclusions are not necessary for mutant huntingtin to induce striatal cell survival. In contrast, we found that the general caspase inhibitor Z-VAD-FMK (Enzyme Systems) did not alter mutant huntingtin nuclear inclusions, but (surprisingly) did improve the survival of the transfected clonal striatal cells. In a separate model, Saudou et al. (1998) reported that entry of an N-terminal fragment of mutant huntingtin (171 residues, 68 glutamine repeats) into the nucleus reduced survival of the transfected primary rat striatal neurons. By placing a nuclear export signal in the mutant huntingtin, the authors discovered that accumulation of the transfected mutant huntingtin was abrogated and cell survival was unaffected. They found that inclusion formation was not requisite for cell death, but that delivery of a mutant huntingtin fragment to the nucleus was needed to induce apoptosis. Similarly, in neuroblastoma cell lines stably expressing mutant huntingtin, Lunkes & Mandell (1998; Lunkes et al., this issue) found that apoptosis was increased by the expression of mutant huntingtin but was not tightly correlated with the formation of nuclear and cytoplasmic inclusions.

The above studies in vitro suggest that nuclear inclusions are not necessary or sufficient to predict striatal cell death, which is reminiscent of the neuropathology seen in...
adult-onset Huntington’s disease. Other compartments that accumulate huntingtin might be more critical for cell death. In striatal hybrid neurons, we found that cell death (marked by significant cell shrinkage) occurred when N-terminal huntingtin fragments of wild-type or mutant proteins accumulated into perinuclear vacuoles (Kim et al. 1999a), not unlike the perinuclear huntingtin accumulation associated with degenerating neurons in the brain with Huntington’s disease (Sapp et al. 1997). That N-terminal fragments of mutant huntingtin can influence cellular toxicity has support from studies in the laboratories of Hayden (Hackam et al. 1998) and Ross (Cooper et al. 1998), who showed that introducing N-terminal fragments of mutant huntingtin into neuroblastoma cell sensitized the cells to apoptosis. We found that a blockade of one N-terminal huntingtin product by caspase inhibition was correlated with increased cell survival (Kim et al. 1999a). N-terminal products of either wild-type or mutant huntingtin are toxic at high levels in non-striatal and striatal cells (Hackam et al. 1998; Kim et al. 1999a). How, then, can N-terminal mutant huntingtin fragments be selectively disruptive to cellular function? Insight comes from crucial studies in which full-length huntingtin was overexpressed in cultured cells (Lunkes & Mandel 1998; Lunkes et al., this issue; Kim et al. 1999a). Under these conditions, N-terminal fragments accumulated only when the protein contained an expanded polyglutamine tract.

4. MULTIPLE PATHOGENIC MECHANISMS IN HUNTINGTON’S DISEASE

We present evidence that mutant huntingtin accumulates in multiple subcellular compartments in selected neurons in Huntington’s disease and is handled differently in the juvenile-onset patient compared with the adult-onset patient. Available information points to two general pathways leading to cell death, with an emphasis on nuclear disruption in juvenile-onset Huntington’s disease and the axon as a major site of pathology in adult-onset Huntington’s disease. In general, the larger is the CAG repeat, the more likely it is that mutant huntingtin will be targeted to the nucleus in addition to its accumulation in the cytoplasm. Neuronal death is exacerbated by the accumulation of N-terminal fragments of huntingtin in either the nucleus or cytoplasm but does not depend on the presence of nuclear or cytoplasmic inclusions. The N-terminal region of huntingtin is cleaved from the full-length protein and robustly amasses in cells when the polyglutamine tract is expanded. Mutant huntingtin’s N-terminal domain interacts abnormally with cytoplasmic proteins involved in vesicle trafficking (HIP1, HAP1, GAPDH and SH3GL3). The early appearance of dystrophic axons enriched in N-terminal mutant huntingtin in adult-onset patients coincides with disturbances of vesicle membrane transport in these axons. Huntington also interacts at its N-terminus with resident nuclear proteins (Faber et al. 1998). Alterations in nuclear function caused by targeting of the cleaved or fully intact protein to the nucleus, with the tendency of huntingtin to form filaments (Perutz et al. 1994; Scherzinger et al. 1997), are especially pronounced in patients with juvenile-onset Huntington’s disease and even pre-empt changes in the cytoplasm. We believe that a single pathogenic event causing Huntington’s disease might prove elusive.

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