Neuron, Vol. 21, 633–642, September, 1998, Copyright ©1998 by Cell Press

Polyglutamine-Expanded Human Huntingtin Transgenes Induce Degeneration of *Drosophila* Photoreceptor Neurons

George R. Jackson,*‖ Iris Salecker,†‖ Xinzhong Dong,† Xiang Yao,‡ Norman Arnheim,‡ Peter W. Faber,§ Marcy E. MacDonald,§ and S. Lawrence Zipursky†#

*Department of Neurology
†Howard Hughes Medical Institute
and Department of Biological Chemistry
University of California, Los Angeles
School of Medicine
Los Angeles, California 90095
‡Molecular Biology Program
University of Southern California
Los Angeles, California 90089
§Molecular Neurogenetics Unit
Massachusetts General Hospital
Charlestown, Massachusetts 02129

Summary

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder. Disease alleles contain a trinucleotide repeat expansion of variable length, which encodes polyglutamine tracts near the amino terminus of the HD protein, huntingtin. Polyglutamine-expanded huntingtin, but not normal huntingtin, forms nuclear inclusions. We describe a *Drosophila* model for HD. Amino-terminal fragments of human huntingtin containing tracts of 2, 75, and 120 glutamine residues were expressed in photoreceptor neurons in the compound eye. As in human neurons, polyglutamine-expanded huntingtin induced neuronal degeneration. The age of onset and severity of neuronal degeneration correlated with repeat length, and nuclear localization of huntingtin presaged neuronal degeneration. In contrast to other cell death paradigms in *Drosophila*, coexpression of the viral antiapoptotic protein, P35, did not rescue the cell death phenotype induced by polyglutamine-expanded huntingtin.

Introduction

Huntington's disease (HD) is characterized by a combination of chorea, cognitive impairment, and affective changes (Martin and Gusella, 1986; Folstein, 1989). It is the most prevalent autosomal dominant neurodegenerative disorder associated with expansion of unstable CAG tracts. These disorders also include spinocerebellar ataxia (SCA) 1 (Chung et al., 1993; Orr et al., 1993), SCA2 (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996), SCA3 (allelic to Machado-Joseph Disease, MJD; Cancel et al., 1995; Haberhausen et al., 1995; Higgins et al., 1996), SCA6 (Jodice et al., 1997; Riess et al., 1997), and SCA7 (David et al., 1997), as well as dentatorubral pallidoluysian atrophy (DRPLA; Nagafuchi et al., 1994; Onodera et al., 1995). Each disease is associated with brain region–specific patterns of neuronal degeneration. Genetic and molecular studies indicate that expression of polyglutamine tracts resulting from expanded CAG repeats imparts novel functions to proteins encoded by the affected loci (Ambrose et al., 1994; Gusella et al., 1985; Duyao et al., 1995; Gutekunst et al., 1995; Jou and Myers, 1995; Nasir et al., 1995; Sharp et al., 1995; Zeitlin et al., 1995; Goldberg et al., 1996a; White et al., 1997). The mechanisms underlying polyglutamine-induced neuronal cell death remain poorly understood.

The product of the HD gene, huntingtin, is a novel 350 kDa cytoplasmic protein (Huntington's Disease Collaborative Research Group, 1993). The unstable CAG repeat within exon 1 gives rise to proteins containing polyglutamine tracts of varying size. Normal alleles have 37 or fewer glutamines; disease alleles range between 37 to in excess of 150 glutamines (Gusella and MacDonald, 1995). Recent studies suggest that cleavage products of huntingtin that include the polyglutamine tract are cytotoxic, and that such cleaved fragments form intranuclear aggregates (Goldberg et al., 1996b; Davies et al., 1997; DiFiglia et al., 1997; Scherzinger et al., 1997; Cooper et al., 1998; Wellington et al., 1998). Similar intranuclear inclusions have been described in HD, DRPLA, SCA1, SCA3, and SCA7, and have been proposed to play a key role in the pathophysiology of glutamine repeat diseases (Roizin et al., 1979; DiFiglia et al., 1997; Paulson et al., 1997; Ross, 1997; Skinner et al., 1997; Davies et al., 1998; Holmberg et al., 1998; Igarashi et al., 1998).

Transgenic mice expressing an amino-terminal fragment of human huntingtin containing 115 or more glutamine residues have a movement disorder and inclusions within neuronal nuclei (Davies et al., 1997). Accumulation of huntingtin immunoreactivity in neuronal nuclei precedes the formation of nuclear inclusions. Inclusion formation and other changes in nuclear ultrastructure, in turn, precede the motor phenotype. Although this murine model recapitulates some features of HD, neuronal cell death is not a prominent feature of the mutant phenotype. In addition, nuclear inclusions and, in some cases, apoptosis have been observed in cells transfected with cDNAs encoding huntingtin fragments containing expanded glutamine tracts (Goldberg et al., 1996b; Ikeda et al., 1996; Paulson et al., 1997; Cooper et al., 1998; Hackam et al., 1998a; Li and Li, 1998; Martindale et al., 1998; Tait et al., 1998; see Discussion).

In an effort to develop a genetic means of dissecting the cell death pathways activated by glutamine repeat diseases, we assessed whether polyglutamine-expanded fragments of human huntingtin could induce degeneration of *Drosophila* photoreceptor neurons. The fly eye has proven to be a favorable system for genetically dissecting various cellular processes, including receptor tyrosine kinase signaling, cell cycle progression, and cell death pathways (Agapite and Steller, 1997; Wolff et al., 1997). We expressed 5′-truncated fragments of the human huntingtin cDNA in the *Drosophila* eye using the eye-specific P element expression vector pGMR (Hay

‖ These authors contributed equally to this work and are listed alphabetically.
To whom correspondence should be addressed.

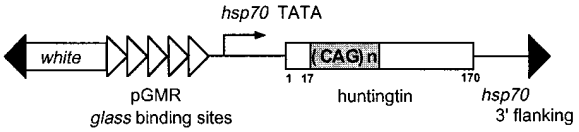

Figure 1. Schematic Representation of the pGMR-Huntingtin Transgenes

The five glass binding sites in pGMR drive expression of downstream genes in the developing and adult eye. The amino acids in all constructs are encoded by exons 1–3 and part of exon 4. Expanded (CAG) repeats encoding the polyglutamine stretches follow amino acid 17. Amino acid positions are indicated (Huntington's Disease Collaborative Research Group, 1993; see Experimental Procedures for details).

et al., 1994). We demonstrate that fragments with expanded repeats induce degeneration of adult photoreceptor neurons, and that neuronal degeneration is preceded by the nuclear accumulation of huntingtin. As in the human disease, longer repeats induced earlier neuronal degeneration.

While this paper was being prepared for publication, Bonini and colleagues described studies in which polyglutamine-expanded fragments from the human MJD protein were expressed in *Drosophila* (Warrick et al., 1998). We address the similarities and differences between their studies and ours in the Discussion section.

Results

Photoreceptor Cell Degeneration Induced by Polyglutamine-Expanded Huntingtin Proteins

Amino-terminal fragments of human huntingtin containing 2 (Q2), 75 (Q75), and 120 (Q120) glutamines were expressed under the control of the eye-specific expression construct pGMR (Figure 1; see Experimental Procedures). Multiple lines of Q2, Q75, and Q120 were analyzed at eclosion and at various times posteclosion (Figures 2 and 3). The fly eye is composed of an array of cell clusters, called ommatidia, each containing eight photoreceptor neurons. These cells elaborate a stack of tightly packed microvilli that form a photosensitive structure, the rhabdomere (see schematic in Figure 3E). The pattern of rhabdomeres can be assessed using a rapid optical technique called the pseudopupil method (Franceschini, 1972). More detailed morphological analyses were carried out using both light and electron microscopy. We examined the morphology of photoreceptor cells in wild-type (*wt*), Q2, Q75, and Q120 lines over the first 10 days after eclosion (Figure 2A). *wt* (Figure 2B) and Q2 flies showed normal ommatidial morphology at eclosion and at 10 days (Figures 2D and 2E). Whereas Q75 flies were indistinguishable from *wt* at eclosion, loss of rhabdomeres was apparent in a small proportion of ommatidia by 10 days. Similarly, Q120 eyes were indistinguishable from *wt* at eclosion (Figure 2F). In contrast to Q2 and Q75, however, degeneration of rhabdomeres and photoreceptor cells in these flies was evident at 2 days and severe by 10 days (Figures 2C and 2G).

The stability of repeats in multiple lines containing Q75 and Q120 was analyzed (data not shown; see Experimental Procedures). A low level of germline instability of

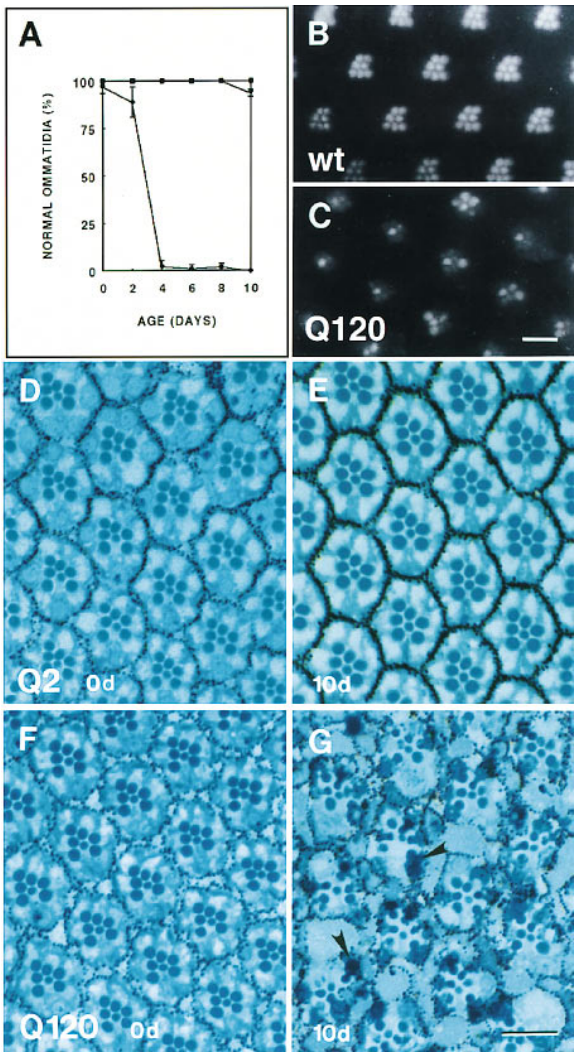

Figure 2. Time- and Polyglutamine-Dependent Degeneration in the Adult Eye

(A) Time course of photoreceptor degeneration as determined by the pseudopupil technique. *wt* (squares), Q2 (open circles), Q75 (triangles), Q120 (closed circles). *wt*, Q2, and Q75 values are superimposed at most time points. Each point shows the mean ± SD for five eyes. Forty to ninety ommatidia were scored from each eye. Values shown without error bars had SDs smaller than the dimensions of the symbol used.

(B and C) Photomicrographs of pseudopupil preparations visualizing the rhabdomere patterns within individual ommatidia. Rhabdomeres are densely packed microvilli containing the visual pigment (see Figure 3E).

(B) A regular trapezoidal arrangement of rhabdomeres is seen in *wt* eyes at 10 days.

(C) The ommatidial structure is severely disrupted in Q120 eyes at 10 days.

(D–G) Toluidine blue–stained semithin sections. Ommatidia in Q2 eyes at eclosion (D) and at 10 days (E) are indistinguishable from *wt* (not shown). Ommatidia in Q120 lines are intact at eclosion (F) but show severe degeneration of photoreceptor neurons at 10 days (G). Arrowheads indicate examples of intense toluidine blue staining in degenerating photoreceptor cell bodies. Eyes from four or five flies were analyzed for each genotype at each time point. Degeneration also was observed in two other independently derived Q120 transgenic lines examined. Genotypes: *wt*, Oregon R; Q2, *yw*;pGMR-huntingtin-Q2/CyO; Q75, *yw*;pGMR-huntingtin-Q75/CyO; and Q120, *yw*;pGMR-huntingtin-Q120/CyO. Scale bars, 10 μm.

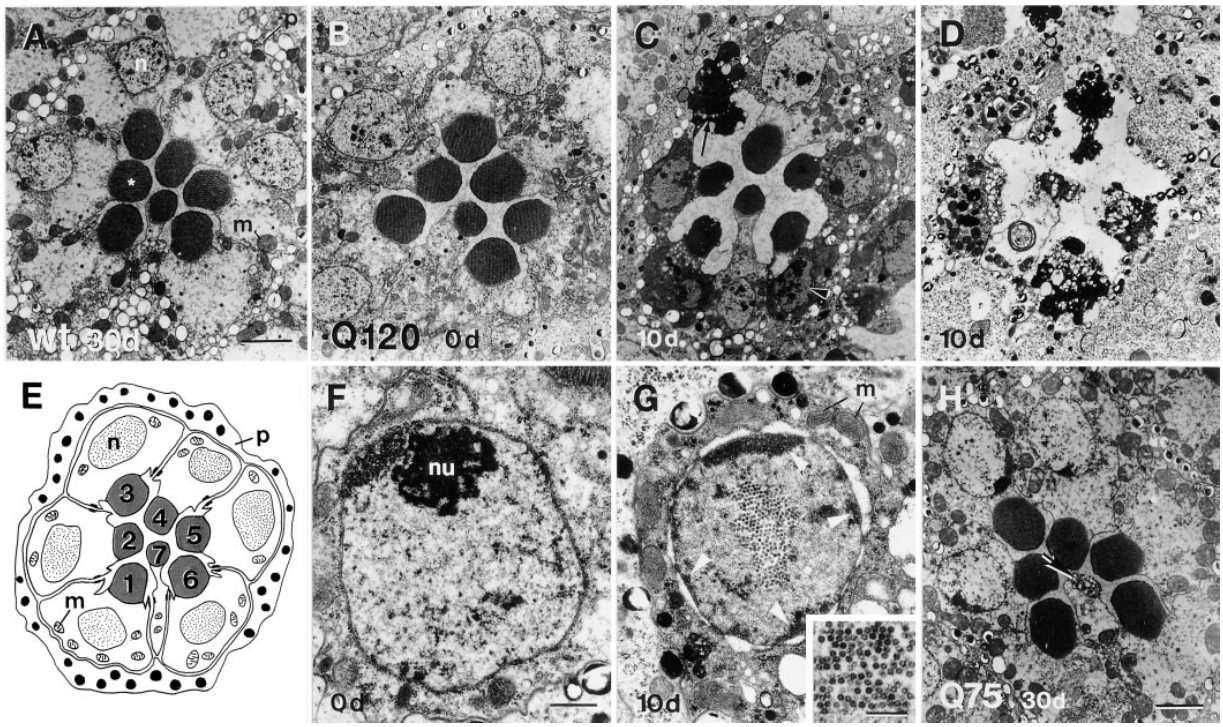

Figure 3. Electron Microscopic Analysis of Polyglutamine-Induced Degeneration of Photoreceptor Neurons

(A and E) Morphology of ommatidia in *wt* at 30 days is normal. Sections of ommatidia were analyzed at a level at which seven of the eight photoreceptor neurons are seen. Asterisk, rhabdomere; m, mitochondrion; n, nucleus; p, processes of pigment cells surrounding photoreceptor neurons in each ommatidium (see schematic representation in [E]).

(B) Normal morphology of Q120 ommatidia is seen at eclosion.

(C) Degeneration with ultrastructural features of apoptosis is evident in Q120 at 10 days. Arrowhead, nucleus with aggregation of condensed chromatin; arrow, shrunken cell with highly osmiophilic cytoplasm.

(D) Severely affected ommatidium in Q120. Some photoreceptor cells are missing, as are the majority of the rhabdomeres in remaining cells.

(F) High magnification view of Q120 shows a normal nucleus at eclosion. nu, nucleolus with adjacent heterochromatin, which appears granular.

(G) Nucleus of a Q120-expressing photoreceptor neuron at 10 days is shrunken and has a more electron-dense nucleoplasm as compared to the nucleus at eclosion (F). Peripheral chromatin condensation adjacent to the inner nuclear membrane (arrowheads) appears compact and highly electron dense in contrast to heterochromatin in a normal nucleus. The nucleus contains densely packed spherical, 40 nm inclusions (see also inset). These are indistinguishable from particles induced by the retrovirus-like *Drosophila copia* element (see text). Inclusions are not seen in all degenerating nuclei. These inclusions do not resemble those associated with HD (see Discussion). m, examples of well-preserved mitochondria.

(H) Mild degeneration is observed in Q75 ommatidia at 30 days. The arrow indicates a degenerating R7 cell.

Three or four eyes of each genotype were analyzed at each time point. Genotypes: (A) Oregon R; (B, C, and F) *yw*;pGMR-huntingtin-Q120/CyO; (D and G) *yw*;pGMR-huntingtin-Q120/TM6b; and (H) *yw*;pGMR-huntingtin-Q75/pGMR-huntingtin-Q75;pGMR-huntingtin-Q75/pGMR-huntingtin-Q75. Scale bars, 2 μm (A–D and H), 0.5 μm (F and G), and 0.2 μm (inset in [G]).

Q75 was observed. However, several Q120 lines showed both somatic and germline mosaicism in repeat length, including contractions to as low as Q35. In contrast, CAG tract expansion was not observed in either Q75 or Q120 lines.

Ultrastructure of Degenerating Photoreceptor Neurons

Morphological features of huntingtin-induced cell death were analyzed by transmission electron microscopy. At eclosion, the ultrastructure of Q120 lines was indistinguishable from that of normal flies (Figure 3B). Hence, despite persistent huntingtin expression throughout development (see below), the eye formed normally. Q120 lines showed massive cell degeneration by 10 days with nuclear and cytoplasmic condensation, chromatin clumping, and increased affinity for osmium (Figures 3C and 3D). Dying cells were clearly demarcated from more healthy neighbors. Subcellular organelles, including mitochondria, appeared intact. These features of cell death in Q120 lines are similar to previously described properties of cells undergoing apoptosis (Kerr et al., 1972; Steller et al., 1994; see Discussion). At 30 days, Q75 flies exhibited a weak photoreceptor degeneration phenotype that was more apparent in flies bearing multiple Q75 insertions (Figure 3H). Degeneration of *wt* eyes was negligible at 30 days (Figure 3A), as reported previously (Kurada and O'Tousa, 1995).

Morphologically distinct nuclear inclusions are readily observed by electron microscopy in glutamine repeat disease brains as well as cultured cells and transgenic mice expressing glutamine repeat–containing proteins. These nuclear inclusions appear as large, circular, electron-hypodense areas that exclude heterochromatin,

and often appear fibrillar in nature. Although multiple, small punctate structures were stained with anti-huntingtin antibodies in nuclei of degenerating cells (see below), large, hypodense inclusions were not seen by electron microscopy in photoreceptor neurons expressing Q120 transgenes. Many nuclei of degenerating photoreceptor neurons, however, contained distinct 40 nm spherical particles (Figure 3G). These are morphologically indistinguishable from virus-like particles induced by the retrovirus-like *Drosophila copia* element, a transposable element found in many fly strains. Such particles have been observed in nuclei of *Drosophila* cells under stress, including neoplastic cells, aging cells, and cells of the *opaque-eye* mutant, as well as cultured cells transfected with a plasmid carrying the cDNAs encoding *copia*-specific transcripts (Akai et al., 1967; Youssef and Gardner, 1975; Gartner and Gartner, 1976; Miyake et al., 1987; Peel et al., 1990). Indeed, *copia* transcripts encode proteins associated with these particles. The role of these virus-like particles, if any, in neuronal cell death induced by huntingtin transgenes requires further analysis.

P35 Does Not Rescue Polyglutamine-Induced Neuronal Degeneration

Programmed cell death, as well as death induced by transgenes such as *hid* (*head involution–defective*), can be blocked in *Drosophila* by expression of the antiapoptotic protein P35, a broad specificity caspase inhibitor, encoded by the baculovirus *Autographa californica* (Clem and Miller, 1994; Hay et al., 1994; Sugimoto et al., 1994; Chen et al., 1996; White et al., 1996). More recently, P35 has been demonstrated to prevent cell death and blindness in *Drosophila* retinal degeneration mutants (Davidson and Steller, 1998). We evaluated the effect of P35 in cell death induced by the Q120 transgene. Q120 flies expressing one (Figure 4B), two, or three copies (data not shown) of pGMR-P35 were indistinguishable at 10 days from Q120 without pGMR-P35 (Figure 4A). Three independently derived Q120 lines were tested with a single P35 line, and, conversely, three P35 lines were tested for their ability to rescue a single Q120 line. In no case was rescue of the Q120 phenotype observed. In contrast, pGMR-P35 completely rescued death induced by pGMR-*hid* (Figures 4C and 4D). These data suggest that Q120-induced cell death may proceed by a novel pathway distinct from cell death pathways previously described in flies.

Age and Polyglutamine Length–Dependent Nuclear Accumulation of Huntingtin

The expression pattern and subcellular localization of Q2, Q75, and Q120 was assessed during larval development, at eclosion, and 10 days posteclosion. pGMR-driven transgene expression begins in the primordium of the eye, the eye imaginal disc, posterior to the morphogenetic furrow (i.e., prior to neuronal differentiation). Accordingly, strong expression of Q2, Q75, and Q120, as visualized by confocal microscopy and Ab1, a polyclonal antibody directed against an amino-terminal epitope of huntingtin (DiFiglia et al., 1995), commenced just posterior to the morphogenetic furrow (Figures 5B–5D). Huntingtin immunoreactivity was predominantly cytoplasmic

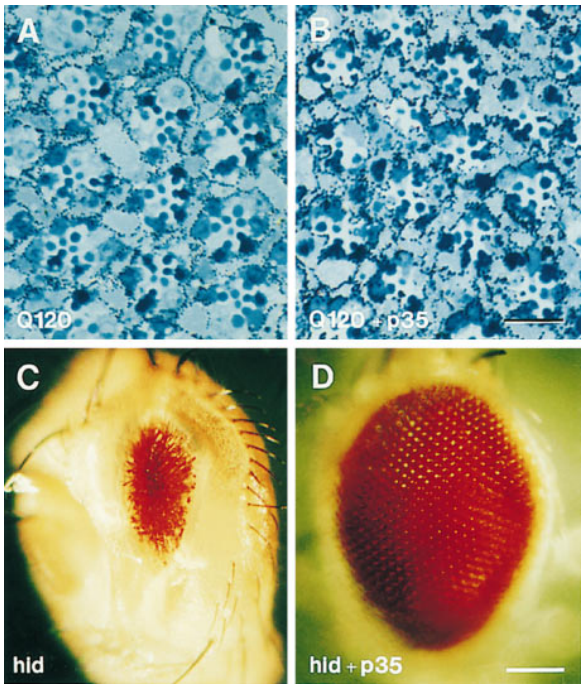

Figure 4. pGMR-P35 Does Not Suppress Q120-Induced Degeneration

(A) Section of Q120 at 10 days.

(B) Section from eye expressing both Q120 and pGMR-P35 is indistinguishable at 10 days from that expressing Q120 alone.

(C and D) The activity of the pGMR-P35 transgene was demonstrated by showing that the cell death–induced phenotype of pGMR-*hid* (C) was suppressed by coexpression of pGMR-P35 (D). Genotypes: (A) *yw*;pGMR-huntingtin-Q120/+; (B) *yw*;pGMR-huntingtin-Q120/+;pGMR-P35/+; (C) *w*;pGMR-hid/+; and (D) *w*;pGMR-*hid*/+;pGMR-P35/+. Scale bar, 10 μm ([A and B], semithin sections stained with toluidine blue), and 100 μm ([C and D], light micrographs).

throughout the developing eye disc regardless of repeat length (shown for Q120 in Figures 5F–5H).

Localization of transgene products in adults was assessed in horizontal cryostat sections using Ab1. At eclosion, Q2 and Q75 were cytoplasmic, whereas Q120 was seen in both the nucleus and the cytoplasm (Figures 6A, 6C, and 6E). At 10 days posteclosion, Q2 immunoreactivity remained cytoplasmic (Figure 6B), Q75 was both nuclear and cytoplasmic (Figure 6D), and Q120 was largely nuclear (Figure 6F). Whereas nuclear staining in Q120 lines at eclosion was diffuse and uniform (Figure 6H), by 10 days staining was punctate (Figure 6I), consistent with the formation of nuclear inclusions.

Discussion

In this paper, we demonstrate that fragments of human huntingtin with expanded polyglutamine tracts induce degeneration in *Drosophila* photoreceptor neurons. Remarkably, the cellular phenotype induced by these fragments shares similarities with neuronal cell death seen in HD brains. Photoreceptor neuronal differentiation is normal, and the onset of degeneration is determined by the length of the polyglutamine tracts. While huntingtin

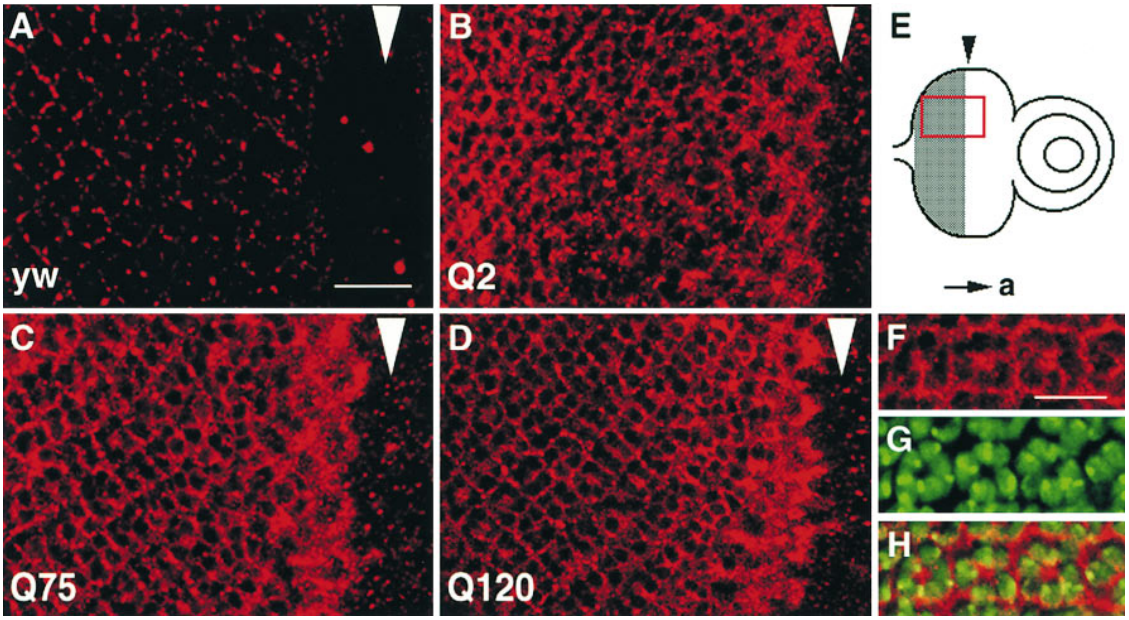

Figure 5. Expression Pattern of Huntingtin Transgene Products in Eye Imaginal Discs of Third Instar Larvae As Assessed by Laser Confocal Scanning Microscopy

(A) through (D), (F), and (H) show staining with anti-huntingtin antibody (Ab1).
(A) *wt*.
(B) Q2.
(C) Q75.
(D) Q120.
All huntingtin transgenes show strong expression posterior to the morphogenetic furrow (arrowhead), consistent with the expected pattern for pGMR-driven expression. A low level of Ab1 cross-reactivity is observed in *wt*.
(E) Schematic representation of the eye-antennal disc indicates the approximate field and orientation of (A) through (D). a, anterior.
(F–H) High magnification views showing clusters of photoreceptor neurons in Q120 approximately three to five rows posterior to the morphogenetic furrow. In these panels, anterior is up. Red, Ab1 immunostaining (F); green, DAPI-stained nuclei (G); merged image (H). Comparison of (F) to (H) indicates that huntingtin is predominantly cytoplasmic in photoreceptor neurons. Similar cytoplasmic staining was observed in Q2 and Q75 imaginal discs (not shown).
Genotypes: *wt*, *yw*; Q2, *yw*;pGMR-huntingtin-Q2/pGMR-huntingtin-Q2; Q75, *yw*;pGMR-huntingtin-Q75/pGMR-huntingtin-Q75; and Q120, *yw*;pGMR-huntingtin-Q120/pGMR-huntingtin-Q120. Scale bars, 5 μm (A–D) and 3 μm (F–H).

with expanded repeats is initially cytoplasmic, the protein relocalizes to the nucleus prior to neuronal death. Degeneration induced by huntingtin transgenes shares features of apoptosis.

Polyglutamine-Containing Huntingtin Fragments Induce Neuronal Degeneration

Fragments of human huntingtin containing expanded repeats were expressed at high levels in photoreceptor neurons. Expression was induced just prior to neuronal differentiation and was continuous into adulthood. Despite this early expression, photoreceptor neurons appeared to differentiate normally as assessed at eclosion using transmission electron microscopy. In the majority of HD cases, disease onset occurs during adulthood, at a mean age of about 40 years (Gusella and MacDonald, 1995). Bates and colleagues have previously described a mouse model upon which our studies were based (Mangiarini et al., 1996; Davies et al., 1997). They demonstrated that an amino-terminal fragment of huntingtin containing 150 glutamines, expressed under the control of the endogenous human promoter, induces a neurological phenotype with some similarities to HD, though this phenotype was not reported to be associated with neuronal pathology. A striking phenotype in mice expressing polyglutamine-expanded proteins in mouse was first described by Orr, Zoghbi, and colleagues (Burright et al., 1995). They demonstrated that the ataxin-1 protein containing 82 glutamine repeats driven by a Purkinje cell promoter induced ataxia and Purkinje cell neuronal degeneration. The onset of behavioral defects occurred within the first month; by 2 months, neuronal degeneration was observed. Similar findings were subsequently reported in a mouse model expressing expanded repeats within the MJD protein (Ikeda et al., 1996).

Although most polyglutamine repeat–containing disease proteins are widely expressed within the nervous system, cell death occurs preferentially in selected subsets of neurons characteristic of each disease. In HD, for example, initial neuronal loss occurs within the striatum. While the basis for this selectivity is not understood, more widespread pathology is observed outside the striatum in advanced stages of HD. This suggests that neurons may show a graded sensitivity to polyglutamines in the context of different proteins. The ability of huntingtin containing expanded repeats to induce cell death in invertebrate neurons raises the intriguing prospect that

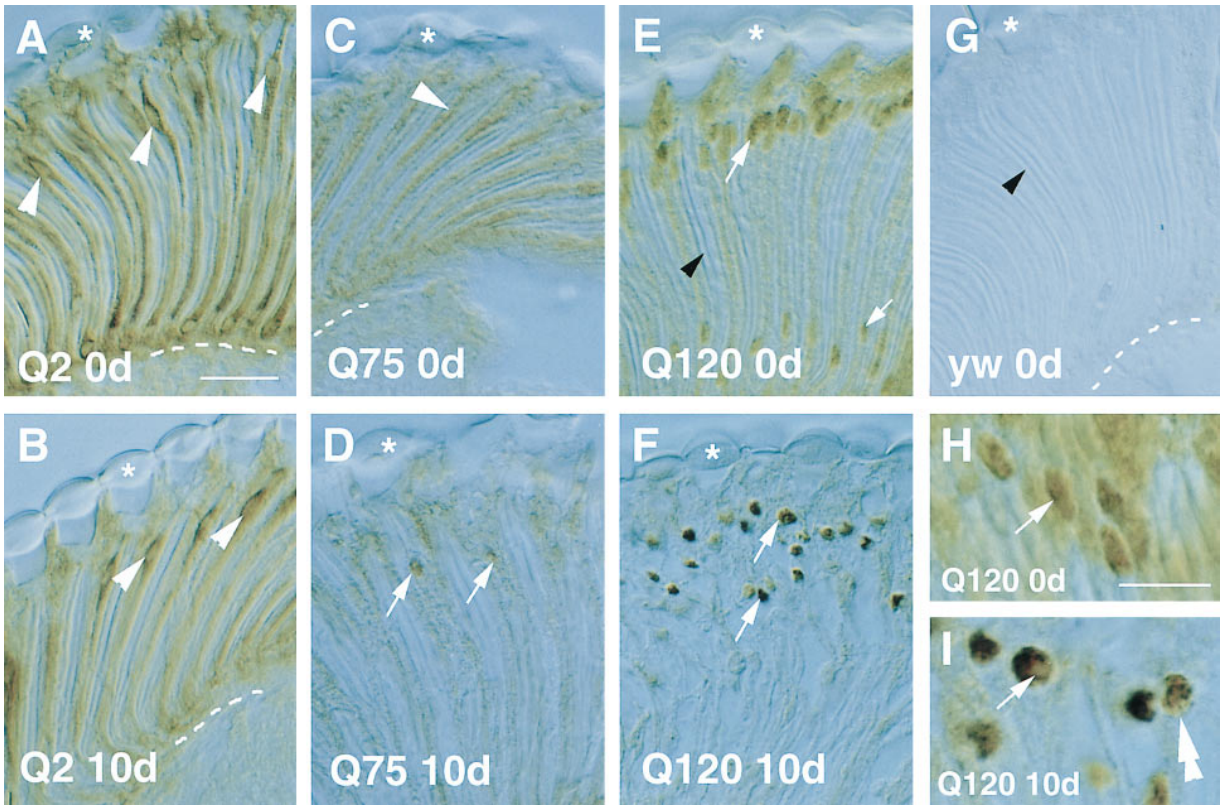

Figure 6. Expression Pattern of Huntingtin in the Adult Retina

Immunolabeling was performed on cryostat sections using Ab1 antibody and visualized with HRP-coupled secondary antibody and diaminobenzidine. Horizontal sections reveal the longitudinal profiles of photoreceptor neurons. These are long, cylindrically shaped cells that extend throughout the thickness of the compound eye. Asterisks indicate the position of lenses. The dashed line demarcates the basal boundary between the retina and the underlying brain. The nuclei of photoreceptors 1–7 are located in the distal region of the eye (i.e., toward the lens), and the R8 nucleus is located more proximally.

(A and B) Q2 ommatidia show strong immunoreactivity at eclosion (0d) and after 10 days (10d). The protein is localized in the cytoplasm of photoreceptor neurons and is excluded from nuclei (arrowheads). Staining at 0 days and 10 days is indistinguishable.

(C and D) In Q75 ommatidia, huntingtin protein is predominantly detected in the cytoplasm at 0 days (arrowhead) but is found in some nuclei at 10 days (arrows).

(E and F) At 0 days, huntingtin immunoreactivity in Q120 photoreceptor neurons is localized in the cytoplasm and nuclei of photoreceptor neurons (arrows). The black arrowhead shows nonimmunoreactive rhabdomeres visible by Normarski optics. Rhabdomeres extend the entire length of one side of each photoreceptor neuron. At 10 days, the protein is largely nuclear (arrows). The morphology of the retina is disrupted, reflecting degeneration of photoreceptor neurons.

(G) Little, if any, cross-immunoreactivity was detected in *wt* eyes. Black arrowhead, rhabdomere.

(H and I) High magnification views of nuclei in Q120 photoreceptor neurons. Huntingtin immunoreactivity is evenly distributed throughout nuclei at 0 days (arrow). At 10 days, the protein is highly concentrated in the nucleus, but it is excluded from the nucleolus (arrow). In some nuclei, the distribution of the protein has a more punctate appearance with multiple spots of high immunoreactivity (double arrowhead). Eyes from 7 to 18 flies were analyzed for all genotypes at both time points. Genotypes: *wt*, *yw*; Q2, *yw*;pGMR-huntingtin-Q2/CyO; Q75, *yw*;pGMR-huntingtin-Q75/CyO; and Q120, *yw*;pGMR-huntingtin-Q120/CyO. Scale bars, 20 μm (A–G) and 10 μm (H and I).

these proteins disrupt an evolutionarily conserved cellular process.

Increased Length of Polyglutamine Tracts Correlates with Nuclear Localization and Age of Onset of Neuronal Degeneration

The age of onset of HD is broadly correlated with the length of triplet repeats: juvenile onset patients encode proteins with repeat lengths greater than ~70, whereas very late adult onset correlates with repeats generally shorter than 40. For any given repeat length, however, age of onset may vary by as much as 18 years (Gusella and MacDonald, 1995). In our study, the age of onset also correlated with repeat length. Most photoreceptor neurons expressing Q120 degenerated within the first 10 days posteclosion, whereas only a small fraction of photoreceptor neurons expressing Q75 showed signs of degeneration at 10 days. Indeed, even at 30 days posteclosion, degeneration was relatively mild in Q75 flies.

The expression and subcellular distribution of the huntingtin transgenes was assessed in developing photoreceptor neurons in the larval eye primordium (i.e., the eye disc), some 5–6 days later at eclosion, and 10 days posteclosion. While Q2, Q75, and Q120 proteins are cytoplasmic in the developing photoreceptor neurons in the eye disc, a progressive nuclear accumulation of Q75 and Q120 protein ensues. At eclosion, Q75 remained largely cytoplasmic, whereas Q120 appeared evenly distributed between the nucleus and cytoplasm.

By 10 days, Q75 was observed in the nuclei of scattered photoreceptor neurons, and Q120 was predominantly nuclear. While Q120 was uniformly distributed within nuclei at eclosion, multiple small aggregates giving a punctate appearance were seen in many nuclei at 10 days. Hence, nuclear accumulation of repeat-containing transgenes correlated with cellular toxicity.

Nuclear accumulation of glutamine repeat–containing huntingtin has been seen in humans, mice, and cultured cells. While huntingtin in post mortem tissue from normal individuals is diffusely localized in the cytoplasm, huntingtin immunoreactivity in post mortem tissue of HD individuals is localized in part to the nuclei of striatal and cortical neurons (Roizin et al., 1979; Hoogeveen et al., 1993; DiFiglia et al., 1995, 1997; Sapp et al., 1997). Nuclear staining is highly distinctive and characterized by a single large intranuclear inclusion, although in some juvenile cases two inclusions are observed (DiFiglia et al., 1997). Furthermore, nuclear inclusions are more frequent in juvenile cases with longer repeat lengths than in adult HD tissue. In the mouse model, some one in five neurons contain a single large inclusion, which appears electron hypodense and excludes chromatin as visualized by electron microscopy (Scherzinger et al., 1997). These inclusions are not seen in newborn mice but accumulate prior to the onset of neurological symptoms. As in the fly model, uniform nuclear localization is seen first, followed by the formation of nuclear inclusions. The formation of these inclusions may be due to effects of the polyglutamine repeats. This is supported by the finding that chimeric proteins containing expanded repeats within hypoxanthine–guanine phosphoribosyl tranferase protein induce similar intranuclear inclusions in transgenic mice (Ordway et al., 1997). Nuclei containing multiple inclusions have been reported in cultured cells expressing amino-terminal fragments of polyglutamine-expanded huntingtin (Cooper et al., 1998). In vitro models for other triplet repeat diseases have also demonstrated preferential nuclear localization of expanded repeat–containing proteins (Paulson et al., 1997; Hackam et al., 1998a, 1998b; Igarashi et al., 1998). Nevertheless, despite the striking correlation between nuclear localization of glutamine repeat–containing proteins and neuronal degeneration, a causal relationship between them has yet to be established.

Photoreceptor Cell Death Associated With Polyglutamine-Containing Huntingtin Shares Features with Apoptosis

Cell death induced by Q120 shares morphological features with apoptosis. These include increased affinity for toluidine blue and osmium, shrinkage of both the cell body and nucleus, and chromatin condensation at the nuclear periphery. Other cell organelles, in particular mitochondria, remained intact and morphologically normal. In addition, dying cells were observed adjacent to unaffected cells. Features commonly seen in apoptotic cells, but not observed in dying cells in Q120 lines, include nuclear fragmentation and phagocytosis by adjacent cells. Nevertheless, the properties of dying cells in Q120 were clearly distinct from features associated with necrosis. Necrosis affects large groups of cells, and necrotic cells typically swell, as do their nuclei and mitochondria (Kerr et al., 1972; Wyllie et al., 1980).

Despite the inherent difficulties in analyzing cell death in post mortem tissues, TUNEL staining and DNA fragmentation observed in HD brains provide some support for the view that death of neurons occurs through an apoptotic pathway (Dragunow et al., 1995; Portera-Cailliau et al., 1995). Although the mouse model described by Bates and colleagues (Mangiarini et al., 1996; Davies et al., 1997) shows a neurological phenotype as well as nuclear inclusions, a description of dying cells has not yet been published. In cell culture, huntingtin with expanded repeats does not induce cell death unless cells are "sensitized" by drugs such as staurosporin, tamoxifen, or camptothecin (Goldberg et al., 1996b; Cooper et al., 1998; Martindale et al., 1998). In contrast, cell death has been reported in cell culture models for DRPLA and MJD (Ikeda, 1996; Paulson et al., 1997; Igarashi et al., 1998).

Comparison between Fly Models for Machado-Joseph Disease and Huntington's Disease

Recently, Bonini and coworkers reported a fly model for MJD, another glutamine repeat disease (Warrick et al., 1998). Similarities include repeat length–dependent degeneration and progressive nuclear accumulation of expanded repeat–containing proteins. While both studies report degeneration of retinal tissue, there are several differences. These include the time course of degeneration and differential effects of P35 rescue on degeneration.

Warrick et al. (1998) expressed a carboxy-terminal fragment of MJD with either 27 or 78 repeats in photoreceptors using a bipartite expression system in which the strong eye-specific expression vector pGMR drives the yeast transcription factor GAL4, which in turn drives the MJD transgenes containing GAL4 upstream activating sequences. Whereas both MJD-Q23 and MJD-Q78 (i.e., MJD protein fragments containing 23 and 78 glutamines) were expressed initially in the cytoplasm, MJD-Q78 but not MJD-Q27 was rapidly localized to nuclear inclusions in photoreceptor cells within the developing eye disc. Retinal degeneration occurred during mid-pupal development. In contrast, we observed nuclear localization and degeneration in fully developed adult photoreceptor neurons. This discrepancy may reflect different levels of expression achieved in the two systems. We used direct fusion of GMR to huntingtin fragments, whereas Warrick et al. (1998) included an additional amplification step through the use of the bipartite GAL4 system. That different levels of repeat-containing proteins may lead to earlier onset is supported by our finding that neuronal degeneration is observed at eclosion by increasing the dosage of Q120 from one to two copies (data not shown).

We have not observed a protective effect of coexpressing the antiapoptotic protein P35 with expanded glutamine–containing proteins. In contrast, Warrick et al. (1998) present data demonstrating that a weak depigmentation phenotype (presumably reflecting death or abnormal development of pigment cells) is partially rescued by coexpression of P35. There are several possible explanations for this discrepancy. First, it may reflect differences between the MJD and huntingtin fragments to which the repeats are fused. Second, the higher levels

of P35 achieved in the bipartite system used by Warrick et al. (1998) may be sufficient for rescue. Third, P35 may rescue developmentally induced pigment cell death occurring during pupal development but not neuronal cell death in adult tissue. It is important to emphasize, however, that neither we nor Warrick et al. (1998) demonstrate a protective effect of P35 on neuronal degeneration induced by polyglutamine-containing proteins.

Concluding Remarks

Fly models for HD and MJD provide powerful genetic systems for dissecting the neuronal degeneration induced by glutamine-containing proteins. The fly is accessible to genetic modifier screens based on both gain-of-function and loss-of-function genetic strategies. These approaches may reveal potential targets for therapeutic intervention.

Experimental Procedures

Molecular Biology

Fragments of huntingtin cDNA were subcloned as blunt-ended NcoI-XhoI fragments into the HpaI-StuI sites of pGMR in the case of Q2 and Q75. Q120 was blunt ended and subcloned into the HpaI site. Each construct contained the first 17 amino acids of huntingtin; a contiguous stretch of 2, 75, or 120 glutamines; an additional 125 amino acids from huntingtin; and different carboxyl termini due to variations in the portion of the parental vector hsp 70 3′ region included prior to the stop codon. The precise amino acid sequences are available upon request.

Germline instability of CAG tract length using PCR amplification of whole body *Drosophila* genomic DNA preparations was assessed by modification of a previously described method (Leeflang et al., 1995). Individual flies were homogenized in 20–40 μl of TE buffer containing 50 mg proteinase K/ml. Samples were then digested at 55°C for 4 hr and denatured at 95°C for 10 min. One round of PCR was applied to 1 μl of sample in a 25 μl reaction volume, using primers IT1 and IT4 with 40 thermal cycles.

Genetics

Standard genetic markers and chromosomes were used as described in Lindsley and Zimm (1992). *wt* flies were the Oregon R strain. We screened nine of the Q2 and eight of the Q75 lines. For Q120, nine lines were examined by the pseudopupil technique. Of these, six lines were selected for further analysis.

Histology

Third instar eye imaginal discs were dissected and fixed as described previously (Van Vactor et al., 1991). For immunostaining, whole mount eye discs were incubated with the polyclonal antiserum Ab1 at 2 μg/ml (generously provided by Dr. Marian DiFiglia). As secondary antibody, Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch; 1:500) was used. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Vector Labs). Samples were viewed on a Bio-Rad confocal laser scanning microscope. Antibody staining of adult eyes (fixed in 4% paraformaldehyde in 0.1 M phosphate buffer) was performed on serial 10 μm thick horizontal cryostat sections; goat anti-rabbit horseradish peroxidase–coupled IgG (Bio-Rad, 1:200) was used as secondary antibody. Toluidine blue–stained 2 μm thick semithin sections for light microsopy and ultrathin sections (~60 nm) for transmission electron microscopy (Zeiss EM 10) were prepared as described previously (Salecker and Boeckh, 1995; Garrity et al., 1996). Details of the protocols are available upon request.

Acknowledgments

The authors thank Drs. B. Hay and H. Steller for providing fly stocks and Dr. M. DiFiglia for providing Ab1. We thank D. A. Gunning and Dr. K. L. Schulze for their expert assistance and K. Ronan for assistance in manuscript preparation. We also thank Dr. R. C. Collins for his support of this project. This work was supported in part by a Jeanne B. Kempner Memorial Scholarship, a Dorothy and Leonard Straus Scholarship in Neuroscience, the Cure HD Initiative (G. R. J.), the Deutsche Forschungsgemeinschaft (I. S.), a Human Frontier Science Program fellowship (P. W. F.), and National Institutes of Health grants NS32765 and NS16367 (M. E. M.). S. L. Z. is an Investigator of the Howard Hughes Medical Institute.

Received August 6, 1998; revised August 14, 1998.

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