Mutant huntingtin aggregates do not sensitize cells to apoptotic stressors

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Received 5 February 2002; accepted 8 February 2002

First published online 25 February 2002

Edited by Jesus Avila

Abstract It has been postulated that neuronal inclusions composed of mutant huntingtin may play a causative role in the pathogenesis of Huntington's disease. To study the putative role of aggregates in modulating apoptotic vulnerability, SH-SY5Y cell lines stably expressing truncated huntingtin with 18 (wild-type) (N63–18Q) or 82 (mutant) (N63–82Q) glutamine repeats were established. Aggregates were observed in ~13% of the N63–82Q cells; no aggregates were observed in the N63–18Q cells. In response to apoptotic stimuli such as staurosporine or hyperosmotic stress, caspase-3 activity was significantly greater in the N63–82Q cells compared to the N63–18Q cells. However, double immunostaining for huntingtin and active caspase-3 revealed that the presence of aggregates did not correlate with the presence of active caspase-3, indicating that aggregates do not contribute to the increase in apoptosis in the N63–82Q cells.

Key words: Huntington's disease; Inclusion; Apoptosis; Caspase-3

1. Introduction

While an abnormal expansion of the polyglutamine sequence in the N-terminal region of huntingtin causes Huntington's disease (HD), the pathogenic mechanisms involved have not been fully elucidated. A possible clue to the pathogenesis of HD came with the discovery of neuronal inclusions composed of mutant huntingtin in HD brain [1–3], as well as in transgenic mice [4,5] and transfected cell models [6–8]. These findings led to the hypothesis that the etiology of HD was at least in part due to the formation of these inclusions [9,10], perhaps due to the sequestration of certain cell survival proteins into the aggregates [11,12]. In support of this hypothesis, the formation of polyglutamine aggregates appeared to increase susceptibility to cell death following apoptotic stimuli [13,14]. However, it has also been suggested that although a conformational change in mutant huntingtin due to the polyglutamine expansion may cause both aggregation and pathogenicity, they occur separately and independently [15–17]. Consistent with this view, studies that uncouple polyglutamine-induced aggregate formation and toxicity have been published [16,17]. In a YAC transgenic mouse model of HD, expression of high levels of mutant huntingtin results in neuropathology and inclusions. Interestingly, lower levels of mutant huntingtin expression also produced HD-like neuropathology but in the absence of inclusions, even at the electron microscopic level [18]. Further, in vitro cell models have shown a reduction of polyglutamine aggregate formation results in greater cell death [15,17,19], suggesting that rather than being pathogenic, the inclusions may actually be protective [15,17].

There is a growing body of evidence that apoptotic processes contribute to the neuronal loss in HD brain. For example, treatment of mice that overexpress a mutant huntingtin transgene with minocycline, which inhibits both caspases-1 and -3, delays the death of the animals [20]. Lymphoblasts from HD patients show increased caspase-9 and caspase-3 activation in response to the apoptotic stressor staurosporine, compared to lymphoblasts from controls [21]. Further, in cell culture models inhibition of caspase cleavage of mutant huntingtin reduces toxicity [14,22]. These and other studies indicate the likely involvement of apoptosis or apoptotic-like processes in the neurodegeneration observed in HD. However, the relationship between the presence of neuronal aggregates and sensitivity to apoptotic stimuli in HD remains unclear.

To examine the relationship between mutant huntingtin aggregates and apoptotic cell death, human neuroblastoma SH-SY5Y cells that stably express N-terminal fragments (N63) of wild-type (N63–18Q) or mutant huntingtin (N63–82Q) protein were established. In this study, we demonstrate that the expression of mutant truncated huntingtin facilitates staurosporine and hyperosmotic stress-induced apoptosis, however, the presence of mutant huntingtin aggregates does not correlate with the presence of active caspase-3. These results suggest that truncated mutant huntingtin sensitizes cells to apoptotic stimuli independently of aggregate formation.

2. Materials and methods

Human SH-SY5Y neuroblastoma cell lines stably expressing myc-tagged N63 wild-type (N63–18Q) and N63 mutant (N63–82Q) huntingtin have been described previously [23]. All experiments were carried out on sub-confluent cultures. Under these conditions, the frequency of cells bearing aggregates was ~13% in N63–82Q cells; no aggregates were observed in the N63–18Q cells.

To measure caspase-3 activity, cells were grown on 60 mm dishes and then incubated with 0.5 M sorbitol for the indicated time periods, or with staurosporine at the indicated concentrations for 3 h. At the appropriate time, cells were harvested in cold phosphate-buffered saline (PBS), collected by centrifugation, resuspended in a homogenizing buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml concentration each of aprotinin, leupeptin, and pepstatin) and sonicated on ice. Protein concentrations were determined using the BCA assay

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Abbreviations: HD, Huntington's disease

0014-5793/02/$22.00
PII: S0014-5793(02)02436-5

(Pierce). Caspase-3 activity was measured in the cell lysates using a fluorometric assay as described previously [24].

For immunoblotting cells were incubated with 0.5 M sorbitol for the indicated time periods, harvested in cold PBS, collected by centrifugation, resuspended in a homogenizing buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml concentration each of aprotinin, leupeptin, and pepstatin) and sonicated on ice. Protein concentrations of the homogenates were determined using the BCA method (Pierce) and diluted to a final concentration of 2 μg/μl with 2× reducing stop buffer (0.25 M Tris–HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, 10% glycerol and bromophenol blue as the tracking dye). Samples (20 μg of protein) were resolved on SDS–polyacrylamide gels, and transferred to nitrocellulose. Blots were blocked in 5% non-fat dry milk in TBST (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) and probed for active caspase-3 (1:500) (Cell Signaling Technology) or the classic caspase-3 substrate poly ADP-ribose polymerase (PARP) (1:2500) (Pharmingen) in the same buffer overnight at 4°C. The membranes were then washed and probed with appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The membranes were then rinsed and developed with the enhanced chemiluminescence method (ECL, Amersham).

For immunocytochemistry cells were seeded on poly-D-lysine-coated cover slips in 24-well plates. After treatment cells were fixed and stained with a monoclonal C-myc antibody (1:200, Zymed) and Texas Red-conjugated anti-mouse IgG (1:200, Jackson Labs) as described previously [23]. To determine the co-localization between huntingtin aggregates and caspase-3 activation, cells were stained with the polyclonal active caspase-3 antibody (1:100, Cell Signaling Technology) and the monoclonal C-myc antibody (1:200), followed by incubation with Texas Red-conjugated anti-mouse IgG (1:100) for huntingtin and FITC-conjugated anti-rabbit IgG (1:200, Jackson Labs) for active caspase-3. Nuclei were counterstained with Hoechst. Cells were viewed using confocal microscopy (Leica) and images were acquired by sequential scanning at the appropriate wavelengths. The cellular distribution of N63–82Q aggregates and caspase-3 activation was assessed by random sweeps at 10× (Nikon). Each cell in a field was scored as having either diffuse or aggregated huntingtin immunoreactivity, and as either negative or positive for active caspase-3 immunostaining. Data are from three independent experiments and values are expressed as mean ± S.E.M.; ~6000 cells were counted for each experiment. Statistical comparisons were made by Student's *t*-test; $P < 0.05$ was considered significant.

3. Results

As previously reported [7,23,25,26], mutant truncated huntingtin (N63–82Q) forms SDS-insoluble aggregates (Fig. 1), which are ubiquitinated and co-localize with heat shock proteins [23], whereas wild-type truncated huntingtin (N63–18Q) is diffuse in cytoplasm with no visible aggregates (Fig. 1). The aggregates in the N63–82Q cells are both nuclear and cytoplasmic, although the majority of the aggregates are found in the cytoplasm. Under basal conditions 13% of the cells have aggregates, and 2% of the N63–82Q cells have nuclear aggregates [23].

Although the level of total caspase-3 expression is not significantly different between the two cell lines, and basal caspase-3 activity is low and equivalent in both cell lines (data not shown), mutant truncated huntingtin expressing cells showed a significantly greater increase in caspase-3 activity compared to wild-type huntingtin expressing cells, when the cells were exposed to apoptotic stressors such as staurosporine (Fig. 2A) or 0.5 M sorbitol (Fig. 2B). Further, the increase in caspase-3 activity in response to staurosporine or 0.5 M sorbitol treatment was concentration- or time-dependent (Fig. 2), respectively. Caspase-3 exists as a pro-enzyme. Cleavage of the pro-form of caspase-3 by activating caspases such as caspases-8 or -9 converts caspase-3 to its active form [27,28]. Immunoblot analysis revealed that the appearance of active (cleaved) caspase-3 occurred earlier in the N63–82Q cells compared to the N63–18Q cells in response to sorbitol stress (Fig. 3). In response to sorbitol stress there was also an earlier increase in the appearance of the 85 kDa PARP proteolytic product, which is a classic indicator of caspase-3 activation, in the mutant truncated huntingtin expressing cells compared to wild-type huntingtin expressing cells (Fig. 3). Further, in the N63–82Q cells no intact PARP was detectable after 60 min of sorbitol stress, while in the N63–18Q cells intact PARP was still present after 120 min of hyperosmotic stress (Fig. 3).

Although it was clear that cells expressing N63–82Q were significantly more sensitive to apoptotic stressors, the relationship between the presence of aggregates in a cell and caspase-3 activation needed to be established. Therefore co-immunofluorescence studies that examined the presence of active caspase-3 and aggregates were carried out. Incubation of N63–82Q cells with increasing concentrations of staurosporine increased the number of active caspase-3 positive cells accordingly. However, the presence of aggregates did not 'predispose' the cells to caspase-3 activation, in that the percentage of aggregate-bearing and non-aggregate-bearing N63–82Q cells that were positive for active caspase-3 was the same (Fig. 4A). These findings suggest that mutant huntingtin aggregates do not facilitate staurosporine-induced apoptosis in this cell model. Similar results were obtained with hyperosmotic stress, in that active caspase-3 was not preferentially located in aggregate-bearing cells (Fig. 4B).

4. Discussion

In this study, stress-induced apoptotic cell death (caspase-3 activation) in cells stably expressing N63–82Q was significantly greater than in cells stably expressing N63–18Q. However, in the absence of stressors, the two cell lines did not differ in viability [23]. In support of these results, selective vulnerability of mutant huntingtin expressing cells to stressors has been reported in lymphoblasts derived from HD patients [21] and in transient transfection studies [7]. Indeed, in transient transfection studies using N2A cells, expression of N171–82Q sensitized cells to staurosporine-induced cell death compared to N171–18Q expression. In these studies ~38% of the cells expressing mutant truncated huntingtin were dead after a 4 h treatment with 0.5 μM staurosporine, while only ~19% of the cells expressing wild-type truncated huntingtin were no longer viable [7]. These studies clearly indicate that expression of mutant huntingtin can sensitize cells to apoptotic stressors. Although the N-terminal fragment of huntingtin used in this study (N63–82Q) has no caspase cleavage sites [22], it may enhance susceptibility to stress-induced apoptotic cell death. For example, expressing a dominant negative caspase-1 in the R6/2 mice (which express exon 1 of huntingtin with ~150 glutamines and develop a pronounced neurological phenotype) delays disease progression [29]. In addition, treatment with minocycline inhibits caspase-1 and -3 expression in the R6/2 mice and delays mortality [20]. The huntingtin construct expressed in the R6/2 mice is approximately the same fragment used in the present study, and thus contains no caspase cleavage sites [5,7,22]. Indeed, in the current study both caspase-8 and caspase-9, as well as caspase-3 were activated to a greater extent in N63–82Q cells compared to N63–18Q cells in response to staurosporine or hyperosmotic stress

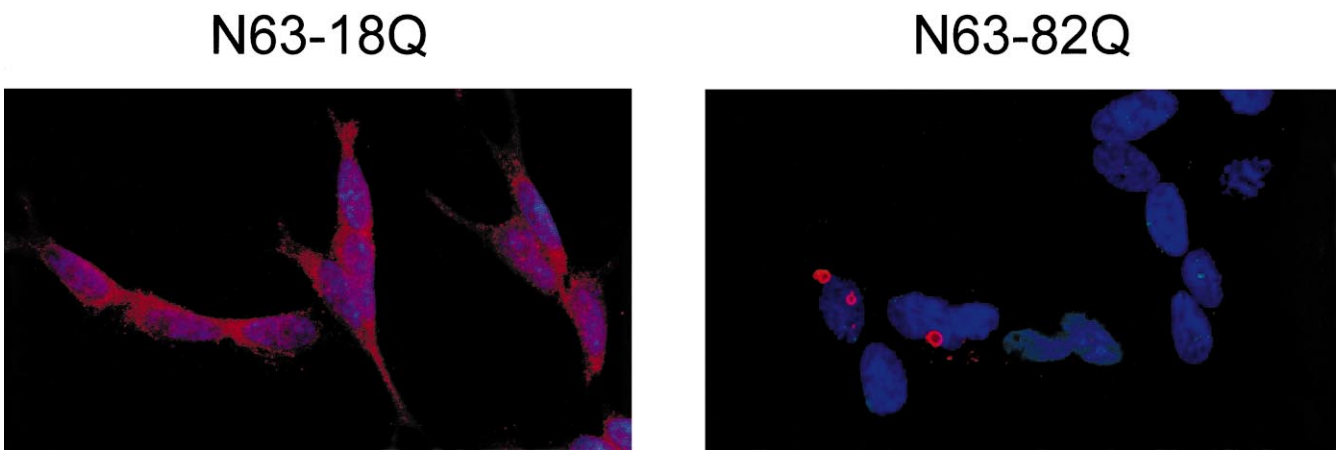

Fig. 1. Huntingtin immunostaining in cells stably transfected with N63–18Q or N63–82Q. Huntingtin was detected with the C-myc monoclonal antibody and nuclei were counterstained with Hoechst. In the N63–18Q cells, huntingtin immunostaining was diffuse throughout the cytoplasm. In contrast, in the N63–82Q cells, huntingtin formed aggregates in cells. Although diffuse mutant huntingtin immunostaining was also present in N63–82Q cells, the intense immunostaining of the aggregates prevented the capture of images showing both aggregates and diffuse staining in the same field.

(data not shown). It has also been demonstrated that the cytotoxicity induced by mutant huntingtin can be inhibited by the anti-apoptotic protein Bcl-X1 and caspase inhibitors in primary striatal neurons [17]. These results suggest that highly truncated mutant huntingtin sensitizes cells to specific stressors resulting in apoptotic cell death.

Although aggregate formation of mutant huntingtin is a characteristic hallmark of the disease [1,4,26], the pathological role of aggregates is still highly controversial. It is not clear whether these aggregates play a causative, protective or just an epiphenomenal role. Originally it was suggested that aggregates cause neurodegeneration [9,30–32]. However, further studies suggested that the conformational change in the protein due to the polyglutamine expansion may result in both aggregation and pathogenicity of the mutant huntingtin, but they are separate and independent events [16–18]. Further several studies have presented data suggesting that the inclusions may actually play a protective role [15,17,19]. In the present study, we found that in response to apoptotic stressors, the percentage of non-aggregate-bearing cells and aggre-

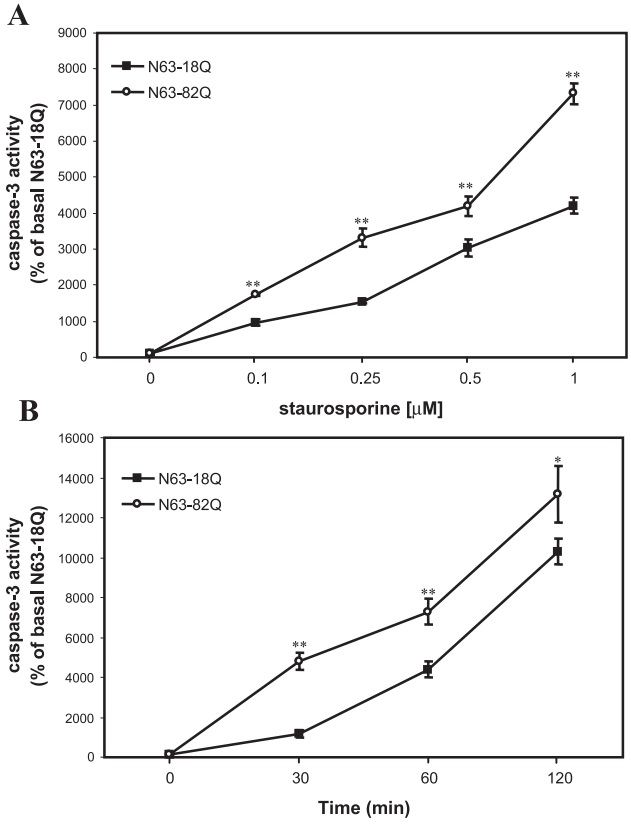

Fig. 2. Potentiation of apoptotic death induced by staurosporine and hyperosmotic stress in mutant huntingtin expressing cells. Cells were incubated with 0.1, 0.25, 0.5, or 1.0 μM staurosporine for 3 h (A) or with 0.5 M sorbitol for 0–120 min (B) and caspase-3 activity was measured. Values are expressed as a percent of basal caspase-3 activity of N63–18Q cells. Data represent three independent experiments and values were expressed as mean percent ± S.E.M.; $^{*}P < 0.05$, $^{**}P < 0.01$.

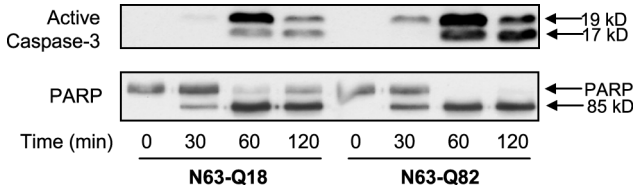

Fig. 3. Caspase-3 activation and PARP cleavage in response to hyperosmotic stress occurs earlier in N63–82Q cells compared to N63–18Q cells. Cells were incubated with 0.5 M sorbitol for 0–120 min, samples were collected at the times indicated and probed for active caspase-3, or the caspase-3 substrate PARP. No active caspase-3 is present in either cell line prior to treatment (0 time). However, in the N62–82Q cells active caspase is present 30 min after sorbitol stress, while in the N63–18Q cells the active caspase-3 is not detected until 60 min after the initiation of treatment. Further the increase in active caspase-3 is more robust in the N63–82Q cells compared to the N63–18Q cells. Both the 17 kDa and 19 kDa immunoreactive bands are active caspase-3. Although the levels of PARP are equivalent in the two cell lines, there is an earlier and more extensive caspase-3-mediated proteolysis of the intact PARP into the 85 kDa breakdown product in the N63–82Q cells compared to the N63–18Q cells in response to sorbitol treatment.

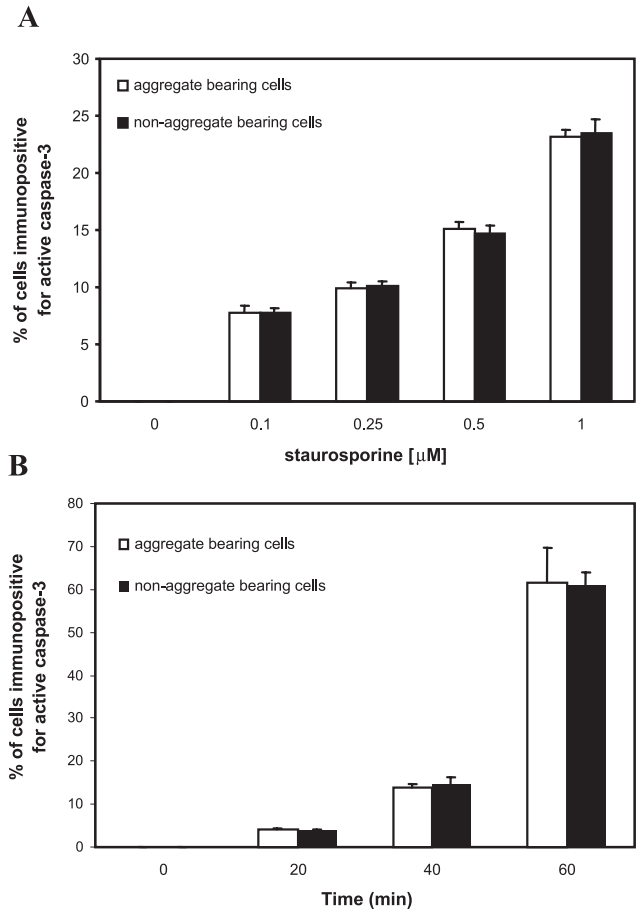

Fig. 4. No significant correlation between the presence of aggregates and caspase-3 activation in N63–82Q cells. N63–82Q cells were incubated with staurosporine for 3 h with the indicated concentrations (A) or 0.5 M sorbitol for the times indicated (B) and double-immunostained for aggregates and active caspase-3. In each field the total number of cells was counted and then each cell was scored as aggregate positive, active caspase-3 positive, aggregate and caspase-3 positive or positive for neither. The percent of active caspase-3 cells with aggregates (white bars), and the percent of active caspase-3 cells without aggregates (black bars) were determined. Data represent three independent experiments and a total of ~6000 cells were counted in each experiment. The data are expressed as mean percent ± S.E.M.

gate-bearing cells that were immunopositive for active caspase-3 was similar. These data suggest that in this model, aggregates neither contribute to the apoptotic vulnerability of cells expressing mutant truncated huntingtin, nor play a protective role. Recent data have suggested that protein aggregation impairs the proteasome system [33], and that polyglutamine aggregates sequester CREB binding protein (CBP), an essential transcription co-activator [11]. These previous findings would suggest that aggregates are toxic to cells, in contrast to what was observed in our model system. However, it is important to note that in the present study we are analyzing the effects of 'macroaggregates' on cell death, i.e. aggregates that can be detected at the light microscopic level. Under basal conditions in the N63–82Q cells only ~13% of the cells have these visible macroaggregates. However, if aggregate formation is a nucleation process as it has been hypothesized [34–36], it is likely that abnormal protein–protein interactions are occurring in almost all the cells expressing the N63–82Q construct. These abnormal interactions of N63–82Q with itself and other proteins may be sufficient to impair proteasome function and/or interfere CBP-mediated transcription and thus sensitize the cells to apoptotic stressors [11,12,33]. This would explain why the entire population of N63–82Q cells was more sensitive than the N63–18Q cells to apoptotic stimuli, independent of the presence of visible aggregates. Further, lymphoblasts from HD patients (which do not contain aggregates) show increased apoptotic cell death compared to lymphoblasts from control subjects. HD lymphoblasts also exhibited a significant increase in mitochondrial depolarization in response to stressors compared to controls, which correlated with increased glutamine repeats [21]. These findings would also suggest that mutant polyglutamine containing proteins might facilitate the apoptotic process independent of the formation of visible aggregates.

Acknowledgements: This work was supported by NIH Grant AG12396.

References

[1] DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Science 277, 1990–1993.
[2] Gutekunst, C.A. et al. (1999) J. Neurosci. 19, 2522–2534.
[3] Becher, M.W., Kotzuk, J.A., Sharp, A.H., Davies, S.W., Bates, G.P., Price, D.L. and Ross, C.A. (1998) Neurobiol. Dis. 4, 387–397.
[4] Davies, S.W. et al. (1997) Cell 90, 537–548.
[5] Mangiarini, L. et al. (1996) Cell 87, 493–506.
[6] Hackam, A.S., Singaraja, R., Zhang, T., Gan, L. and Hayden, M.R. (1999) Hum. Mol. Genet. 8, 25–33.
[7] Cooper, J.K. et al. (1998) Hum. Mol. Genet. 7, 783–790.
[8] Persichetti, F., Trettel, F., Huang, C.C., Fraefel, C., Timmers, H.T., Gusella, J.F. and MacDonald, M.E. (1999) Neurobiol. Dis. 6, 364–375.
[9] Ross, C.A. (1997) Neuron 19, 1147–1150.
[10] Davies, S.W., Beardsall, K., Turmaine, M., DiFiglia, M., Aronin, N. and Bates, G.P. (1998) Lancet 351, 131–133.
[11] Nucifora Jr., F.C. et al. (2001) Science 291, 2423–2428.
[12] Steffan, J.S. et al. (2000) Proc. Natl. Acad. Sci. USA 97, 6763–6768.
[13] Hackam, A.S., Singaraja, R., Wellington, C.L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M. and Hayden, M.R. (1998) J. Cell Biol. 141, 1097–1105.
[14] Wellington, C.L. et al. (2000) J. Biol. Chem. 275, 19831–19838.
[15] Klement, I.A., Skinner, P.J., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (1998) Cell 95, 41–53.
[16] Kim, M. et al. (1999) J. Neurosci. 19, 964–973.
[17] Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Cell 95, 55–66.
[18] Leavitt, B.R., Wellington, C.L. and Hayden, M.R. (1999) Semin. Neurol. 19, 385–395.
[19] Cummings, C.J. et al. (1999) Neuron 24, 879–892.
[20] Chen, M. et al. (2000) Nat. Med. 6, 797–801.
[21] Sawa, A. et al. (1999) Nat. Med. 5, 1194–1198.
[22] Wellington, C.L. et al. (1998) J. Biol. Chem. 273, 9158–9167.
[23] Chun, W., Lesort, M., Tucholski, J., Ross, C.A. and Johnson, G.V. (2001) J. Cell Biol. 153, 25–34.
[24] Krishnamurthy, P.K., Mays, J.L., Bijur, G.N. and Johnson, G.V. (2000) J. Neurosci. Res. 61, 515–523.
[25] Sieradzan, K.A., Mechan, A.O., Jones, L., Wanker, E.E., Nukina, N. and Mann, D.M. (1999) Exp. Neurol. 156, 92–99.
[26] Lunkes, A. and Mandel, J.L. (1998) Hum. Mol. Genet. 7, 1355–1361.
[27] Green, D.R. (1998) Cell 94, 695–698.
[28] Hengartner, M.O. (2000) Nature 407, 770–776.

[29] Ona, V.O. et al. (1999) Nature 399, 263–267.
[30] Scherzinger, E. et al. (1999) Proc. Natl. Acad. Sci. USA 96, 4604–4609.
[31] Martindale, D. et al. (1998) Nat. Genet. 18, 150–154.
[32] Moulder, K.L., Onodera, O., Burke, J.R., Strittmatter, W.J. and Johnson Jr., E.M. (1999) J. Neurosci. 19, 705–715.
[33] Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Science 292, 1552–1555.
[34] Jarrett, J.T., Berger, E.P. and Lansbury Jr., P.T. (1993) Ann. N.Y. Acad. Sci. 695, 144–148.
[35] Lansbury Jr., P.T. (1997) Neuron 19, 1151–1154.
[36] Wanker, E.E. (2000) Biol. Chem. 381, 937–942.