Loss of normal huntingtin function: new developments in Huntington's disease research

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Huntington's disease is characterized by a loss of brain striatal neurons that occurs as a consequence of an expansion of a CAG repeat in the huntingtin protein. The resulting extended polyglutamine stretch confers a deleterious gain-of-function to the protein. Analysis of the mutant protein has attracted most of the research activity in the field, however re-examination of earlier data and new results on the beneficial functions of normal huntingtin indicate that loss of the normal protein function might actually equally contribute to the pathology. Thus, complete elucidation of the physiological role(s) of huntingtin and its mode of action are essential and could lead to new therapeutic approaches.

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1993 marked a major breakthrough in research into Huntington's disease (HD) with the discovery that the disease-causing mutation is the expansion of a variable stretch of CAG triplets (to more than the maximum 35 repeats normally present) in the first exon of the *HD* gene[1], which encodes a widely expressed 348 kDa cytoplasmic protein named huntingtin[1]. CAG translates into glutamine (gln), and the resulting mutant huntingtin protein is therefore characterized by an aberrant poly-gln expansion. As a consequence of the presence of this expanded protein, the striatal medium-sized spiny neurons undergo selective degeneration accompanied in HD by progressive chorea and dementia[2].

An early key discovery that followed the identification of the gene was that normal huntingtin is widely distributed in the body, with the highest levels in the brain, but in HD patients normal and mutant huntingtin have a similar distribution and expression [3,4]. The current hypothesis is that the damage in HD results from a gained activity as a result of the extended CAG. CAG itself is indeed toxic: expression of CAG alone[5] or in the context of a small part of the huntingtin protein[6,7], or even in a protein with no relevance to HD (Ref. 8) causes neurological symptoms in animals.

Although an expanded CAG repeat could play a major role in the disease, the possibility of subtle dysfunctions of the normal allele in HD cannot be ruled out. Suggestions that loss of normal huntingtin function(s) might contribute to the disease arise from recent work in cells and in mice, in which the absence of normal huntingtin was shown to decrease the survival and phenotypic stability of CNS cells[9–11]. In addition, mutant huntingtin can recruit normal huntingtin into insoluble aggregates both *in vitro* and *in vivo*[12–14], suggesting that the toxic effect of mutant huntingtin might lie in sequestration of normal huntingtin or of its functions. In addition, depletion of full-length endogenous huntingtin is reported in an animal model of HD and not in the controls[15]. Finally, a correlation between the inhibition of the cleavage of endogenous wild-type huntingtin and prolonged survival has been reported[15,16].

In this review we propose that HD pathogenesis results from a combination of both gain and loss of huntingtin function, thereby influencing therapeutic approaches.

Molecular pathology of Huntington's disease

The presence of intranuclear and cytoplasmic aggregates of mutant huntingtin in HD brains and in animal models of the disease has been widely reported[17]. Aggregates have also been found in dystrophic neurites and in the neuropil of postmortem HD brains[18–20]. The role of these aggregates in HD pathogenesis remains controversial (reviewed in Refs 21,22), but recent evidence obtained in mice that have either a mutant full length human huntingtin or a pathological CAG insertion into the mouse *HD* gene, indicates that N-terminal fragments formed from the mutant protein accumulate in the nucleus and neural processes, including in axonal terminals[23,24]. N-terminal huntingtin fragments traverse the nuclear pore to the nucleus from the cytosol[23], and a soluble form of mutant N-terminal fragment interacts with synaptic vesicles inhibiting their glutamate uptake *in vitro*, thus showing toxicity regardless of whether aggregates are formed[24].

One popular hypothesis states that the full-length mutant protein is cleaved by caspases to yield the N-terminal fragment[25–27], and studies in cultured CNS cells show that deletion of the caspase 3 cleavage site in huntingtin reduces toxicity and aggregate formation[28]. Inhibition of caspase 1 also slows disease progression in an HD mouse model[15].

Various authors have shown that transcription factors such as CBP [CREB (cAMP-response element binding protein)-binding protein][14], TATA-binding protein[12] and Sin3a (Ref. 29) can be recruited into the intranuclear aggregates, suggesting a role for

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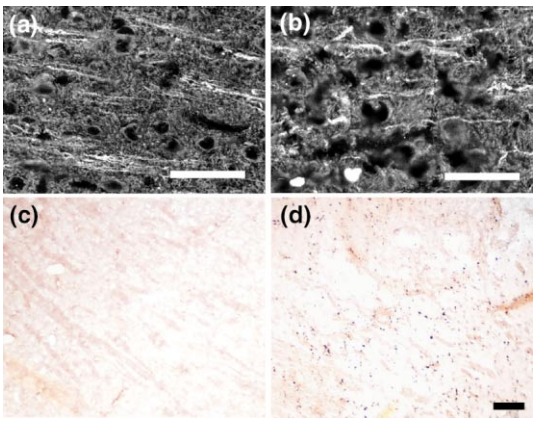

Fig. 1. (a) and (b) Sections from the frontal cortex of a control (a) or 10-month-old mice with a conditional inactivation of wild-type huntingtin selectively (b). Altered MAP2 staining is present in mutant (b) with respect to control (a) mice, indicative of fibres disorganization. (c) and (d) *In situ* end-labeling of DNA nicks on sagittal sections through the striatum of a control (c) and 10-month-old mutant brain shows DNA breaks in the mutant striatum. Reproduced, with permission, from Ref. 11.

transcriptional dysregulation in the pathology. Mutant huntingtin was found to affect the expression level of several genes, including neurotransmitter receptor genes and intracellular signalling proteins (reviewed in Ref. 30). In addition to transcriptional activities, general cellular functioning might also be perturbed by mutant huntingtin via either sequestration into the aggregates of factors that are important for protein turnover (reviewed in Ref. 30) or via blockage of the normal proteosomal apparatus[31]. Finally, it has been reported that mutant huntingtin affects the coupling of neurotransmitter receptors, such as the adenosine A_{2A} receptor subtype, with relevant intracellular transducers[32].

All these effects are clearly indicative of a gained activity of mutant huntingtin, they do not provide clues as to the role of wild-type huntingtin in the same events.

Analyses of the gain-of-function hypothesis in HD: evidence from studies in patients

Most of the compelling evidence against a simple loss of molecular function in HD is derived from the genetic studies and from the fact that deletion of one huntingtin allele (in the Wolf–Hirschhorn syndrome) does not result in HD (Ref. 33). Thus, one mutant allele is necessary for disease manifestation, and heterozygous HD patients exhibit the full spectrum of phenotypes. However, neither of these pieces of evidence excludes a contribution to the disease from the loss of normal huntingtin.

Other earlier evidence indicated that homozygous HD is clinically indistinguishable from the heterozygous condition[34–36]. If this is true, that is, that the normal allele in a heterozygote does not improve the disease phenotype, this would consequently exclude a role for normal huntingtin in the disease. In other triplet diseases, such as the dominant ataxias[37–41], the homozygous condition does enhance phenotypic severity, but in HD, the relationship between genotype and phenotype severity has not been definitively assessed. For example, some of the earlier evidence preceded discovery of the HD gene, and thus came from subjects who were considered only by linkage as probable homozygotes[34]. Later reports compared HD homozygotes and heterozygotes on the basis of age at onset[35,36]. However, this assessment has its own difficulties because several factors, other than the triplet expansion, influence the age at onset of HD (Refs 42,43). Indeed, there is a much stronger correlation between CAG repeats and age at onset in diseases such as the cerebellar ataxias (r2 of 0.70–0.80 in SCAs and DRPLA) compared with HD (r2 0.50)[42,43,44]. Furthermore the overlap of psychiatric, cognitive and motor manifestations, typical of the first stages of HD, represents an additional confusing clinical element possibly representing a bias for age at onset assessment[44,46].

Thus, the equivalence between homozygous and heterozygous HD subjects is far from established, and preliminary data comparing homozygotes to heterozygous siblings support the opposite (Squitieri, F. personal comunication). For example, homozygous transgenic mice expressing mutant *huntingtin* cDNA have a shorter life span compared with heterozygous mice by ~two months[47]. If homozygotes have a more severe phenotype compared with heterozygotes, this could be the result of either a double content of mutant huntingtin or the loss of the normal allele, the latter possibility indicating a loss-of-function contribution to the disease.

In conclusion, the clinical genetics data do not convincingly rule out a contribution of loss of normal huntingtin function in the disease. In addition, data from earlier knockout mice studies were viewed in favor of an exclusively gained activity of mutant huntingtin. However, the implication of these data in HD should also be re-evaluated in view of more recent findings obtained in conditional knockout mice.

Evidence from huntingtin knockout mice

The fact that the embryos of huntingtin homozygous knockout mice die by day 7.5 (Refs 48–50) had always been considered as proof of the gain-of-function hypothesis in HD. Two heterozygous knockout mice, with only half the normal level of huntingtin, pass the development stage and reach adulthood, in which a normal phenotype was observed[48,50]. In another knockout model (in which the mice produced a truncated N-terminal fragment of the protein), the heterozygotes showed increased motor activity, cognitive deficits, neuronal loss and degeneration in the subthalamic nucleus and globus pallidus[9,49]. On the basis of these observations it was suggested that huntingtin plays an important role in normal functioning of the basal ganglia.

The dosage effect of huntingtin during development was evaluated by White *et al.*[51] by breeding knock-in animals that had either a normally expressed allele with 50 CAG repeats or a variant with 50 CAG repeats and reduced transcriptional activity (generated by the insertion of a neo cassette that gives a 60% decrease in the amount of translated wild-type protein), with wild-type or knockout heterozygous mice. These studies showed that a single copy of the *huntingtin* gene is

Table 1. Huntingtin-interacting proteins

Interacting protein[a]	Method of identification	Dependence of interaction from CAG-length	Region of huntingtin involved	Function of the protein	Ref.
Calmodulin	Co-elution by gel filtration	Stronger with mutant huntingtin	Indirect interaction, Ca^{2+} -dependent	Ca^{2+}-binding regulatory protein	56
CREB-binding protein and mSin3a (2)	GST pull-down	Weak interaction; little increase with increasing CAG length	Poly-Pro region	Transcriptional coactivator and corepressor, respectively	57
Cystathionine B-synthase	Yeast two-hybrid system	No	N-terminal (aa 1–171)	Metabolic enzyme	58
GAPDH	Affinity chromatography	Stronger interaction with increasing CAG length	Poly-Gln stretch	Glycolitic enzyme, translational regulator	59
Grb2 and RasGAP (3)	Co-immunoprecipitation	Not known	Not known		60
HAP-1 (1)	Yeast two-hybrid system	Stronger interaction with increasing CAG length	N-terminal (aa 1–230) aa 171–230 are essential for the interaction	Involved in membrane trafficking	61
HAP-40 (?)	Co-immunoprecipitation	Not known	C-terminal	Not known	62
HIP-1 (1)	Yeast two-hybrid system	Weaker interaction with increasing CAG length	N-terminal (aa 9–294)	Pro-apoptotic, homolog to Sla2p in yeast, protein involved in actin organization and endocytosis	63,64
HIP-2 (1)	Yeast two-hybrid system	No	N-terminal (aa 1–540)	Ubiquitin-conjugating enzyme	65
HYP-A, -B,-C (2)	Yeast two-hybrid system	Stronger interaction with increasing CAG length	Poly-Pro region	WW-domain proteins. HYP-A/FBP-11 and HYP-C are involved in mRNA splicing; HYP-B is a transcription factor	66
MLK2 (3)	Co-immunoprecipitation and GST pull-down	Weaker interaction with increasing CAG length	First three exons	JNK activator	67
N-CoR (2)	Yeast two-hybrid system	Stronger interaction with increasing CAG length	N-terminal (aa 1–171)	Nuclear receptor co-repressor	29
p53 (2)	GST pull-down	No	Poly-Pro region	Transcription factor	57
SH3GL3 (3)	Co-immunoprecipitation and co-localization	Stronger with mutant huntingtin	Poly-Pro region	Clathrin-mediated endocytosis and recycling of synaptic vescicles	68
Shc and EGF receptor (3)	Co-immunoprecipitation	Not known	Indirect interaction	Signalling proteins	60

[a]The search for a huntingtin-interacting partner that could explain the toxicity of the expanded Gln and the selective vulnerability has led to the discovery of a large number of huntingtin-interacting proteins. However, as yet, not one of these can, by itself, explain the pathology observed in Huntington's disease. Known huntingtin-interacting proteins fall into three main subgroups. Members of subgroup 1 [(1) in the table] are represented by proteins involved in membrane trafficking, clathrin-mediated endocytosis and recycling of synaptic vesicles. Proteins involved in transcriptional events and transcriptional regulation belong to subgroup 2 [(2) in table]. As an example, huntingtin is found to specifically bind the nuclear receptor co-repressor (N-CoR) in a CAG length-dependent fashion[29], after which this protein remains mainly in the cytoplasm instead of migrating into the nucleus of the cortex and striatum of HD brain[29]. In a cell model, exon-1 huntingtin has also been shown to interact with p53, and when the CAG is extended this represses transcription of p53-regulated promoters[57]. Subgroup 3 [(3) in table] includes interactors involved in intracellular signalling. Other proteins that do not fall into these three categories are also indicated.

Abbreviations: aa, amino acid; CREB, cAMP response-element binding factor; EGF, epidermal growth factor; FBP-11, formin-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grb2, growth factor receptor-bound protein 2; GST, glutathione S-transferase; HAP-1, -40, huntingtin-associated protein-1, -40; HIP-1, -2, huntingtin interacting protein-1, -2; HYP-A, -B, -C, huntingtin yeast partners -A, -B, -C; MLK2, mixed-lineage kinase 2; N-CoR, nuclear receptor corepressor; RasGAP, Ras GTPase activating protein; JNK, Jun N-terminal kinase; Shc, Src-homologue and collagen homologue; SH3GL3, SH3-containing Grb2-like protein.

sufficient for correct brain development, regardless of the length of the CAG. However, a further reduction of the quantity of huntingtin leads to abnormal brain development and perinatal death, the severity of the phenotype being strictly dependent on the protein dosage. Thus, mutant huntingtin can substitute for wild-type huntingtin during development, consistent with the fact that homozygous HD patients do not present developmental defects.

More recently, a conditional inactivation of wild-type huntingtin selectively in brain (and testis) has allowed examination of the normal function of the protein in adult mice[11]. These animals showed a progressively more severe limb-clasping-upon-tail suspension, a feature also observed in transgenic HD mouse mutants [7,23,47,52]. Motor deficits were also present and at 10–12 months the animals were hypoactive and exhibited a slight tremor. Mutant mice survived for, at most, 13 months. Histological examination of the brains revealed tissue degeneration in the striatum (Fig. 1a,b) and cortex that, at ten months, was accompanied by

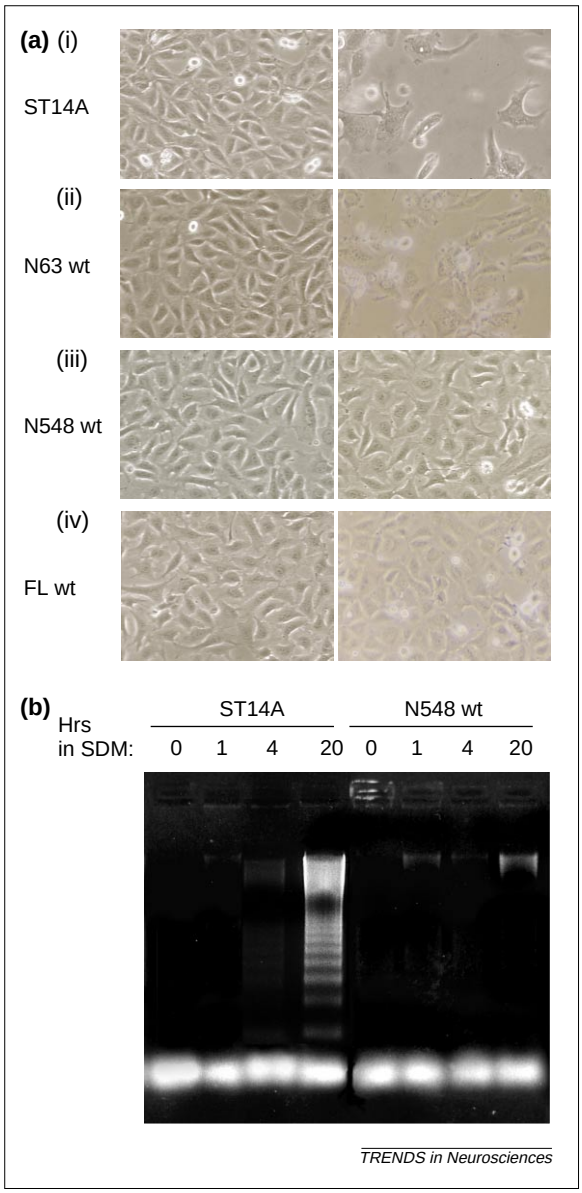

Fig. 2. (a) Huntingtin supports the survival of CNS-derived cells under stress. Left panels show normal growth condidtions; right panels show postmitotic conditions. ST14A cells derived from rodent striatum (i) and subclones stably expressing the N63 (N63wt) (ii), N548 (N548wt) fragments of wild-type huntingtin (iii), or the full-length protein (FLwt) (iv) were exposed to serum-free medium (at a temperature of inactivation of the immortalizing oncoprotein, allowing cells to become post-mitotic). In these conditions parental striatal cells (i) tended to differentiate but eventually began to die (right panel); cell death was reduced in clones expressing N548wt or FLwt (iii and iv), whereas cells expressing N63wt were not protected (ii). Reproduced, with permission, from Ref. 10. (b) Anti-apoptotic effect of wild-type huntingtin. Cells were exposed to serum deprivation for the time indicated and then examined for the presence of DNA laddering. DNA fragmentation gradually increased in parental ST14A cells, but was prevented in cells expressing N548wt even after 20 hr under these conditions. Abbreviation: SDM, serum-deprived medium. Reproduced, with permission, from Ref. 10.

degeneration of axon fibres and apoptosis (Fig. 1c,d)[11]. This phenotype is similar to those in currently used models of HD, suggesting that loss of huntingtin function might strongly contribute to the phenotype.

Thus, it is clear from these data that huntingtin has important roles during embryogenesis in addition to roles in the mature neurones of the brain. Direct evidence of the function of normal huntingtin has also begun to accumulate and benefiting from a large, although not conclusive, literature indicating its ability to interact with various key cellular proteins.

In search of the function of normal huntingtin

In spite of early recognition that normal huntingtin function is required for embryonic development, evidence of its function in brain cells over the whole lifetime was lacking. Interestingly, although the sequence of the gene is highly conserved phylogenetically, there are striking differences in the number of triplet repeats carried by the normal gene in different species. Whereas the human huntingtin gene carries a normal polymorphic CAG stretch ranging from 9 to 35 repeats[53], the rat and mouse genes have 8 and 7 triplets, respectively [54,55], the pufferfish has only 4 repeats[33] and *Drosophila* (GenBank Accession number AF177386) does not have any. Thus, because this region has not been conserved through evolution, these data suggest that the CAG repeat is not crucial for normal huntingtin function.

Several proteins interact with huntingtin, most of which interact with both normal and mutant proteins (reviewed in Ref. 30; Table I). Among them, HAP1 (Ref. 61) and HIP1 (Refs 63,64) are associated with membrane vesicles and interact with cytoskeletal proteins, suggesting a role for huntingtin in endocytosis, intracellular trafficking and membrane recycling (reviewed in Ref. 69). Indeed, huntingtin is found in axons and axon terminals that are associated with vesicle membranes and microtubules[3,70,71]. Huntingtin also co-distributed with intracellular and plasma membranes that contain clathrin[72], a coat protein involved in endocytosis, and its association with endosomes is increased by activation of adenylyl cyclase and stimulation of dopamine D1 receptors[73].

Another set of investigations implies a role in retrograde and fast axonal transport[74]. Although the mechanism is not clear, results in HD brains and mice[18,23,24], and in the conditional huntingtin knockout mice[11], show degeneration of axon fibres, compatible with the above hypothesis of a function for huntingtin in cellular trafficking.

A striking feature in the structure of huntingtin is the presence of a long polyglutamine stretch followed by a polyproline domain. These structures are primarily associated with transcriptional regulatory proteins[75], and their length negatively influences the transcriptional activity of the protein. Although huntingtin does not seem to have a DNA-binding domain, evidence suggests that it normally interacts with transcriptional regulatory proteins and has a role in their transport within the cytoplasm and inside the nucleus (Table 1). Furthermore, the only known structural domains in huntingtin are multiple HEAT domains[76], which have also been found in nuclear shuttle proteins, such as the importins[77]. This would further support a function for huntingtin as a HEAT domain nuclear–cytoplasmic transport protein[78].

Huntingtin also interacts with a family of WW domain proteins closely related to binding-nuclear proteins[79]. On the basis of confocal microscopic analyses showing huntingtin co-localizing with specific nuclear structures, it has been suggested that huntingtin is implicated in the transport and processing of mRNA (Ref. 80).

The interactions here described between huntingtin and key cellular proteins indicate the possibility of a pivotal role of huntingtin in cell functioning. More recently, direct evidence for an influence of huntingtin on cell viability has been reported, setting the ground for a more thorough

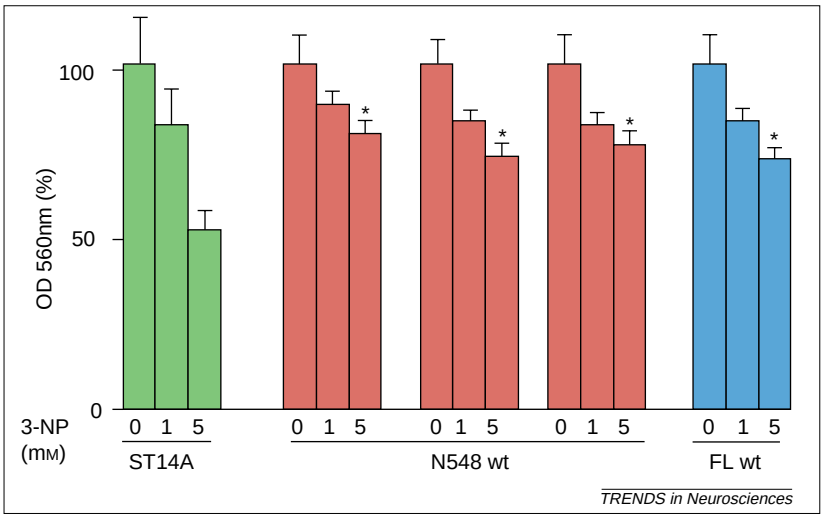

Fig. 3. Parental striatal ST14A cells, three subclones expressing N548 wt huntingtin and one clone expressing FL wt were exposed to the indicated doses of 3-nitropropionic acid (3-NP) under normal passaging conditions and the number of viable cells was estimated by an MTT assay. The N548 fragment and full-length huntingtin exerted an anti-apoptotic effect. Reproduced, with permission, from Ref. 10.

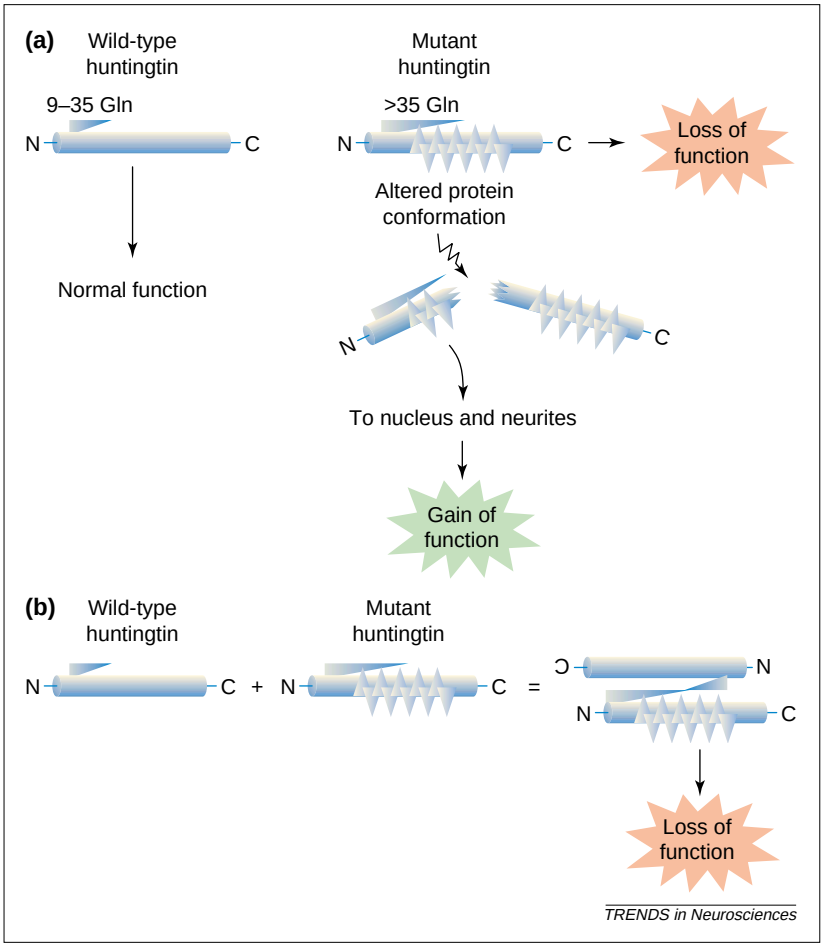

Fig. 4. Potential mechanism of cell death in Huntington's disease. Processing of mutant huntingtin would generate an amino-terminal fragment that translocates into the nucleus and a C-terminal portion that remains in the cytoplasm. Some of the full-length protein might also move into the nucleus. The generation of amino-terminal fragments would coincide with increased toxic activity in cells. At the same time, extension of the CAG would cause a loss of function in the mutant protein (Fig. 4a) and/or the mutant protein could act negatively on the functions of the normal one (Fig. 4b). Finally, loss of huntingtin function might result as a consequence of decreased protein levels and stability. To this respect, Ona *et al.*[15] showed decreased levels of full-length endogenous huntingtin in R6/2 HD transgenic mice.

assessment of how loss of normal huntingtin activities might contribute to the disease phenotype.

Direct evidence of a function for huntingtin in cell survival and neuronal stability

Studies by Rigamonti *et al.*[10] have provided the first direct evidence for a role of huntingtin on the survival of CNS cells[10]. Striatal cells that were engineered to express wild-type huntingtin were indeed resistant to the lethal effect of stresses such as serum deprivation (Fig. 2a), exposure to 3-nitropropionic acid (Fig. 3) (a mitochondrial toxin that, when injected systemically into animals, gives a similar pattern of neurodegeneration compared with that observed in HD, reviewed in Ref. 83) or transfection of death genes. In particular, we reported that huntingtin is an anti-apoptotic protein (Fig 2b).

The anti-apoptotic effect of huntingtin action lies downstream of pro-apoptotic BCL2 members and upstream of caspase 3 activation[10]. Both full-length normal huntingtin and its 548-amino acid terminus (N548) were equally protective for cells, whereas a shorter N-terminus (N63) had no effect (Fig. 1), suggesting that huntingtin is organized in several domains. Additional protective domains might also be present towards the C-terminus, because mutant N548 cDNA is more toxic compared with the full-length mutant protein[10].

Further evidence for the anti-apoptotic effect of huntingtin is derived from a study of cells in the embryonic ectoderm of homozygous knockout mice, which show characteristics of programmed cell death[50]. The phenotype observed in the conditional huntingtin knockout[11] is also compatible with a role for huntingtin in cell survival. Huntingtin also clearly affects neuronal stability because, as previously mentioned, its deletion causes degeneration of axon fibres[11].

In agreement with the observations of the beneficial functions of huntingtin, increasing the expression of wild-type huntingtin in transgenic mice protects against the toxic effects of mutant huntingtin[84]. A recent report[85] also indicates that the anti-apoptotic effects of huntingtin occurs via sequestration of HIP1, a pro-apoptotic molecule containing a novel death-effector domain, which was previously found to interact efficiently with wild-type huntingtin but not with mutant huntingtin[66,64]. Given the specific distribution of HIP1 in the brain (although expression is not restricted to striatum), Hackam *et al.*[85] suggested that the inability of mutant huntingtin to modulate HIP1 toxicity contributes to the amplification cascade of cell-death signals in HD through the same pathway identified by Rigamonti *et al.*[10].

Thus, it appears that huntingtin has crucial roles in cells, potentially via different domains, and that loss of the antiapoptotic functions of huntingtin might contribute to HD (Fig. 4a,b). As such HD might now be viewed as a double disease, that is, caused by both a

new toxic property of the mutant protein and by a loss of the neuroprotective activity of normal huntingtin. How these modifications affect the selective vulnerability observed in the disease remain unclear.

Selective vulnerability

An issue remaining unexplained is the specific vulnerability of striatal neurones in HD. Following the cloning of *huntingtin* it was suggested that the specificity of cell loss was as a result of a pathogenic interaction of mutant huntingtin with striatum-specific molecules. However, an extensive search for such molecules has identified brain-specific, but not striatum-specific, proteins with which mutant huntingtin preferentially interacts. Another possible explanation for the selective vulnerability might rely on a striatum-specific anti-apoptotic effect of huntingtin that is lost in the disease. However, although huntingtin inhibits an apoptotic pathway, which is, probably, the most active death pathway in CNS cells, it is not striatal specific. Huntingtin can recruit and oppose the lethal action of other proteins, for example HIP1, but this is also not striatum-specific. Alternatively, striatum-specific transcriptional regulatory proteins could be more vulnerable to huntingtin dysfunction resulting in a progressive transcriptional dysregulation.

Two studies[23,24] have found a selective accumulation of huntingtin N-terminal fragments in striatal neurons and their axonal projections in knock-in mice and full-length HD mice. These findings have resulted in the hypothesis that striatal vulnerability in HD might rely on striatum-specific processing of the full-length protein[24].

Finally, it is also possible that the selective vulnerability might depend on the loss or gain of activity of mutant huntingtin in brain areas that normally project to the striatum. Histological examination of striatal and cortical neurons indicates that huntingtin is highly localized in all corticostriatal neurons[86], which suggests that a defect in huntingtin function or the acquired activity of mutant huntingtin could render corticostriatal neurons destructive as oppose to rendering striatal neurons vulnerable.

Concluding remarks

This review has re-examined the evidence that a loss of huntingtin function might contribute to HD. In practical terms, identifying all possible routes through which a disease is manifested broadens our therapeutic perspectives. In HD, whereas one approach aims at blocking the aberrant activity that is caused by the lengthened CAG repeat, an additional strategy might be to restore normal huntingtin function. With current technologies this might be achieved either via gene therapy approaches or through molecules that influence the levels of normal huntingtin.

We might also postulate that similar loss-of-function events occur in some of the other inherited triplet-repeat diseases in which there is CAG expansion in genes of known or unknown function. Whereas the CAG domain always evokes cell death, the different proteins in whose backbones the CAG is expressed identify the neurones that will die. If such proteins have crucial functions for the neurones that die in the disease, the resulting selective neuronal death might be directly attributable to the loss of those functions.

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