

Huntington's disease: new hope for therapeutics

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Huntington's disease (HD) is one of eight progressive neurodegenerative disorders in which the underlying mutation is a CAG expansion encoding a polyglutamine tract. There are currently no cures or even effective therapies for HD. Effective strategies have remained elusive because little is known about either the mechanisms of expansion or the mechanism of polyglutamine-mediated neuronal death. However, recent advances in understanding the basic mechanisms of expansion and toxicity have renewed hope that a therapeutic strategy might someday be possible. Strategies effective in the treatment of HD are likely to be relevant in the treatment of a range of neurological and neurodegenerative disorders.

Huntington's disease (HD) is one of eight dominantly inherited and progressive neurodegenerative disorders in which the underlying mutation is caused by a CAG expansion within the coding sequence of the affected gene (Fig. 1)^{1,2}. Expansion causes disease when the CAG triplet in the mutated allele exceeds that of the normal range¹⁻³. However, a feature that distinguishes trinucleotide expansion is the non-Mendelian form of inheritance³; the repeat number can grow in each successive transmission (Fig. 1a). Each CAG triplet codes for the amino acid glutamine. As the CAG repeat number grows, the growing polyglutamine tract produces an HD gene product (called huntingtin) with increasingly aberrant properties that cause the death of brain cells controlling movement, memory and behavior¹⁻³ (Fig. 1b). Above 36 CAG repeats, loss of brain cells (primarily in the striatum and cortex) causes the personality changes, cognitive decline and uncontrolled muscle movements (termed 'chorea') that are characteristic of the disease¹⁻³ (see also the Hereditary Disease Foundation's website at <http://www.hdfoundation.org>). However, onset and severity of symptoms are strongly influenced by the number of CAG repeats in the disease allele¹⁻³. In individuals with 36 CAG triplets, symptoms can go unnoticed or develop mildly late in life. As the CAG triplet number grows, those affected develop more severe features at a younger age (Fig. 1a). Once symptoms begin, death usually occurs within 15–20 years¹⁻³.

There is currently no cure, or even an effective therapy, to offset the decline in mental and motor capacity suffered by those affected by HD. However, recent advances in understanding have provided new hope that a therapeutic strategy might one day be possible.

Therapy at the DNA level

The strong dependence of the character of the disease on the CAG repeat length has raised the possibility that stopping expansion at the DNA level might be an effective therapeutic strategy. Recent studies in mice have suggested that the inherited distribution of CAG repeat lengths of expanded transgenes remains stable after birth. However, at around 11 weeks of age, somatic expansion is observed in almost every tissue of the body and continues throughout the lifetime of the animal⁴. Age-dependent expansion is particularly prominent in the brain^{4,5}. Thus, it is possible that tissues preferentially affected in disease contain huntingtin gene products with more and longer polyglutamine tracts. Although this phenomenon has not been confirmed in humans, there is intense interest in determining whether the age-dependent increases in the polyglutamine tracts length are related to the onset of disease.

Early efforts to stop expansion were hampered because the genetic mechanism was poorly understood. Recent findings, however, have confirmed that CAG

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expansion occurs during the repair of strand breaks and implicates the mismatch repair system in the mutation process⁴.

Mechanism of expansion and possible therapeutic implications

It had been speculated that expansion occurs by polymerase slippage during cell proliferation or meiotic recombination (in sperm). By following CAG expansion in transgenic R6/1 mice, however, it has been observed that expansion occurs during post-mitotic phases in the brain and in the sperm of male animals. R6/1 mice harbor a single integrated copy of a transgene containing a CAG repeat in exon 1 of the human huntingtin gene (hHD)⁵. In germ cells, expansion occurs late in sperm development – at the point when cells called spermatids differentiate into mature sperm⁴. This is significant because spermatids are haploid cells that are both post-mitotic and post-meiotic. Therefore, CAG expansion cannot depend on mitotic replication or meiotic recombination and must arise from the repair of strand breaks. Break repair is also implicated as the mechanism in neurons – the age-dependent expansion is observed in the adult mouse brain at a time when neurons are also post-mitotic^{4,6}.

Expansion occurs when CAG/CTG loops are trapped in the DNA after gap repair synthesis (Fig. 2)⁴. It has long been recognized that expansion depends in some way on the ability of expansion-capable repeats to form secondary structures³. Then, at the site of the break, gaps can arise when CAG repeats form stable hairpin loops (Fig. 2). Repair of the gaps traps DNA loops, which are the precursors for expansion. DNA loops can also be trapped by slippage within the repeat region during repair-dependent, gap-filling synthesis⁴ (Fig. 2).

A big surprise was the discovery that the mismatch repair system, the normal function of which is to remove mispaired bases and loops from DNA, plays a causative role in the mutation^{4,7}. As shown by mating hHD (R6/1) animals with Msh2^{-/-} animals, the absence of Msh2 completely abolished germ line expansion and age-dependent, somatic expansion^{4,7}. It is suspected that an Msh2 complex binds and stabilizes the mispaired bases in the stems of the CAG hairpins⁴. Hairpins that form from CAG repeats contain an A–A mismatch base every third position between two Watson–Crick C–G pairs³. Stable hydrogen bonding within a contorted DNA template could abort a crucial step of recognition or coupling of the mismatch repair system⁸.

The finding that loss of Msh2 attenuates expansion in animals provided the first evidence that expansion can be stopped *in vivo*, and has raised the hope that a therapeutic

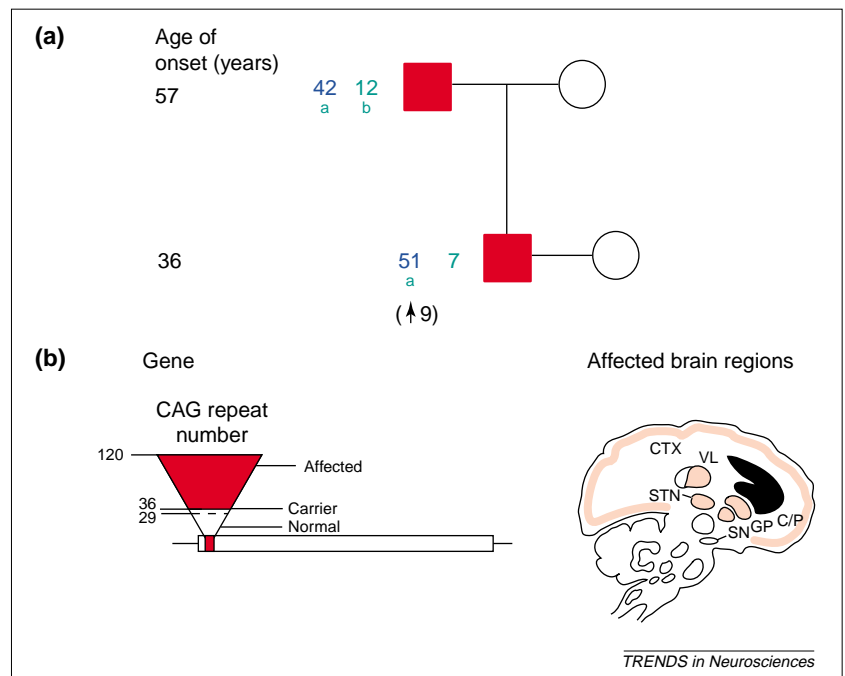


Figure 1. Effects of CAG expansion in the huntingtin gene

(a) Expansion in huntingtin decreases the age of onset in those affected. A representative two-generation pedigree of an HD family. Squares are males; circles are females. Red boxes indicate affected individuals. Open circles are unrelated spouses. Green numbers represent CAG repeat number in each allele of the affected family members. Small green letters indicate the alleles present in father-to-son transmission. The unlettered repeat number (blue) is a normal allele inherited from the mother. The number in brackets represents the size of the CAG expansion during inheritance. Age of onset of symptoms is indicated on the left in years. (b) The relationship between HD pathophysiology and CAG repeat number (left). In this schematic representation of the HD gene, the open bar represents the coding region of the Huntington's gene (called huntingtin); the lines indicate the non-coding portions of the gene; the small red bar indicates the position of the CAG repeat stretch located within the N-terminal portion of the coding sequence. The inverted triangle represents an increasing number of CAG repeats. The base of triangle, in white, represents unaffected individuals with 6–29 CAG repeats; dotted lines indicate unaffected carriers for disease with 29–35 CAG repeats and the red part of the triangle indicates affected individuals with 36–120 CAG repeats. Regions of neuronal loss in HD are shown on the right. Red regions indicate the major areas of neuronal loss in HD patients with 36–120 CAG repeats; these brain regions control movement. Abbreviations: C/P, caudate/putamen; CTX, cortex; GP, globus pallidus; STN subthalamic nucleus; VL ventrolateral thalamic nucleus; SN substantia nigra.

intervention of a repair complex could be used to attenuate, or at least delay, onset of disease. Although complete elimination of the Msh2 repair function is unlikely to be beneficial, the mechanism by which Msh2 is involved in expansion might reveal pathways and points of intervention that could be useful in a potential therapy. Additionally, it has been suggested that the mutational 'load' caused by age-dependent expansion could initiate a DNA damage response resulting in cell death. If this is correct, inhibition of damage-induced apoptosis could be therapeutic (see 'Caspase inhibition' later in the review).

Therapy at the protein level: managing the toxic effects of the mutant huntingtin protein
Interfering with huntingtin-mediated aggregation
 Stopping CAG expansion, even if successful, will at best prevent disease progression and diminish disease severity.

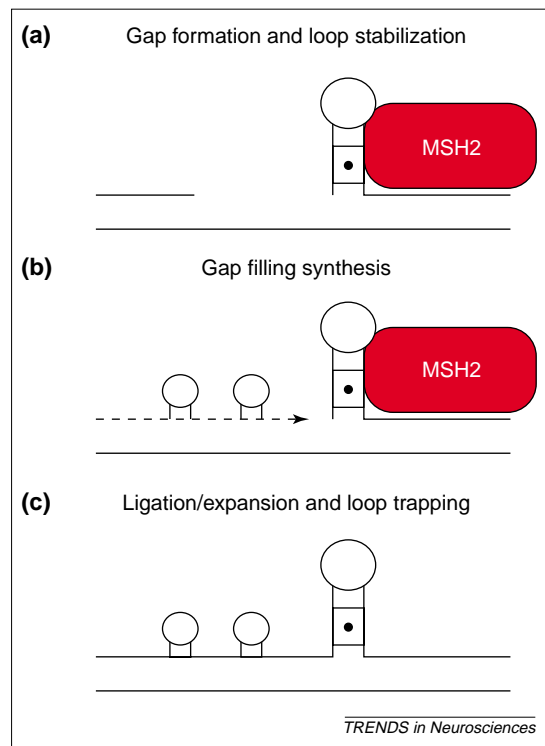


Figure 2. Model for expansion by gap repair

(a) After a strand break, gaps can form by folding of CAG/CTG repeats into hairpins. Hairpins comprising CAG repeats form a repeating unit of an A–A mismatch base every third position (dot in the hairpin stem) between two Watson–Crick C–G pairs (lines in hairpin stem). Msh2 might stabilize loop structures.

(b) Consequently, Msh2 binding prevents the reannealing of the complementary strands at the break site and increases the lifetime of the gap, forcing its repair. As haploid cells contain only one complement of the chromosomes, repair is not possible through genetic exchange with a homologous chromosome or a sister chromatid. Repair can occur through a simple 'fill-in' reaction or by gene conversion in which the DNA loop invades a CAG repeat region in a non-homologous chromosome. Loops of DNA can be introduced into the DNA either at the site of the break by folding of CAG/CTG repeats into secondary structure or at the break or during gap repair synthesis (b). (c) Gap filling synthesis followed by ligation results in loop trapping and gain of DNA at the site of the break (bottom).

Therefore, more emphasis has been placed on developing therapeutic strategies to offset or prevent toxicity induced by the mutant huntingtin protein. Mutation analysis and transgenic animal models for disease have unequivocally identified the expanded polyglutamine tract as key in toxicity^{1–3}. As with the expansion, the mechanisms by which the polyglutamine tracts kill neurons are unclear and the function of huntingtin protein is unknown. Polyglutamine regions self-associate to form polar zippers (β -strands that assemble into sheets or barrels by hydrogen bonding)⁹, promoting aggregation. In HD-affected areas of the caudate and cortex, immunochemical detection reveals that mutant huntingtin protein forms high molecular weight complexes and inclusion bodies^{1–3,5}. Despite an incomplete

understanding of toxicity, it is generally accepted that aggregation and sequestration of cellular targets are causative factors^{1–3,9}. Consequently, antibodies and a number of small molecule inhibitors are being developed to block aggregation. For example, when co-expressed with a truncated form of the mutant huntingtin protein, single-chain sFv antibodies have been shown to reduce the number of visible aggregates in mammalian cells¹⁰. Similarly, small molecules and peptides inhibitors^{11–13} of huntingtin-mediated aggregation are being developed that specifically bind to huntingtin, reduce aggregation and improve cell survival. To date, the testing of new molecules has been limited to *in vitro* culture systems. However, future *in vivo* studies will determine whether modulation of aggregates represents a viable approach to treatment for HD.

Identification of specific targets of huntingtin-mediated aggregation has also suggested new avenues for therapy. Many cytoplasmic¹⁴ and nuclear proteins¹⁵ are reported to associate with normal and mutant huntingtin. Cytoplasmic targets include vesicular trafficking motors, ubiquitin-conjugating enzymes, actin-organizing proteins, and microtubules. Nuclear targets include p53 and CREB binding protein. Although loss of any of these targets might be deleterious to cell function, one in particular has generated intense interest. The mutant huntingtin protein can sequester its normal counterpart in the cell. Huntingtin protein has essential functions during development¹⁶ and throughout the life of an animal¹⁷. For example, it has recently been reported that huntingtin can regulate transcription of brain-derived neurotrophic growth factor (BDNF)¹⁸. BDNF is particularly important for the growth of striatal neurons; consequently, loss of BDNF, owing to huntingtin-mediated aggregation, could contribute to neuronal death. These data suggest that treatment with BDNF could improve neuronal survival. In addition, sequestration of normal huntingtin could abolish a recently reported anti-apoptotic function of the normal protein that blocks activation of procaspase-9 (Ref. 19). Therefore, loss of the normal huntingtin protein by the mutated gene product could result in a 'functional knockout'. Indeed, conditional knockout in mice of the normal huntingtin protein after birth in mice results in a progressive neurodegenerative phenotype similar to HD (Ref. 17). If correct, future efforts could focus on how to replace the function of the normal huntingtin in a manner that is not defeated by aggregation of the mutant protein.

Cellular processes suspected of being altered by polyglutamine-dependent aggregation have resulted in the testing of several therapeutic strategies for HD. All of these have met with modest success.

Replenishing energy metabolism owing to oxidative stress

Disruption of mitochondrial function and glucose metabolism has been proposed to mediate neuronal death in many neuropathological diseases, including HD (Ref. 20). In human HD patients, magnetic resonance imaging confirms that creatine (which is a free-radical scavenger, a substrate for the enzyme creatine kinase and a precursor for ATP) is depleted²¹. Similarly, in cells expressing the mutant huntingtin protein, mitochondria do not readily take up cationic dyes that depend on intact charge gradients²². These data indicate that mitochondrial membrane potential is impaired because of expression of the polyglutamine protein. In rats, systemic administration of the mitochondrial complex II inhibitor, 3-nitropropionic acid, causes neurobehavioral and pathological abnormalities consistent with HD (Ref. 23) and, in HD patients, the caudate has severe deficiencies in mitochondrial complexes II and III (Ref. 24). Finally, in affected striatal and cerebral regions of the brain, glucose metabolism is decreased and precedes bulk tissue loss in HD patients²⁴. Taken together, these data point to impairment of mitochondrial function as contributing factor in HD.

If mitochondrial deficits and ATP depletion play a role in HD, then replenishing impaired energy metabolism might offset toxicity by restoring ATP levels. Indeed, the survival rate of animals treated with dietary creatine does increase in both R6/2 transgenic mice²⁵ (which are the same as R6/1 mice but with a larger CAG tract⁵) and in mice subjected to systemic administration of 3-nitropropionic acid²⁶. In both sets of animals, creatine not only improved survival but also delayed striatal atrophy and the formation of neuronal inclusions^{25,26}. Despite improvements in the animals, supplementation with creatine has not yet proven effective in offsetting disease progression in human clinical trials²⁷. Efficacy was also absent in an earlier study of another free-radical scavenger, OPC-14117 (Ref. 28). In both cases, however, the compounds were well tolerated with no adverse side-effects. Studies with larger patient groups are planned for creatine and clinical trials are now underway to evaluate the efficacy of co-enzyme Q, another free-radical scavenger that improves energy production in mitochondria²⁶. However, even if these agents are protective they are unlikely to be a permanent cure or therapy, but might prove efficacious in combination with other treatments.

Glutamate excitotoxicity

Among the affected cells in HD are specialized brain cells in the striatum called medium spiny neurons²⁹. These neurons contain many small 'spines' that are rich in

excitatory N-methyl-D-aspartate (NMDA) receptors. As expression of mutant huntingtin has recently been reported to enhance excitotoxic death in cultured cells³⁰, trials are now underway to study the effects of glutamate receptor blockers in HD patients. Clinical trials for lamotrigine have already been completed³¹. Lamotrigine is an anti-epileptic drug that blocks voltage-gated sodium channels, thus inhibiting glutamate release^{31,32}. Although lamotrigine was successful in reducing chorea in patients, it failed to affect disease progression^{31,32}. Currently, trials are underway to test the efficacy of remacemide³², another blocker of this receptor-mediated, excitatory pathway.

Caspase inhibition

Damaged cells can be removed by initiating a programmed pathway to cell death mediated by caspase activation³³. It has recently been suggested that huntingtin-mediated aggregation might induce activation of important initiator caspases 9 (Ref. 19), -8 and -10 (Ref. 34). Activated caspases 8 and 10 appear to be recruited to the insoluble fraction in homogenates derived from HD brains³⁴. Initiator caspases are responsible for cleavage and activation of downstream effector caspases, such as caspase 3 (Ref. 33). Interestingly, caspase 3 cleavage of the mutant huntingtin protein generates a small, N-terminal peptide containing the expanded polyglutamine region, and cells transfected with this N-terminal fragment form nuclear inclusions and undergo apoptosis³⁵. Similarly, mice expressing a truncated N-terminal fragment of huntingtin display inclusions and a progressive neurological phenotype, although neuronal loss is not necessarily observed^{5,36}. These data have given rise to the 'toxic peptide' theory of pathogenesis in which a small N-terminal fragment containing the expanded polyglutamine region must be generated to mediate toxicity. Unambiguous proof that caspase cleavage of huntingtin occurs *in vivo* has not yet been demonstrated and the full-length mutant protein is not easily degraded in transgenic animals²⁹. Thus, it is still not known if it is the full length or truncated form of huntingtin that initiates early events of HD pathophysiology.

If caspase activation occurs early enough in disease progression then disease onset could be blocked by the use of caspase inhibitors, which might provide protection by blocking a general cell death pathway, by preventing the formation of toxic N-terminal fragment, or both. Indeed, several attempts to rescue the HD phenotype in mice by using caspase inhibitors have been reported. For example, Ona and colleagues reported that caspase 1 is activated in the brains of both mice and

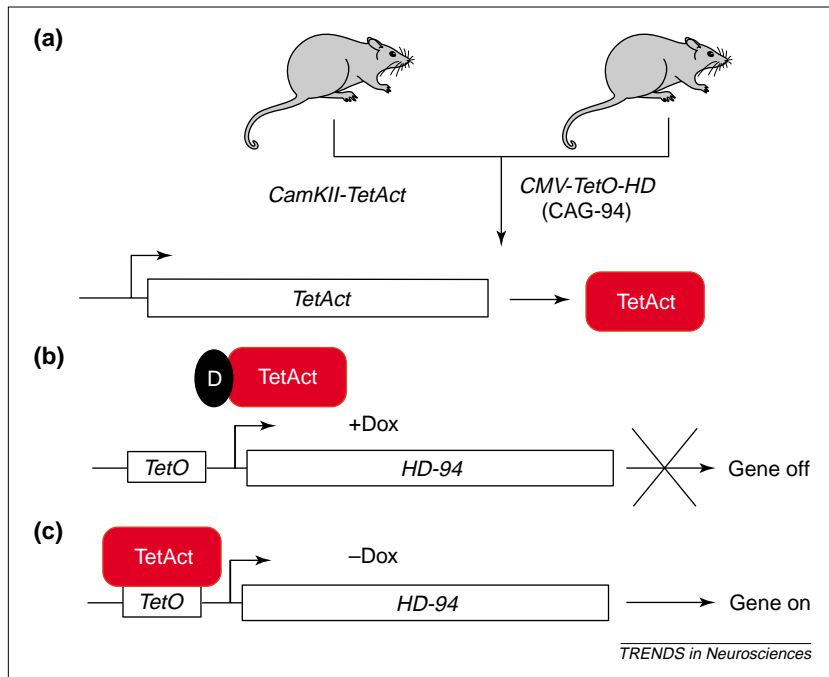


Figure 3. 'Tet-off' regulation of huntingtin in the mouse

(a) Two mouse lines were generated and bred. The first contained the tetracycline transactivator (TetAct) under control of the calmodulin kinase II promoter (CamKII). TetAct is a fusion protein between the tetracycline-responsive repressor and the activation domain of the herpes simplex VP-16 transcriptional activator. TetAct binds to the tetracycline-responsive operator sequence and activates a linked gene. The second mouse contained a truncated, recombinant HD transgene (CMV-TetO-HD). The transgene contained only exon 1 of the human huntingtin gene, with a CAG repeat of 94 driven by a cytomegaloviral promoter (CMV) engineered to contain a tetracycline-responsive operator sequence (TetO). Progeny of the breeding contain both transgenes and are capable of antibiotic regulation of the HD transgene expression. (b) To regulate HD expression, progeny mice are exposed to doxycycline (Dox) in the drinking water (+Dox). Dox is a stable derivative of tetracycline that binds to the TetAct and causes dissociation from the TetO. As TetAct is required for CMV-TetO-HD expression, dissociation abolished production of the toxic huntingtin gene product (Gene off). (c) When Dox is removed from the drinking water (-Dox), TetAct binds to the operator and enables expression of the toxic huntingtin gene product (Gene on). The black circle is doxycycline (D); open bars represent the cDNAs of the respective genes; lines represent the promoter elements; arrows represent the transcriptional start sites.

humans with HD (Refs 37,38). R6/2 transgenic mice treated by intracerebroventricular injection of caspase 1 inhibitors³⁷ or 3-propionic-acid-challenged animals that express a dominant-negative caspase-1 (Ref. 38), displayed improved motor function and significantly delayed onset of symptoms and mortality. The N-terminal human huntingtin fragment present in R6/2 mice is an approximately 75-amino-acid protein, in addition to the glutamines, and contains no caspase 1 cleavage sites. Therefore, caspase 1 inhibition cannot be acting by preventing N-terminal cleavage of the mutant huntingtin protein.

It has therefore been speculated that blocking a general programmed death pathway might improve survival of affected brain cells. Indeed, clinical trials are planned to test the efficacy of minocycline, an inhibitor of cell death pathways that has improved survival in animals³⁹. Minocycline is a derivative of the antibiotic tetracycline

that crosses the blood-brain barrier and inhibits caspases 1 and 3 (Ref. 39).

Like creatine treatment, caspase inhibition appears promising as a therapeutic approach, it merely delays disease progression in animals, being unable to prevent it. Thus, these compounds are not expected to cure HD; rather, it is hoped that they will improve symptoms and survival.

The approaches discussed above have in common a strategy to offset or to manage the toxic effects produced by the expanded huntingtin protein. Recently, however, several approaches have been aimed at cure or prevention of disease.

Transplantation

Polyglutamine aggregation leads to the death of several neurons. To offset the severe phenotype that ensues, surgical strategies have been developed in which transplantation of embryonic stem cells replaces lost neurons in the striatum⁴⁰. Embryonic grafts placed in quinolinic-acid-treated animals improved motor functions such as paw reaching. Furthermore, neural precursors develop synaptic connections and express neural antigens and many markers of mature differentiation⁴¹. These studies suggest an exciting avenue for therapeutic intervention in severe cases. The success of grafting is sensitive to the age of the donor, the time of the graft placement and the degree of neuronal loss in the host^{40,41}, and can be improved by treating animals with caspase inhibitors⁴². This approach is highly invasive and will be useful only at later stages of disease progression. However, if effective, defective neurons would be replaced with normal ones that lack the mutant gene.

Inhibition of protein expression from the mutant allele

An ideal approach would be to eliminate the expression of the mutant protein before toxic effects occur. Such an approach could prove effective because function does not appear to be sensitive (within limits) to the amount of expressed gene product in HD patients. Individuals can vary by as much as 50% in their huntingtin protein content without developing disease (Ref. 36 and references therein). Because normal huntingtin is required for development^{16,17}, however, inhibition of the mutant allele is expected to be beneficial only if expression from the normal allele is preserved. Few anti-gene or antisense strategies have been reported^{43,44}. Attempts using oligonucleotides specifically to reduce the expression levels of the mutant allele have been limited to cell culture and have met with limited success. For example, antisense oligonucleotides targeting the methionine initiation codon and exon 1 (the -25 to +35 region of the

promoter) can inhibit expression of a stable incorporated green-fluorescence-huntingtin in PC-12 cells to roughly half that of untreated cells⁴³. Although these studies report successful reduction in the protein levels, whether a reduction of 50% is sufficient to rescue cell survival during long-term culture remains to be seen. A larger issue (that also remains to be tested) is whether this approach will be effective in targeting neural tissue in whole animals or humans.

Although testing of the antisense and anti-gene approaches is in its infancy, recent studies confirm that selective inhibition of the expanded Huntington's allele is likely to be an effective strategy³⁶. Using a 'tet-off' regulatable huntingtin transgene (Fig. 3), Yamamoto and colleagues³⁶ were able to control the expression of the mutant truncated protein in mice. In the absence of doxycycline in the drinking water ('gene on' condition), these animals developed inclusion bodies, progressive claspings response, tremors, general brain atrophy, an increased ventricular size and reactive astrogliosis³⁶. All of these effects evolved between 3 and 18 weeks of age. However, restoration of doxycycline (2 mg ml⁻¹; 'gene off' condition) at 18 weeks of age had, by 34 weeks, reversed the claspings phenotype, reduced the incidence of inclusions to nearly that of control mice, and attenuated or improved neuropathology. These data in mice provide important proof of principle that specific inhibition of the expanded huntingtin allele can indeed reverse the disease phenotype if the normal gene expression is maintained. However, the identification of a small molecule that can effect selective inhibition *in vivo* still remains to be achieved. This might be no easy task because the mutant and normal huntingtin alleles have few sequence variations to target outside of the CAG tract length.

Carriers of the expanded HD allele can be genetically identified long before clinical symptoms develop. Early detection and late onset of disease render HD patients particularly well suited for effective therapeutic intervention if this is developed. Although effective therapy is not yet possible, rapid advances in the understanding of the basic mechanisms of disease are leading to expanded approaches towards therapeutic strategies, and to renewed hope for a cure.

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