Chapter 16 – Preclinical Experimental Therapeutics in Huntington’s Disease

Gillian P. Bates and Christian Landles

Dept. Medical and Molecular Genetics, King’s College London, 8th Floor Tower Wing, Guy’s Hospital, Great Maze Pond, London SE1 9RT, UK.
Introduction

The isolation of the Huntington’s disease (HD) mutation in 1993 (Huntington’s Disease Collaborative Research Group, 1993), made it feasible to generate models of HD in any of the organisms amenable to genetic manipulation including yeast, nematodes, fruit flies, zebrafish and a wide range of mammals: mouse, rat, sheep, pig and non-human primates. The availability of model systems has overcome the limitations of studying HD post mortem brain material, allowing the onset and progression of phenotypes to be tracked, and has led to major advances in our understanding of the molecular pathogenesis of HD. Mammalian models have been of fundamental importance to ensure that discoveries made in single cell systems and invertebrates are relevant in the context of the mammalian brain and physiology. They are also essential for the preclinical screening of potential therapeutic agents to determine which approaches should be taken forward for clinical evaluation. The mouse genome can be manipulated with ease and this, together with a comparatively short generation time, has meant that mouse models have dominated the field. They have been the models of choice for the investigation of disease mechanisms and preclinical therapeutic validation. However, the use of the rat is likely to become more prominent with the advent of Zn$^{2+}$ finger (Le Provost et al., 2010) and transcription activator-like effector nuclease (TALEN) (Tesson et al., 2011) technologies, which promise to make the genome of any organism amenable to sophisticated manipulations.

Before discussing therapeutic target validation and preclinical screening, this chapter will begin by reviewing the mammalian models of HD that have been generated. It will discuss the limitations in generating models of a mid-life onset disease and the means by which these have been overcome.
**Strategies Employed to Generate Mouse Models of HD**

The human (HTT) and mouse (Htt) huntingtin genes each contain 67 exons and produce large proteins (huntingtin/HTT) of approximately 350 kDa (Ambrose et al., 1994; Barnes et al., 1994). There are two basic approaches by which the mouse genome has traditionally been manipulated. Transgenic animals are generated by the injection of DNA into the pronucleus of a single cell embryo. In the case of HD the injected DNA has included fragments of the human HTT gene (Laforet et al., 2001; Mangiarini et al., 1996; Schilling et al., 1999), a cDNA copy of the human HTT gene (Reddy et al., 1998) or the entire genomic sequence containing the human HTT gene in the form of either a yeast artificial chromosome (YAC) (Hodgson et al., 1999; Slow et al., 2003) or bacterial artificial chromosome (BAC) (Gray et al., 2008). This DNA inserts into the mouse genome at a position that is distinct from the location of the mouse Htt gene on murine chromosome 5. Therefore all of these transgenic animals express two copies of Htt as well as the human transgene. Because HD is an autosomal dominant disease, the introduction of the mutation as a transgene in this manner could be predicted to cause a disease phenotype.

The alternative approach is to manipulate the mouse Htt locus which encodes for the murine HTT protein with seven glutamine residues (Barnes et al., 1994; Lin et al., 1994), encoded by a (CAG)$_2$CAA(CAG)$_4$ repeat. This manipulation can be engineered either to prevent the expression of a gene (knock-out) or to alter the gene sequence of the expressed gene (knock-in). Mice that are knocked-out for Htt show embryonic lethality, demonstrating that the HTT protein is essential for life (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). In the context of HD, knock-in mice are those in which a long stretch of glutamine residues has been inserted into one of the mouse Htt loci. Heterozygous HD knock-in mice express one copy of the Htt gene encoding for a protein with seven glutamines and one in
which this has been replaced with an expanded glutamine tract in the disease causing range (Levine et al., 1999; Lin et al., 2001; Menalled et al., 2003; Shelbourne et al., 1999; Wheeler et al., 2000). Therefore knock-in mice more precisely recapitulate the genetics of human HD than the transgenic models.

The onset of the adult form of HD is generally in mid-life and a major concern when attempting to model a mid-life onset disease is that phenotypes will not occur within the lifetime of the laboratory mouse (approximately two years). However, the age of symptom onset for HD can range from early childhood to extremely old age. CAG expansions of 70 and above invariably result in an onset in childhood or adolescence (Myers, 2004) and therefore, it could be predicted that the use of very long repeats might sufficiently accelerate the onset of phenotypes in mice to generate useful mouse models.

A second approach to accelerate phenotype onset is to increase the level of mutant protein by the use of a strong promoter, however, the possibility of inducing non disease-relevant phenotypes by this approach cannot be excluded. Finally, there is considerable evidence to indicate that small, highly pathogenic N-terminal fragments of HTT are present in HD patient tissue. The process by which they are generated might represent one of the first steps in the pathogenesis of HD (DiFiglia, 2002; Sathasivam et al., 2013) and the use of truncated constructs might by-pass this initiating event. As mentioned above, the ‘knock-in’ strategy most precisely replicates the genetics of HD. However, it is the least flexible approach with respect to generating a mouse with an early phenotype onset. The only options available are to use long CAG repeats and to increase the expression of the mutant protein by breeding to homozygosity.
Mouse Models of Huntington’s disease

The mouse models of HD that are currently in use are summarised in Table 16.1, which details the HD construct used, the length of the CAG / polyglutamine (polyQ) repeats, the promoter under which the mutant gene is expressed and the mouse strain background.

The N-terminal HTT fragment models of HD, include the R6 (Mangiarini et al., 1996), N171-82Q (Schilling et al., 1999) and HD (Laforet et al., 2001) lines. The R6/2 mice are transgenic for a genomic fragment of the HTT gene that contains promoter sequences, exon 1 (initially carrying ~150 CAGs) and a portion of intron 1. It has now been shown that the R6 lines are a model of the mis-splicing that occurs in HD which produces an exon 1 HTT protein (Sathasivam et al., 2013). They are the most widely used HD mouse model as the early phenotype onset and rapid disease progression offers many advantages. HTT aggregates appear in the brains of these mice prior to other phenotypes (Davies et al., 1997; Li et al., 1999). They develop motor (Carter et al., 1999) and cognitive (Lione et al., 1999) impairments, deficits in synaptic plasticity at hippocampal CA1 synapses (Murphy et al., 2000), electrophysiological alterations in the cortico-striatal pathway (Cepeda et al., 2003), decreases in brain volume (Zhang et al., 2010) as well as a failure to gain weight (Davies et al., 1997) prior to the appearance of overt neurological phenotypes such as hind-limb clasping. These changes correlate with alterations in global transcription (Cha et al., 1998; Luthi-Carter et al., 2000) and altered signalling mechanisms (Bibb et al., 2000; Menalled et al., 2000). The CAG repeat in the R6 lines is unstable (Mangiarini et al., 1997) with the result that specific R6/2 colonies now have considerably different CAG repeat sizes, influencing disease onset, progression and longevity (Cowin et al., 2012; Menalled et al., 2009). Surprisingly, highly expanded repeats of greater than 330, resulted in a milder phenotype as a result of decrease in mutant HTT levels and the exclusion of much of the aggregate
pathology from the nucleus (Dragatsis et al., 2009; Morton et al., 2009). The N171-82Q mice develop similar phenotypes with a slightly later onset and more protracted course of disease (Schilling et al., 1999).

The shortstop mice expressing a 117 amino acid fragment (exon 1 and 2) with 128Q were reported to exhibit robust inclusion pathology without developing the phenotypic abnormalities seen in the R6/2 and N171-82Q models (Slow et al., 2005). However, a comparative analysis of the appearance of aggregate / inclusion pathology across a range of mouse models indicated that this appears much later in the short-stop mice than in the R6/2 or N171-82Q lines (Figure 16.1), most likely accounting for the delayed appearance of phenotypes (Wang et al., 2008a). In support of this interpretation, mice transgenic for a 118 amino acid fragment with 82Q developed inclusion pathology and neurological phenotypes, failure to gain weight and early death, consistent with that observed in the R6/2 and N171-82Q lines (Tebbenkamp et al., 2011b).

The YAC128 (Slow et al., 2003) and BACHD (Gray et al., 2008) models express a transgene containing the entire human genomic HTT gene (Table 16.1). The expanded polyQ tract in the BACHD mice is encoded by a CAG repeat that is interrupted with CAA codons to stabilise the repeat. The YAC 128 mice were initially reported to develop motor deficits from 3 months of age, cognitive impairments as well as striatal atrophy and neuronal loss (Slow et al., 2003; Van Raamsdonk et al., 2005). More recent studies have extended the behavioural analysis to indentify additional impairments (Brooks et al., 2012a), including a motor-learning deficit (Brooks et al., 2012e) and cognitive impairments that correlated with the appearance of aggregates (Brooks et al., 2012c). Aggregate pathology and neuronal inclusions develop throughout the brain and by end stage disease there is widespread neurodegeneration (Bayram-Weston et al., 2012a). The BACHD mice also develop motor
deficits, synaptic dysfunction and striatal atrophy but the aggregate pathology was predominantly extranuclear (Gray et al., 2008). The BACHD mice have been crossed to YAC mice carrying 18 CAGs (YAC18) on an Hdh-/- knock-out background to generate a fully humanised HD mouse model that is heterozygous for the HD mutation (Hu97/18 line) (Southwell et al., 2013). The Hu97/18 mice display motor, psychiatric and cognitive deficits as well as canonical neuropathological abnormalities and will be particularly useful for testing strategies to lower the levels of HTT as discussed later in this chapter.

Knock-in models have been generated by two strategies. The first was to insert an expanded CAG repeat into the mouse Htt gene (Heng et al., 2010; Lin et al., 2001; Shelbourne et al., 1999) and the second to replace mouse exon 1 Htt with a mutated version of human exon 1 HTT creating a hybrid protein (Levine et al., 1999; Menalled et al., 2003; Wheeler et al., 2000). The lines that have been characterised most extensively include the ‘Detloff’ lines with either ~150 CAG or ~200 CAG inserted into mouse Htt (HdhQ150; HdhQ200) (Heng et al., 2010; Lin et al., 2001) and the ‘Zeitlin’ lines with ~140 CAGs (CAG140) (Menalled et al., 2003) or ~190 CAGs (zQ175) (Menalled et al., 2012) that were generated as a human/mouse chimeras. Motor impairments such as poor rotarod performance, abnormal gait, abnormal locomotion and exploratory behaviour, cognitive deficits, striatal shrinkage, neuropathological changes and global transcriptional dysregulation have been observed in the CAG140 and/or HdhQ150 lines, (Bayram-Weston et al., 2012b; Brooks et al., 2012b; Heng et al., 2007; Hickey et al., 2008; Kuhn et al., 2007; Menalled et al., 2003; Rising et al., 2011; Woodman et al., 2007). However, the identification of robust molecular, behavioural and / or neuropathological phenotypes in heterozygous animals that can be used for the purposes of testing therapeutic interventions remains a challenging and on-going endeavour (Heikkinen et al., 2012; Menalled et al., 2012).
The HD research community is fortunate in that so many complementary mouse models of HD are available and detailed characterisation is predicting which models are most appropriate for any given application. Broadly speaking, there is a remarkable similarity between the major phenotypes that they develop (Brooks et al., 2012d; Cepeda et al., 2010; Menalled et al., 2009; Woodman et al., 2007). However, one notable exception is that although muscle atrophy and a failure to gain weight is a feature of the fragment and knock-in models, as it is of the human disease, the BACHD and YAC128 mice gain weight, which has been shown to be consequence of the expression of more than two copies of full length HTT (Van Raamsdonk et al., 2006).

Comparisons between models can only usefully be made when performed in parallel in the same laboratory by the same individuals, of which there are few examples. A side-by-side comparison of motor and cognitive abilities in the R6/2, BACHD, YAC128 and the HdhQ111 knock-in mice confirmed the reproducibility of the R6/2 phenotypes and showed that BACHD mice developed a robust and progressive phenotype, whilst that in the YAC128 mice was milder and those of the HdhQ111 line were extremely mild (Menalled et al., 2009). An independent comparison of the YAC128 and BACHD lines indicated that although they displayed similar behavioural phenotypes, the aggregate pathology in the BACHD mice was much less prominent than in the YAC128 mice and although the levels of HD-associated genes were decreased in YAC128 mice, they were unaltered in the BACHD model (Pouladi et al., 2012), potentially reflecting the lack of nuclear pathology. A careful comparison of the appearance of aggregate pathology in the striatum and cortex between a wide range of models showed that this correlated with the age of onset and severity of the phenotypes that developed (Wang et al., 2008a) (Figure 16.1). The Cardiff group have been performing an extremely detailed behavioural, neuropathological and molecular longitudinal
characterisation of several HD mouse models. Of those studied, they conclude that the 
HdhQ150 demonstrates the most similarity to the functional deficits observed in human HD 
(Brooks et al., 2012d).

One comparison that presented surprising results was the similarity between 
symptomatic R6/2 mice at 12 – 14 weeks and HdhQ150 knock-in mice at 22 months of age. 
Aggregate pathology was present throughout the entire brain (Woodman et al., 2007) and 
periphery (Moffitt et al., 2009) in both models indicating that this widespread pathology in 
line R6/2 had not arisen as a consequence of expressing an N-terminal fragment, as had 
been assumed. Striatal gene expression profiles were highly comparable to each other and 
to HD post mortem brain (Kuhn et al., 2007) and both models developed an impairment in 
their ability to induce a heat shock response (Labbadia et al., 2011). The main difference 
between the models is the age at phenotype onset and the rate of phenotype progression, 
consistent with the hypothesis that it is an N-terminal fragment of HTT that is the pathogenic 
species in the knock-in mice (Landles et al., 2010).

It has now been shown that exon 1 of the mutant huntingtin gene does not always 
splice to exon 2 resulting in a small exon 1 – intron 1 polyadenylated transcript that is 
translated to produce an exon 1 HTT protein. The presence of the mis-spliced product could 
be detected in all knock-in mouse models, in the YAC128 and BACHD transgenic lines as well 
as in patient tissue. Therefore, all HD mouse models that carry a full length version of the Htt 
or HTT gene express an exon 1 HTT protein (Sathasivam et al., 2013).

Mammalian HD models (non-mouse)

HD models have now been generated using a range of mammalian species including rats 
(von Horsten et al., 2003; Yu-Taeger et al., 2012), sheep (Jacobsen et al., 2010), pigs (Yang et
al., 2010) and the rhesus macaque non-human primate (Yang et al., 2008) and are summarised in Table 16.2.

A rat transgenic model of HD expressing a truncated protein of approximately 654 amino acids with 53 glutamines was developed (von Horsten et al., 2003). These animals fail to gain weight and a clear aggregate pathology can be detected at six months of age, which precedes motor deficits (Nguyen et al., 2006). The HD rats develop cortical and striatal pathology (Kantor et al., 2006) and decreased spontaneous activity of striatal neurons has been reported (Miller et al., 2010). A wide variety of phenotypes have been documented including a deterioration in reaction time (Cao et al., 2006), mild cognitive deficits (Brooks et al., 2009), sleep disturbances (Bode et al., 2009), memory deficits (Zeef et al., 2012) and altered emotional and motivational processing (Faure et al., 2011). However, a recent detailed study reported that although it was possible to observe modest but clear cut deficits in motor performance (more pronounced in males), a robust cognitive impairment on a range of tasks sensitive to frontostriatal function, as would be required for testing therapeutics, could not be identified (Fielding et al., 2012). More recently, a BACHD transgenic rat has been generated that expresses full length human HTT with 97 glutamines (Yu-Taeger et al., 2012). In contrast to the YAC and BACHD mouse models, the BACHD rats do not gain weight. They displayed early-onset and progressive motor deficits and anxiety-related symptoms with the early detection of neuropil aggregates in the cortex and amygdala, and neurodegeneration apparent at by 12 months of age (Yu-Taeger et al., 2012).

A transgenic sheep model was generated by microinjection of a human HTT cDNA construct containing 73 glutamines under the control of human HTT promoter sequences (Jacobsen et al., 2010). The transgene is expressed in CNS and non-CNS tissues and there was evidence for a reduction in DARPP-32 staining in the striatum of a 7 month old
transgenic sheep (Jacobsen et al., 2010). Next generation sequencing has recently been performed on DNA from the five founders indicating that complex rearrangements have occurred at the integration site and only three of the five founders would be expected to express the transgene (Chiang et al., 2012). The developmental pattern of the ovine basal ganglia and cortex is similar to analogous regions of the human brain and it is anticipated that these animals will be particularly useful for the assessment of neuropathology and of the delivery of agents designed to lower HTT levels. In preparation for the behavioural assessment of the HD sheep, it has been shown that sheep can perform ‘executive’ cognitive tasks that are an important part of the primate behavioural repertoire (Morton and Avanzo, 2011).

Pigs that are transgenic for an N-terminal fragment of HTT with 105Q have been generated by nuclear transfer (Yang et al., 2010). Of five transgenic piglets, the three with the highest transgene expression died within 60 hours of birth. Examination of the brains showed evidence for apoptosis. Similarly, the generation of non-human primates expressing an exon 1 construct with 84Q under the control of the ubiquitin promoter was quite severe (Yang et al., 2008). Of five newborn monkeys, one survived for one month and two for less than a day. The four expressing transgenes with expanded glutamine repeats displayed neurological phenotypes and an aggregate pathology was present at post mortem (Yang et al., 2008).

**Mouse models designed to investigate specific disease mechanisms**

In addition to generating mouse models that recapitulate the human disease, genetically modified mice have been designed to probe specific pathogenic mechanisms.
In order to discover what would happen to the course of HD-related phenotypes in a mouse if the mutant protein was removed at specific stages, a mouse model in which it was possible to switch off an exon 1 transgene was generated (Yamamoto et al., 2000). Consistent with previous models, mice expressing the mutant HTT transgene developed a progressive neurological disorder with motor impairment, striatal atrophy, decreased dopamine receptor binding and an aggregate pathology. At a specific stage of disease, the transgene was switched off in one group of mice, whilst the disease was allowed to develop further in a second matched group. Switching off the transgene had remarkable consequences. In less than three weeks, the aggregate pathology had cleared from the brain and the motor impairment was no longer present (Martin-Aparicio et al., 2001; Yamamoto et al., 2000). This was the first indication that there might be stages of the disease at which symptom reversal could occur in response to a suitable therapeutic intervention.

Experiments in cell culture had suggested HTT exerts its pathogenic effects in the cell nucleus (Saudou et al., 1998). Two groups tested this hypothesis in vivo and examined the extent to which nuclear and extranuclear mutant huntingtin might contribute to specific phenotypes in HD (Benn et al., 2005; Schilling et al., 2004). Schilling et al. showed that directing the N171-82Q construct to the nucleus generated similar phenotypes to N171-82Q mice (that had both nuclear and extranuclear pathology), and concluded that disruption of nuclear processes could account for many of the disease phenotypes (Schilling et al., 2004). Benn et al. generated a more extensive set of transgenic lines expressing the R6 exon 1 construct that had been tagged with a nuclear localisation signal (NLS), nuclear export signal (NES) or non-functional versions of these tags as controls (Benn et al., 2005). Surprisingly, an NLS-tagged exon 1 transgene with 20Q was never seen in the nucleus, confirming the presence of a strong nuclear export signal in the first 17 amino acids (Cornett et al., 2005).
However, mutant versions of exon 1 were retained in the nucleus because they formed detergent insoluble complexes. These studies revealed that driving mutant HTT into the nucleus accelerates phenotype onset, that exon 1 HTT is only present in the nucleus as an aggregated form of the protein and that nuclear mutant HTT is sufficient to cause axonal degeneration. However, the presence of mutant HTT in the cytoplasm was necessary for the expression of all of the HD-related phenotypes in mouse (Benn et al., 2005).

It had been shown that HTT serine residues S13 and S16 in could be phosphorylated in cell culture (Thompson et al., 2009). To explore the relevance of this in vivo, mice that were transgenic for BAC clones in which S13 and S16 had either been mutated to alanine or aspartate (to mimic phosphorylation) residues were generated (Gu et al., 2009). The neurological and neuropathological phenotypes were abolished in the aspartate mutated mice but retained in the alanine mutants. The authors showed that mutation of S13 and S16 to aspartate inhibited aggregation and fibril formation in vitro (Gu et al., 2009) suggesting that this might underlie the beneficial effects observed in vivo.

The processing of HTT to generate pathogenic HTT fragments has been proposed to be an early event in HD pathogenesis and mouse models have been used to determine which fragments might be pathogenic. One study investigated whether huntingtin processing at caspase 3 sites (513 and 552) or at the caspase 6 site (586) are important disease initiating molecular events (Graham et al., 2006). YAC transgenic lines were generated in which either both of the caspase 3 sites or the caspase 6 site had been mutated to render them inactive. Mutation of the caspase 3 sites had no effect on the onset and progression of disease phenotypes. However, mice expressing mutant huntingtin that is resistance to cleavage by caspase 6 maintained normal neuronal function and did not develop striatal neurodegeneration (Graham et al., 2006). Following on from this, the HTT-
586 caspase 6 fragment was expressed as a transgene under the control of the prion promoter in two independent studies (Tebbenkamp et al., 2011a; Waldron-Roby et al., 2012). In both cases, the caspase 6 fragment caused neurological and neuropathological phenotypes, large cytoplasmic aggregates were observed in the brain and the caspase 6 product was subject to further proteolysis, confirming that this fragment can be pathogenic. However, two subsequent studies have demonstrated that the pattern of proteolytic cleavage products does not change in either the BACHD mice (Gafni et al., 2012) or the HdhQ150 knock-in mice (Landles et al., 2012) when mutant HTT is crossed onto a caspase 6 knock-out background, raising the question as to whether the HTT-586 fragment is generated by a protease other than caspase 6.

Finally, mice in which an exon 1 HD transgene can be switched on in specific neuronal or non-neuronal cell populations has used to ask whether neuronal dysfunction and/or neuronal cell death occurs through cell autonomous processes or through cell-cell interactions. In two elegant studies, these mice were used to demonstrate that neuronal dysfunction is dependent upon cellular interactions (Gu et al., 2007; Gu et al., 2005). They show that a pathological interaction between cortical interneurons and pyramidal cells may be important for the cortical manifestation of the disease (Gu et al., 2005). Expression of mutant HTT in only the striatal medium spiny neurons is not sufficient for striatal pathogenesis (Gu et al., 2007) and the authors propose a “two-hit” model of HD in which both cell-autonomous toxicity and pathological cell-cell interactions are critical to HD pathogenesis. These results were confirmed by an independent experiment in which the expression of mutant HTT in striatal neurons was used to dissect cell autonomous and non-cell autonomous aspects of the pathogenic process (Kim et al., 2011). Finally, expression of mutant HTT with 160Q in astrocytes alone was shown to cause a neurodegenerative
phenotype (Bradford et al., 2009) whereas as the glial expression of HTT with 98Q did not show obvious abnormalities (Bradford et al., 2010). However crossing the 98Q mice to the N171-82Q HD model exacerbated the phenotype thereby underlining the importance of the contribution of glia to the pathogenesis of HD (Bradford et al., 2010).

**Are the mouse models relevant to the human disease?**

In all cases, the widely-used mouse models carry CAG repeat sizes that would cause an early childhood form of the HD in humans. Might they therefore, be models of juvenile rather than adult onset HD? Juvenile HD presents with Parkinsonian features e.g. rigidity, bradykinesia and tremors known to be associated with a more widespread neuropathology (that includes the cerebellum) than the adult onset form of the disease (Squitieri et al., 2003). Both the R6/2 and the HdhQ150 knock-in models develop a very widespread aggregate pathology that is distributed throughout the entire brain and periphery (Moffitt et al., 2009; Woodman et al., 2007). Similarly, at the molecular level, both the R6/2 and HdhQ150 knock-in mice have a highly comparable profile of cerebellar transcriptional dysregulation (Bates and Luthi-Carter, unpublished data) but there are very few transcriptional changes in the cerebellum of adult onset HD brains (Hodges et al., 2006). Might the cerebellar profiles of childhood HD cases better correlate with the mouse data? It is very difficult to answer these questions, as so little is known about the molecular pathology of juvenile HD.

One criterion by which the success of a disease model might be judged is whether it has predicted aspects of the human disease, of which the scientific community was previously unaware. To date, most insights into HD pathogenesis have come through the study of the R6/2 mouse predominantly because the rapid onset and progression of the
phenotype in this model has facilitated a large number of studies, described in more than 200 research papers.

Impairments in neurotransmission and in mitochondrial function are aspects of HD pathogenesis that were known prior to the identification of the HD mutation and it was not a great surprise to find that these functions are also disturbed in HD mouse models (Beal, 2005; Cepeda et al., 2007). However, it was remarkable that proteinaceous inclusion bodies were not described in HD patient brains (DiFiglia et al., 1997) until after they were discovered in the R6 mice (Davies et al., 1997). This is most likely because, unlike in the case of Alzheimer’s disease and Parkinson’s disease, they cannot be observed with classical histological stains and their immunohistochemical detection requires the use of an antibody raised against the extreme N-terminus of HTT. Transcriptional dysregulation was unknown as a pathogenic process in HD until it was proposed by Jang Ho Cha in 1998 after he discovered the selective down regulation of genes encoding specific neurotransmitters in the brains of R6/2 mice (Cha et al., 1998). This is now recognised as an early and highly reproducible molecular pathology in HD (Luthi-Carter and Cha, 2003) of which both the R6/2 and knock-in mice have proven to be excellent models (Hodges et al., 2006; Kuhn et al., 2007). A disturbance in hypothalamic-pituitary-adrenal (HPA) axis has been identified in R6/2 mice (Bjorkqvist et al., 2006) and although hypothalamic atrophy was described in HD post mortem brains many years ago (Timmers et al., 1996), this had not been studied further. The analysis of mouse models has the potential of unraveling the hypothalamic and neuroendocrine disturbances that occur in HD (Petersen and Bjorkqvist, 2006; Petersen et al., 2005). Finally, circadian rhythm disturbances in the R6/2 mice were found to be one aspect of sleep disturbance in HD patients (Morton et al., 2005). It seems highly probable that phenotypes that develop in the HD mouse models might lead us to more subtle, but
potentially highly relevant aspects of the human disease. However, one aspect of HD that the mouse models do not replicate, is the extensive striatal cell death present in most post-mortem brains, and although cell loss has been reported in the R6/2, R6/1, YAC128 and 
HdhQ150 lines (Brooks et al., 2012d; Slow et al., 2003; Stack et al., 2005), it only represents a fraction of that seen in the human disease.

**Preclinical Validation of Therapeutic Targets for HD**

**Identification of Therapeutic Targets for Huntington’s Disease**

The development of HD models provided a means by which approaches to modulating HD-related phenotypes could be explored. Single cell (S. *cerevisiae* and mammalian cell-culture) and invertebrate (C. *elegans* and D. *melanogaster*) models are amenable to high-throughput analyses using whole-genome genetic screens and / or pharmaceutical compound libraries (Table 16.3). *In vitro* approaches have been used to identify proteins that interact with HTT (Goehler et al., 2004; Kaltenbach et al., 2007), some of which have been shown to modify HD phenotypes in a *Drosophila* model (Kaltenbach et al., 2007). These studies, combined with the results of hypothesis-driven genetic and / or pharmacological manipulations (e.g. histone deacetylases (Pallos et al., 2008)) have generated in the order of 100 potential HD-therapeutic targets (Kwak et al., 2011) which when manipulated in single cell of invertebrate HD-models have beneficial effects on an HD-related phenotype.

The clinical assessment of disease-modifying treatments for HD is complex and extremely expensive. The combination of a slowly progressing disease, considerable patient-to-patient variability and subjective assessment measures has meant that the phase III clinical efficacy trials that have been performed to date have been considerably
underpowered (Huntington Study Group, 2001; Landwehrmeyer et al., 2007). Initiatives to develop quantitative assessment measures and to improve the power of clinical efficacy trials for HD are in progress (see Chapter 17). However, irrespective of the extent to which these clinical studies can be optimised, the expectation that clinical-grade therapeutics could be developed to test the validity of each of these targets in HD patients is completely unrealistic. The process of target validation must take place in the context of the mammalian brain and therefore, rodent models of HD have the potential to provide an intermediate step in this process. The ease with which it has been possible to manipulate the mouse genome in recent years means that many sophisticated tools can be accessed for this purpose. Once therapeutic targets have been validated in HD rodent models, the decision as to whether a target should be selected for clinical development will depend on a combination of the potential disease-modifying properties of a target and its tractability in drug development terms (Kwak et al., 2011).

**The importance of standardising preclinical assessments**

It is of major concern that preclinical studies for a wide range of neurodegenerative diseases have failed to predict efficacy in the clinic. It is certainly true that in some cases, this is the consequence of the use of an inappropriate model system. However, inadequately designed preclinical studies are also a major contributing factor (Brunner et al., 2012). This has led to calls by the federal funding agencies in both the US and UK for the rigorous standardisation and transparent reporting of preclinical trials (Kilkenny et al., 2010; Landis et al., 2012).

The problem was highlighted in the paper ‘Design, power, and interpretation of studies in the standard murine models of ALS’ (Scott et al., 2008). Although more than 50 publications had reported therapeutic agents that extend the lifespan of the SOD1$^{G93A}$
transgenic mice, that carry a mutation that causes familial amyotrophic lateral sclerosis (FALS), riluzole remains the only agent that has shown clinical efficacy. Over a period of five years, Scott et al. screened 70 drugs in 18,000 mice across 221 studies. They then used computer modelling and statistical analysis of the 5429 SOD1^{G93A} mice tested in their efficacy trials to quantify the impact of several critical confounding variables that must be controlled for when designing and interpreting efficacy studies. These included: litter of origin, the number of copies in the transgene array, gender and deaths due to non-ALS causes. Based on these data, they optimised a study design for the SOD1^{G93A} mice, retested compounds that had been reported to show efficacy in previous studies (minocycline, creatine, celecoxib, sodium phenylbutyrate, ceftriaxone, WHI-P131, thalidomide, and riluzole) and found no survival benefit for any of these compounds at the previously reported efficacious routes and doses. (Riluzole also provided no improvement in survival, but given the very small extension of lifespan that this bestows on ALS patients, the mouse studies could only have been underpowered to detect a comparable effect). The failure of these drugs to demonstrate efficacy, in repeat studies that were adequately designed and powered, led the authors to conclude that the majority of published effects were measures of noise in the distribution of survival means as opposed to a drug effect. They were able to recommend a minimum study design that would largely eliminate this inherent noise and should facilitate more meaningful and reproducible results.

It is therefore essential that potentially confounding variables for the preclinical studies that are performed in HD mouse models are understood and controlled for. A number of factors should be considered when distributing mice across treatment groups. Litter of origin has been reported to be a source of variability in the R6/2 line (Hockly et al., 2003) and therefore, as with the SOD1^{G93A} mice, it is prudent to distribute mice from the
same litter between groups. The length of the CAG repeat is a known modifier of phenotype
onset and progression and therefore, the mean and standard deviation of the CAG repeat
size for each of the groups must always be stated, and if possible should be matched. For
both the R6/1 and R6/2 lines, there is a single functional copy of the transgene at the
integration site (Mangiarini et al., 1996), however, in models where this might not be the
case, the possibility of differences in transgene copy number must be taken into
consideration. If possible, all of the mice that will be entered into behavioural trials should
be born over a period of two to three days to ensure that the assessment of all mice can
occur simultaneously. Although the study design may not include the treatment of wild type
mice, a wild type vehicle group should be included, so that in the event of an improvement,
the effect size can be determined. It goes without saying that a study should be adequately
powered so that there is a realistic possibility of detecting a statistically significant
improvement for any given outcome measure. Obviously, the number of animals required
will be dependent on the variability between animals in performing a particular task and so
will need to be powered for the measure that requires the largest number of animals.

The administration of the therapeutic agent should be performed by someone other
than the person performing the efficacy assessment to ensure that the operator is blind to
treatment. Throughout a longitudinal preclinical trial, the housing and husbandry should be
standardised such that each mouse has the same food and water provision. In the case of
R6/2 mice, they ‘do better’ when housed with wild type mice than if housed singly, or with
other R6/2 animals, therefore all cages should contain an equivalent mix of genotypes.
Environmental enrichment is the one intervention that has been shown to have beneficial
effects across multiple mouse models of HD in several laboratories (Hockly et al., 2002;
Nithianantharajah and Hannan, 2006; van Dellen et al., 2000; Wood et al., 2011). Therefore,
it is essential that mice in all treatment groups are exposed to the same degree of environmental enrichment and handling. Given that phenotypic improvements can be achieved by modest changes to the home-cage environment, it would seem sensible to test interventions on environmentally enriched mice, although this has been a matter of debate. Finally, the potential of circadian influences should be taken into consideration and the assessment of mice in each treatment group should be distributed throughout the day (or dark cycle) as appropriate.

All preclinical compound studies should include a pharmacokinetic (PK) analysis of the compound in question in the stable lines of mice that will be used for the study. The PK profile of compounds can be very different in mice compared to that in humans (Andes and Craig, 2002) and so a PK profile that is known for humans cannot be extrapolated to mice. All studies should minimally provide the brain and plasma concentrations of compound over a time course given via the intended route of administration. If possible, pharmacodynamic (PD) studies will be performed to demonstrate that the compound achieves the required biological response in the target tissue e.g. a decrease in phosphorylation in response to a kinase inhibitor or an increase in chromatin acetylation in response to a class I HDAC inhibitor. Without PK and PD data it is not possible to interpret a negative (or positive) result.

**HD mouse models used for preclinical studies**

The models that are most frequently used for preclinical therapeutics include the R6/2, R6/1 and N171-82Q HTT fragment models. Of these the R6/2 mice are the most widely used reflecting the fact that R6/2 phenotypes are highly reproducible between studies and between laboratories (Carter et al., 1999; Hickey et al., 2005; Hockly et al., 2003; Lione et al.,
1999; Menalled et al., 2009; Rattray et al., 2013). However, adequately powered studies can also easily be performed in the R6/1 and N171-82Q mice provided attention is paid to the potentially confounding variables as discussed above.

Preclinical studies in full-length HTT models have been largely restricted to the YAC128 line and CAG140 knock-in model. Although some investigations have indicated that the YAC128 line would have limitations for preclinical work (Menalled et al., 2009), others have used it in a number of studies. In most cases, the age at which statistically significant improvements were reported to occur was at approximately 12 months of age (Pouladi et al., 2012; Van Raamsdonk et al., 2005), although improvements in motor performance have been reported from 9 months onwards (Wang et al., 2010). Efficacy trials in the CAG140 mice have mostly focussed on relatively subtle motor impairments in young mice (Hickey et al., 2012a; Hickey et al., 2012b) and the search for robust phenotypic read-outs in knock-in models of HD with good power to detect improvements is still in progress (Heikkinen et al., 2012; Menalled et al., 2012).

Transcriptional dysregulation is a pathogenic consequence of the HD mutation that occurs early in disease progression (Cha, 2007) and HD-related dysregulated transcriptional signatures have been well-defined and are consistent between HD patient brains and a variety of HD mouse models (Kuhn et al., 2007). The gene that encodes the dopamine D2 receptor is consistently down-regulated and a line of mice that presents an accurate reporter assay for the level of expression of the mouse Drd2 gene has recently been developed (Crook and Housman, 2012). The system uses a fluorescent-activated cell sorting (FACS) assay as a highly quantitative readout for this pathology in mouse neurons expressing a GFP reporter under the control of the Drd2 promoter in the context of mutant HTT. It has very good statistical power to measure improvements in the dysregulation of Drd2 of as little
as 20% or less in response to HD therapeutics, not only in R6/2 and R6/1 mice, but also in the CAG140 and HdhQ111 knock-in mouse lines (Crook and Housman, 2012). This is the first screening system of its kind that will allow the opportunity to rapidly and systematically screen compounds in vivo at multiple doses and time points.

Genetic approaches to the validation of therapeutic targets for using HD mouse models

One approach to testing the therapeutic potential of a target is to manipulate the levels of the gene of interest in HD mouse models and look for phenotypic modifications. Table 16.4 summarises the genetic crosses that have been performed for this purpose. A vast range of well-characterised constitutive and conditional knock-out lines, as well as transgenic mice, are already available through mouse repositories such as the Jackson Labs (http://www.jax.org/) or through individual collaborations. Initiatives are in progress, under the auspices of the International Knock-out Mouse Consortium (IKMC) to mutate all protein-coding genes in the mouse through combination of gene trapping and gene targeting methodologies in C57BL/6 mouse embryonic stem cells (http://www.knockoutmouse.org/about). Therefore, going forward, there will be a reasonable probability that a conditional knock-out mouse for any ‘gene of interest’ will already exist, or that that the targeted ES cells will be in place with which to create one, thereby obviating the expensive and time consuming hurdle of creating the resources with which to manipulate the levels of specific genes in HD mice.

There are both conceptual and practical issues associated with the use of constitutive knock-out mice for this purpose. The possible phenotypic outcomes of the constitutive knock-out of a particular gene can range from early embryonic lethality to no discernible effect. In the first instance, it will not be possible to transfer the mutated Htt gene onto a
null background and therefore, transfer onto a heterozygous knock-out background may be the only available option. This might be considered a good strategy, as the ‘knock-down’ of the levels of a gene product are more likely to correlate to the effects of a pharmacological intervention than a complete knock-out. However, prior to initiating the breeding for such experiments, it is prudent to ensure that both the gene and protein in question are expressed at reduced levels in the heterozygous knock-out mice. In some cases, autoregulation of the heterozygous allele, e.g. *Hdac1*, ensures that it is expressed at wild type levels (Schuettengruber et al., 2003).

In the event that the knock-out of a gene does not result in a phenotype, it will always be of concern that other related genes are providing a functional redundancy. These concerns can be alleviated if the expected biological consequences of the knock-out or knock-down of a gene of interest can be detected, i.e. the equivalent of a pharmacological dynamic read-out in response to a pharmacological intervention. For example, *Hdac6* knock-out mice appear to be indistinguishable from wild type mice (Zhang et al., 2008). HDAC6 is a tubulin deacetylase, and although in the absence of *Hdac6*, the levels of tubulin acetylation were found to increase by approximately five-fold throughout the brain, this had no effect on the formation of aggregates and did not modulate any other phenotypes in the R6/2 mice (Bobrowska et al., 2011). We can state with some confidence that the lack of phenotype modification in *Hdac6* knock-out mice is not due to compensatory effects and that tubulin acetylation does not influence HTT aggregation (Bobrowska et al., 2011). However, the function of a gene in wild type mice is not always understood with certainty. SIRT2 had been proposed from experiments in cell culture to be a tubulin deacetylase (North et al., 2003) and to regulate the expression of genes that are involved in the cholesterol biosynthesis pathway (Luthi-Carter et al., 2010). *Sirt2* knock-out mice are indistinguishable from wild
type, and the absence of SIRT2 did not change the level of acetylated tubulin in either brain or liver and had no effect on the expression level of the cholesterol synthesis genes (Bobrowska et al., 2012). Are these observations the result of functional redundancy, or because the function of SIRT2 is not understood? Whichever of these might be the case, the lack of an effect of Sirt2 knock-out on HD-phenotypes is difficult to interpret. In this case, the experiment was complemented with a pharmacological intervention (Bobrowska et al., 2012) to avoid the potentially confounding compensatory effects that might occur during mouse development, although crossing to a conditional knock-out Sirt2 line could have achieved the same purpose.

Conditional knock-out mice are designed such that a portion of the gene is deleted in the presence of Cre, or an alternative recombinase, and therefore these targeted alleles can be used to generate either constitutive or conditional knock-out mice dependent on the Cre driver used (e.g. the European conditional mouse mutagenesis program (EUCOMM) http://www.knockoutmouse.org/about/eucomm). A constitutive knock-out can be generated by crossing to mice that express Cre in the germ line. Mice transgenic for Cre expressed under the control of cell-specific promoters can be used to generate cell or tissue-specific knock-outs, thereby circumventing embryonic or perinatal lethality by avoiding gene knock-out in cells for which the expression of the gene is essential. Finally, the expression of Cre can be induced pharmacologically. Fusion of the Cre recombinase to a mutant form of the mouse estrogen receptor (CreERTM), which does not bind its natural ligand (17β-estradiol) at physiological concentrations but will bind synthetic estrogen receptor ligands (e.g. tamoxifen) (Feil et al., 1996), provides a means of generating knock-outs in adult mice, thereby avoiding the establishment compensatory effects during development. There are now more than 500 Cre driver lines (Nagy et al., 2009) and it should be possible to design
genetic experiments that will be informative as to the relevance of potential HD therapeutic targets. However, genetic crosses that involve the segregation of three independent alleles: mutant Htt (hemizygous), a specific Cre transgene (hemizygous) and a targeted gene of interest (that must be homozygous) are long and expensive, and require the production of many mice to ensure that sufficient numbers of each of the desired genotypes are recovered. It would be wise to determine whether constitutive knock-out approaches could be informative for a ‘gene of interest’, and if not, whether there might be a pharmacological agent that could be used prior to embarking on a conditional knock-out strategy.

As in all preclinical efficacy experiments, the breeding and assessment of mice must be rigorously standardised such that each mouse is exposed to a comparable environment and levels of handling (see above). The breeding strategy should be designed so that all of the genotypes to be studied are born as littermates and mice should be weaned into cages so that they contain an equivalent distribution of genotypes. The inbred strain background of the various lines must also be taken into consideration to ensure that any effects observed are a consequence of the manipulation of the ‘gene of interest’ and are not due to the skewed distribution of inbred strain-specific modifier alleles. The simplest means of avoiding strain effects is to ensure that all of the lines involved are on the same inbred background (usually C57BL/6). However, this is not necessarily desirable because of the deleterious phenotypes frequently associated with inbred strains and, for this reason, the Banbury Conference on Genetic Background in Mice (Silva et al., 1997) recommended that neurological mutants should be studied on F1 rather than inbred backgrounds. It is also often not practical to standardise strain backgrounds, in which case steps should be taken to avoid an unequal distribution of inbred-strain alleles as follows: (1) specific colonies should be maintained by backcrossing to inbred strains or F1 hybrids to avoid fixing alleles, for this
reason, brother – sister matings should always be avoided (2) when establishing a genetic cross to provide the genotypes for analysis, the use of a small number of males for breeding should be avoided (3) a large number of litters from which mice can be selected should be generated (4) in the event of phenotypic improvements, the entire experiment should be repeated at least once to ensure that the results can be replicated. The ES cell lines that were traditionally used for generating knock-out mice were derived from 129 inbred sub-strains and once germ line transmission for the knock-out allele had been established, mice were generally backcrossed to C57BL/6 mice. However, irrespective of the number C57BL/6 backcross generations, the targeted allele for the ‘gene of interest’ is likely to reside in a segment of 129 DNA. Therefore, in this situation, beneficial effects that occur when breeding to mouse models of HD, could either be due to the intended manipulation of the ‘gene of interest’ or to a closely linked 129-modifier. This possibility can be ruled out through the identification of single nucleotide polymorphisms (SNPs) that are linked to the gene-targeted allele of the ‘gene of interest’ between, in this case, the 129 and C57BL/6 substrains. If the HD mouse model is bred to the relevant 129 substrain and the presence of the linked SNP allele does not confer benefit, a 129 modifier effect can be ruled out.

The use of transgenic or genetically manipulated mouse lines to validate therapeutic targets is a low-throughput, slow procedure. In order to accelerate this process, viral vectors have been used to either overexpress or knock-down genes of interest in the brains of HD mouse models (Table 16.5). The constructs are generally injected into the striatum and the extent of the brain that is targeted by this approach can be quite limited, often not extending much beyond the striatum itself. This can be a powerful method of assessing the effect of manipulating a ‘gene of interest’ on brain region and cell specific molecular or histological read-outs. Interpretation of the effects of these interventions on behaviour
should be treated with more caution, as the neuropathological correlates of the disease-specific behaviours observed in mouse models of HD are generally not known. These studies could also be considered as preclinical evaluations of gene therapies although progression to the clinic is complicated by issues related to gene delivery as discussed in the following section on HTT lowering strategies.

**Preclinical Evaluation of Pharmacological and Biological Agents**

The comprehensive list of compounds that have been assessed for efficacy in HD mouse models is detailed in Table 16.6. These have predominantly been tested in the R6/2 or N17182Q mouse models that are transgenic for N-terminal fragments of HTT. A small number have been assessed in the YAC128 line and CAG140 knock-in mice (see above).

The preclinical testing of potential therapeutic compounds in mouse models of HD has been in progress for more than 12 years, and yet serious candidates for disease modifying treatments have not been identified and are not waiting in the wings to be assessed in clinical trials. Why should this be the case if so many compounds have been reported to show beneficial effects, and what degree of preclinical evidence is required to support the development of a drug development program for a particular target? It has been previously suggested that efficacy should be demonstrated in more than one HD mouse model and that this should be replicated in a separate laboratory (Bates and Hockly, 2003). The requirement for a treatment to be efficacious in at least two mouse models seems reasonable. Until a disease-modifying treatment for HD has been identified, there are no tools with which to assess the predictive value of our mouse models. However, the similarity in the late-stage phenotypes between R6/2 and HdhQ150 knock-in mice and the recent discovery that all of the models express an exon 1 protein (Sathasivam et al., 2013) may
indicate that the mechanistic basis of HD pathology in all of the models is more similar than was once anticipated. The replication of beneficial effects in an independent laboratory is essential, but is difficult to co-ordinate in an academic setting and this aim can best be served by establishing a highly standardised platform (Scott et al., 2008), most likely operated within a contract research organisation.

Scanning Table 16.6 reveals a number of instances in which multiple trials of the same compound have been resulted in conflicting outcomes and two examples of this will be discussed below. In 2003 it was reported that the systemic treatment of R6/2 mice with Congo red caused a reduction in the number of striatal inclusions and an improvement in motor symptoms and survival (Sanchez et al., 2003). These results were surprising as Congo red is known not to cross the blood brain barrier. A careful study not only failed to replicate these results, but also extended the analysis to include cognitive measures, also with negative outcomes (Wood et al., 2007). The preclinical evaluation of minocycline has been performed by a number of laboratories. In the first report of minocycline efficacy, it was administered by intraperitoneal injection to R6/2 mice at 5 mg/kg daily resulting in improvements in motor performance (n = 12 / group) and survival (n = 7 / group) (Chen et al., 2000). A follow up study using the same dosing strategy for minocycline alone or in combination with coenzyme Q₁₀ (n = 10 / group) confirmed the improved motor performance and survival as well as documenting neuropathological benefits (Stack et al., 2006). Following on from an organotypic slice culture study, R6/2 mice (n = 13 / group) were treated with minocycline, as an aggregation inhibitor, in the drinking water and despite achieving brain concentrations of 20 µM, no beneficial effects were observed (Smith et al., 2003). A more comprehensive study was finally provided by Menalled et al., applying their best-practise procedures (Menalled et al., 2009). R6/2 mice were treated with minocycline
by intraperitoneal injection at 5 mg/kg/day (n ≥ 16 / group) or in the food at 0.1% or 0.375% (n ≥ 22/ group). There were no beneficial effects, and mice receiving minocycline in the food had a shortened lifespan (Menalled et al., 2010). The many factors that may contribute to the different outcomes in these studies are discussed at length in the Menalled paper and will not be reiterated here. Minocycline has been studied in HD patients in clinical trials. In one, 11 patients who were monitored for 24 months showed amelioration of psychiatric symptoms and a stabilisation of motor and neuropsychological function (Bonelli et al., 2004). Another study showed that 200 mg/kg per day minocycline is well-tolerated (Thomas et al., 2004). Finally, a double-blind multi-centre study (DOMINO) did not find a meaningful slowing of the rate of functional decline and concluded that further study of minocycline at 200 mg/kg/day is not warranted (http://www.huntington-study-group.org/ClinicalResearch/CompletedClinicalTrials/DOMINOResults/tabid/104/Default.aspx).

Finally, the importance of complementing preclinical efficacy studies with pharmacokinetic data cannot be emphasised too greatly. The effects reported for the HDACi4b compound (Thomas et al., 2008) were brought into question, when a detailed pharmacokinetic analysis of this compound found that its physicochemical properties, metabolic and pglycoprotein (Pgp) substrate liability rendered it suboptimal for use in vivo through oral administration (Beconi et al., 2012).

Replication studies for many of the compounds that have been reported to show benefit in HD mouse models have been performed on a standardised best-practice platform at Psychogenics Inc. with negative results in most cases (Brunner et al., 2012). It is possible that many of the preclinical evaluations of therapeutic approaches in HD mouse models could be measures of noise, as was the case for ALS (Scott et al., 2008). On a positive note, the research community is now much more aware of the factors that are important in
preclinical trial design than it was more than 10 years ago when the preclinical evaluation of compounds in genetic models of HD was initiated. Minocycline was one of the first compounds reported to show beneficial effects in HD mice, and the enthusiasm for progressing this to the clinic, without first replicating the results in a much larger study on a standardised platform would be unlikely to happen today.

**Strategies aimed a lowering HTT levels**

The *HTT* mutation results in the dysregulation of multiple molecular and cellular pathways very early in the disease process (Chapter 13). It is possible that targeting specific pathways will be insufficient to slow the course of the disease and provide a significant clinical benefit. In contrast, directly targeting the mutant gene or protein holds the promise of delaying the onset or slowing the progression of the entire spectrum of HD symptoms. The advent of oligonucleotide approaches such as RNA interference (RNAi) and antisense oligonucleotides (ASOs) provide one set of therapeutic strategies that can be developed toward this aim (Boudreau and Davidson, 2012; Sah and Aronin, 2011). Alternatively, the HTT protein can be directly targeted through the development of intrabodies (Butler et al., 2012).

**Oligonucleotide Approaches**

RNAi approaches exploit the endogenous microRNA (miRNA)-based mechanism for the regulation of gene expression (Figure 16.2). Endogenous miRNAs are small non-coding RNA molecules that are derived from a stem loop structure encoded by nuclear DNA. The stem loop is processed by the Drosha complex to a pre-miRNA structure that is exported from the nucleus and further processed to miRNA by the DICER complex. miRNAs function via base-pairing with complementary sequences within mRNA molecules, usually resulting in
gene silencing via translational repression or target degradation. The miRNAs generally exhibit only partial complementarity to their mRNA targets and target specificity is governed by a seed region of approximately 6-8 nucleotides at the 5’ end of the miRNA. The mature miRNA becomes part of an active RNA induced silencing complex (RISC), through which one strand of the miRNA interacts with the target mRNA resulting in either the direct cleavage of the target transcripts or the recruitment of additional proteins to achieve translational repression. This system can be harnessed for therapeutic purposes by the viral delivery of shRNAs to the nucleus, where they are expressed and as a pre-miRNA, exported and processed by Dicer. Alternatively, siRNAs enter the cell where they are incorporated into the RISC complex. ASOs are single strand oligonucleotides of approximately 15-25 nucleotides that bind to their complementary mRNA and decrease gene expression by causing the RNase-H mediated degradation of the target mRNA or by physically blocking its translation (Bennett and Swayze, 2010).

The development of oligonucleotide therapies for HD requires that issues relating to safety and the effective delivery to the brain are resolved. The safety issues are both generic to the therapeutic strategy and specific to HD. The absence of Htt expression during mouse development results in embryonic lethality (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995) and the reduction in Htt expression to less than 50% during development results in brain abnormalities (White et al., 1997). The extent to which HTT can be knocked down in adult mice has not been investigated in depth, although decreasing Htt expression at a point between 5 and 60 days of age suggested that HTT has an essential function in the adult brain (Dragatsis et al., 2000). Therefore silencing of the HTT gene must ensure that total HTT is maintained at safe levels. This could be achieved either by selectively silencing the mutant allele or by simultaneously lowering both alleles to reduce the expression of mutant HTT.
sufficiently for therapeutic benefit whilst ensuring that total HTT levels are adequate for normal physiological function. One strategy to specifically lower mutant HTT targets SNPs within the HTT gene and the clinical development of siRNAs against a small number of allele specific targets may be sufficient to treat close to 90% of the HD population (Lombardi et al., 2009; Pfister et al., 2009; Warby et al., 2009). Following a similar strategy, an allele specific ASO has been developed, predicted to target 49% of the HD population, that is selective for the target SNP in informative transgenic mouse models (Carroll et al., 2011). Alternatively, allele-specific targeting of the mutant CAG repeat using mismatch siRNAs (Hu et al., 2010; Hu et al., 2012) is also proving to be a promising approach.

The in vivo assessments that have been performed for oligonucleotide based therapeutics for HD are listed in Table 16.7. Early proof of concept studies demonstrated that RNAi approaches directed against mutant HTT transgenes improved neuropathological and behavioural phenotypes in HD mouse models and that the knock-down of wild type Htt in adult HD mouse models can be tolerated for a number of months (Boudreau et al., 2009; DiFiglia et al., 2007; Drouet et al., 2009; Harper et al., 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005). The development of further shRNA constructs uncovered an unexpected toxicity associated with the striatal injection of AAV encoded shRNAs that was mitigated when the same RNAi sequences were packaged as miRNA expression systems (McBride et al., 2008). More recently, the transient infusion of ASOs into the CSF of symptomatic HD mice, not only delayed disease progression but also mediated a sustained reversal of the disease phenotype that persisted for longer than the HTT knock-down (Kordasiewicz et al., 2012). This sustained reversal was also achieved when together with the mutant HTT transgene, wild type levels were reduced to 75% of endogenous Htt. These data indicated that a transient rather than continuous decrease in HTT levels might constitute an effective
therapeutic strategy (Kordasiewicz et al., 2012). However, the simplicity of single stranded (ss) ASOs has recently been combined with the efficiency of RNAi to generate chemically-modified ss-siRNAs that are potent and allele-selective inhibitors of mutant HTT. The ss-siRNAs efficiently entered the RNA pathway inhibiting mutant Htt expression after introduction into HD-patient derived fibroblasts and the intraventricular infusion into the CSF of HdhQ150 knock-in mice (Yu et al., 2012).

The safety of RNAi approaches can be hampered by off-target gene-silencing, the ability of siRNAs or ASOs to bind to unintended mRNAs and reduce their expression. This can be minimized by the selection of the target mRNA sequence (Bennett and Swayze, 2010). In the case of siRNAs, this primarily occurs when the seed region (nucleotides 2 – 8 of the small RNA) pairs with sequences in the 3'UTRs of unintended mRNAs, a phenomenon that can be predicted in advance and used for the design of ‘safe-seed siRNAs (Boudreau et al., 2011). Aptameric effects, or interaction with proteins, can be a source of toxicity affecting processes such as coagulation, complement activation or immune cell activation (Bennett and Swayze, 2010). These effects tend to depend on the chemistry of the oligonucleotides and can be mitigated once these toxicities have been characterised for a given class (Aronin et al., 1995; Bennett and Swayze, 2010; Sah and Aronin, 2011).

The effective delivery of siRNA and ASO therapeutic candidates to the CNS remains a major challenge as these molecules are not suitable for oral administration and do not cross the blood brain barrier. They must be directly introduced into the CNS via either intraparenchymal or intraventricular routes, both of which result in different limited distribution profiles and, is a factor for concern as many areas of the brain are affected in HD (Sah and Aronin, 2011). The delivery of ASOs into the cerebral spinal fluid of nonhuman primate via intrathecal infusion was recently shown to effectively lower HTT primarily in the
cortex, and to a lesser extent in many brain regions affected by HTT pathology (Kordasiewicz et al., 2012). siRNAs are injected directly into the striatum and a number of modifications have been tested to enhance distribution including the utilisation of convention enhanced delivery (Stiles et al., 2012), cholesterol conjugation (DiFiglia et al., 2007), complexing to rabies virus glycoprotein peptide (Kumar et al., 2007), linkage to a transferrin receptor antibody (Xia et al., 2007) and complexing with β-cyclodextrin nanoparticles was recently demonstrated to significantly increase the intracellular delivery of siRNAs (Godinho et al., 2013). AAV delivery of sRNAs is also parenchymal. AAV vectors have many serotypes provides the potential to extend neurotropism, although at present multiple injections into the brain would probably be needed for the desired coverage (Sah and Aronin, 2011). Whether adequate distribution any of these oligonucleotide therapies, and especially for those requiring intraparenchymal delivery, could be achieved for clinical benefit is yet to be established.

**Intrabodies**

As an alternative to targeting HTT mRNA, anti-HTT intracellular antibodies (intrabodies) are being developed to decrease levels of the mutant HTT protein (Table 16.8). Fulfilling this predication, adenoviral delivery of intrabody scFvEM48 into the striatum of HD mouse models increased the ubiquitylation and degradation of mHTT resulting in a decrease in neuropil aggregates and cytotoxicity and an improvement in neurological symptoms (Wang et al., 2008b). Patterson and colleagues have developed intrabodies that bind to either the first 17 amino acids of HTT or to the proline rich region (Southwell et al., 2008). Targeting the proline region *in vivo* conferred benefits in multiple mouse models of HD (Southwell et al., 2009) and was shown to reduce the level of soluble mutant HTT be
enhancing its clearance (Southwell et al., 2011). The intrabody that targeted amino acids 1-17 increased nuclear aggregates and accelerated the phenotype in R6/2 mice (Southwell et al., 2009), whereas in contrast, an alternative intrabody raised against amino acids 1-17 had beneficial effects when administered R6/1 mice (Snyder-Keller et al., 2010), potentially due to an alteration in HTT post-translational modifications (Butler et al., 2012). Delivery to the brain remains one of the major challenges facing the development of intrabody therapies, as discussed above.

**Concluding remarks**

Excellent *in vivo* models have been developed with which to unravel the molecular pathogenesis of HD and that can be used for the preclinical assessment of therapeutic strategies. Over the past 10 years a great deal of knowledge as to the best-practice design of preclinical-efficacy studies has been accumulated by the scientific community. This should ensure that greater confidence can be placed in the outcomes of the preclinical therapeutic evaluations and that the limitations of these studies are better understood. This is an exciting time. There are real possibilities that strategies designed to lower the levels of HTT will progress to clinical evaluation in the not too distant future. In parallel, improvements in our understanding of the molecular basis of HD increases the likelihood that small molecule therapies will be developed against targets that are proximal to the mutation and that have good chance of providing disease-modifying benefit for people suffering from Huntington’s disease.
References


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Aberrant splicing of HTT generates the pathogenic exon 1 protein in Huntington disease.


Figure Legends

**Figure 16.1**
Micrographs of EM48 immunohistochemistry. (A) EM48 staining of 12-month old WT or HD mice expressing full-length mutant HTT. KI = HdhQ150 knock-in; YAC = YAC128 transgenic mice; C6R = caspase-6-resistant YAC transgenic mice. (B) EM48 staining of transgenic mice expressing N-terminal mutant HTT at the age of 3, 4 or 12 months as indicated. SS = shortstop transgenic mice; Ctx = cerebral cingulate cortex; Str = dorsomedial striatum; WM = white matter in the middle portion of the corpus callosum. Scale bars, 20 μm. Reproduced with permission from Wang et al. Human Molecular Genetics 17, 2738-2751, 2008.

**Figure 16.2**
siRNA, miRNA, and shRNA cellular pathways. With successful delivery, siRNAs enter the cell and gain access to the cytoplasmic compartment, where they are incorporated into RNAi silencing complex (RISC). The RISC complex with the active guide strand (in red) binds the complementary sequence within the target mRNA, resulting in Argonaut 2–mediated cleavage and subsequent mRNA degradation. Endogenous miRNAs are derived from miRNA genes that are transcribed to primary miRNAs (primRNA) that are cleaved by Drosha to hairpin precursors (pre-miRNAs) within the nucleus. Viral delivery of shRNAs requires entry into the nucleus, where promoter-directed expression of pre-miRNA occurs. These pre-miRNAs are exported to the cytoplasm by Exportin-5, and then the hairpin precursor is cleaved by Dicer to form miRNAs that are incorporated into RISC, leading to mRNA binding and cleavage. Reproduced with permission from Sah and Aronin Journal of Clinical Investigation 121, 500-507, 2011.