PRODUCTION AND CHARACTERIZATION OF
YAC TRANSGENIC MICE EXPRESSING THE NORMAL
AND MUTANT HUNTINGTON'S DISEASE GENE

by

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B.Sc., The University of Guelph, 1994

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

MEDICAL GENETICS GRADUATE PROGRAM

DEPARTMENT OF MEDICAL GENETICS

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

January 1999

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Date 4/27/99
ABSTRACT

Insights into how polyglutamine (Q) expansion in the Huntington’s disease (HD) protein, huntingtin (htt), results in neurotoxicity in vivo can be achieved with the development of an animal model. No such model exists for HD with accurate spatial and temporal expression of a full-length mutant human protein. To address this, a Yeast Artificial Chromosome (YAC) transgenic approach was adopted to produce mice that express normal (18Q) and mutant (46Q and 72Q) htt. The normal and mutant human proteins are expressed in a temporal and spatial manner identical to that of the endogenous mouse protein. Mice expressing 46Q htt show no obvious neurological impairment up to 20 months. Behavior in home cages, coordination, and activity, were no different to controls, and no obvious pathological abnormalities were noted in the brain up to 20 months. Electrophysiological analyses in the hippocampal CA1 region at 6 and 10 months revealed a significant reduction in LTP, a form of synaptic plasticity involved in learning and memory. Two transgenic lines expressing 72Q htt were generated, 1 of which has integrated a higher number of YAC copies (3-5X). This increase in YAC copy number was associated with a 2-3X increase in protein expression levels. The high expressing founder (2498) exhibited a neurological phenotype at 6 wks, characterized by hyperactivity, lateralized circling, and impaired motor control, which were not seen in the lower expressing founder (2511). Analyses of brain tissue showed a dramatic increase in htt nuclear staining in the lateral striatum of mouse 2498 with accumulation of neuronal nuclear htt microaggregates and evidence for neurodegeneration. Neurodegeneration was also evident in founder 2511 without obvious presence of nuclear htt aggregates. These results demonstrate that a behavioral, neurophysiological and neuropathological phenotype can be achieved in YAC transgenic mice expressing full-length mutant htt consistent with that observed in HD patients.

Additionally, CAG trinucleotide instability was assessed in the YAC transgenic lines as the trinucleotide repeat was passed from parent to offspring. These results show that size of the CAG repeat was a significant factor in influencing trinucleotide instability with more unstable meioses
occurring as CAG size increased. Sex of transmitting parent and age of transmission did not
significantly affect instability rates. Transmission of the repeat in a DNA mismatch repair (MSH2)
deficient background also significantly increased repeat instability.

The YAC transgenic mice presented in this thesis represent an important milestone in the
development of an animal model for HD that closely represent the human condition. They provide an
important tool for gaining insight into the in vivo mechanisms involved in polyglutamine mediated
neuronal cell death and CAG trinucleotide instability, as well as a model on which to test therapeutic
strategies designed to alleviate or eliminate disease progression in HD.
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ACKNOWLEDGEMENTS

I would like to take the time to acknowledge those individuals who have made this thesis become a reality. Firstly my family, Brian, Elizabeth, and Kathryn Hodgson, for providing tremendous support and encouragement at all times during my studies, but mostly for those times when things seemed overwhelming. Secondly, to Dr. Michael Hayden, who set the stage for this thesis and provided an intellectual and challenging forum that allowed me to flourish and develop as a scientist. His support and understanding during critical stages of this thesis will not soon be forgotten. And also to my supervisory committee, Dr. Carolyn Brown, Dr. Jan Friedman, Dr. Frank Jirik, and Dr. Wendy Robinson for guidance and intellectual input throughout my graduate studies.

Of critical importance to this thesis has been the tremendous technical and intellectual support that has been provided by many individuals. Kerrie Nichol and Krista McCutcheon were invaluable for assistance with DNA, RNA, and protein work. Dr. Nagat Bissada, a most valuable colleague whose diligent help in management of the mouse colony, always with a smile, kept me sane. Dr. Gregory Lee for his guidance and help with development of the antibodies. Dr. Eddy Rubin and Dr. Des Smith for their role in training me and developing my understanding of YAC transgenic technology. Dr. Tony Philips for intellectual support and Fred LePiane for the behavioral analyses of the YAC mice. Of special mention are Dr. Nadia Agopyan for the neurophysiological analyses, and Dr. Claire-Anne Gutekunst for pathological analyses of the mice. And to Dr. Jamal Nasir, Dr. Paul Goldberg, and Dr. Hakan Telenius for their guidance during the early years of my degree.

Finally, the friendships I established here in Vancouver have made such a difference to my life. I cannot help but smile and laugh when I think of Tommy-Boy, Disco Pauly, DG, Sergio, Duva, Peanut, Shorty, Singaraja, Beecake, Squire, and Bad boy. The memories of our times together will never fade. Cheers.
CHAPTER 1 - HUNTINGTON’S DISEASE
1.1 Clinical Presentation, Epidemiology, and Natural History

Huntington's disease (HD) is a slowly progressive neurological disorder that usually manifests in mid adult life (Hayden and Kremer, 1995). The disease is inherited as an autosomal dominant trait and affects ~ 1/10,000 individuals in North American and European populations with other populations such as Japanese and African blacks showing a lower prevalence at ~ 1/100,000. Onset in both males and females usually occurs in mid adult life with a mean age of onset between 43 and 48 years. The initial stages of HD are insidious, often making accurate clinical diagnosis difficult in the early stages (Martin and Gusella, 1986). Clinical onset of HD is gradual, proceeding from an asymptomatic phase, through a transitional phase in which accurate diagnosis is often difficult, and finally to a stage where obvious HD signs and symptoms have manifested. Behavioral and mood disturbances (aggressiveness, irritability, anxiety), memory impairment, and minor motor control problems (e.g. clumsiness) are often the first signs of HD. As the disease progresses involuntary movements known as chorea present, the hallmark clinical feature of adult onset HD. This disturbance in motor control gradually worsens over the course of the illness, followed by onset of severe rigidity and dystonia (slow abnormal movements) in the latter stages of the disease. Global decline in cognitive abilities (dementia) also occurs and is a prominent feature in the late stages of the illness. Typical duration of HD (from age at onset to death) is usually around 15 years.

Approximately 10% of all patients manifest with symptoms before the age of 20, and are considered to manifest the juvenile form of the disease. The progression of the disease is more rapid and aggressive in this cohort of patients. The duration of chorea is much shorter than that seen in adult onset HD, and rigidity is the predominant form of motor disturbances. Cognitive decline is also observed.
1.2 Genetics

The HD gene (IT15), located on the short arm of chromosome 4 (4p16.3), was cloned and found to contain 67 exons, spanning a distance of ~210kb of genomic DNA (Ambrose et al., 1994). A CAG trinucleotide repeat was identified in exon 1 and an association was found between the number of consecutive CAG repeat units and the appearance of HD (HDCRG, 1993). The CAG repeat length is highly polymorphic in the general population ranging from 10 to 35 (median 18) consecutive repeats (Kremer et al., 1994). In HD patients, the repeat size is expanded beyond 35 repeats (range is 36-121) and a strong inverse correlation exists between CAG repeat length and age of onset (Andrew et al., 1993; Duyao et al., 1993). The largest CAG lengths are seen in individuals with juvenile onset of the disease.

HD is a dynamic mutation disorder whereby transmission of the CAG repeat through the germline is highly unstable on HD chromosomes. Studies on affected parent-child and normal parent-child pairs show that intergenerational CAG repeat size changes are much more frequent on HD chromosomes (~70%) than on normal chromosomes (<1%) (Kremer et al., 1995). Sex of the transmitting parent influences the level of instability. There is a significantly greater likelihood of CAG expansions if the allele is transmitted through the paternal germline, and can be attributed, in part, to the fact that large expansions (> 7 repeat units) are almost exclusively limited to paternal transmission (Kremer et al., 1995). Additionally, molecular analysis of juvenile HD showed that the large alleles seen in juvenile cases are due to expansion of the paternal allele (Telenius et al., 1993). Transmission through the maternal germline shows a significantly greater chance of being stable or contracting when compared to paternal transmissions, although small CAG expansions (< 7) are observed in ~40% of maternal meioses. This overall tendency of the CAG to expand upon transmission
through the germline accounts for the phenomenon of anticipation; the observation that disease severity worsens and onset is earlier as the disease is passed on in HD families.

New mutations causing HD were previously believed to be exceedingly rare (Hayden, 1981). However, molecular analysis of the CAG repeat showed that a total of 21 sporadic cases were identified from a total of 650 families studied indicating that new mutations may account for up to 3% of affected individuals (Goldberg et al., 1993). Sporadic cases arise due to unstable expansions from a subset of alleles, termed intermediate alleles (IAs). These are defined as alleles with CAG repeat lengths greater than that usually seen in the general population but less than that observed in HD, thus establishing the range for IAs between 29 and 35 CAG repeats. Thus far, new mutations have only been observed upon transmission through the paternal germline.

1.3 Gene Expression Analysis

The HD gene produces two transcripts of 10.3 and 13.6 kb that differ with respect to alternate 3’ polyadenylation, with the larger transcript being more predominant in brain tissue (Lin et al., 1993). Sequence analysis of the cDNA revealed an open reading frame expected to produce a protein of ~350kD in size (HDCRG, 1993). The CAG trinucleotide encodes the amino acid glutamine, thus producing a protein with a polyglutamine tract of varying size, depending on the length of the CAG repeat. Despite the fact that polyglutamine stretches have been identified in a number of transcription factors including the TATA binding protein (Kao et al., 1990), sequence analysis outside of the polyglutamine tract reveals no homology to any known proteins. Detailed mRNA expression analyses show that the HD transcript is expressed in most brain regions including hippocampus, cerebellum, cortex, thalamus, and striatum, as well as in peripheral tissues (Strong et al., 1993; Landwehrmeyer et al., 1995). Expression is higher in neurons than in glial cells. Additionally, no differences in expression patterns between HD patients and controls have been observed (Li et al., 1993; Landwehrmeyer et al.,
The HD protein, huntingtin (htt), is also widely expressed in the brain and peripheral tissues and is not enriched in the caudate or putamen (Sharp et al., 1995; Trottier et al., 1995). Thus the regional specificity of neuropathology observed in HD cannot be attributed to the pattern of HD gene expression.

1.4 CAG Repeat Disorders

HD is a member of an ever-increasing list of diseases caused by polyglutamine expansions in the coding region of each disease's respective gene (Table 1.1). All polyglutamine expansion disorders are inherited in an autosomal dominant fashion (with the exception of SBMA, which is X-linked) and show anticipation. Interestingly a relatively selective neuronal loss is observed in each disease, despite widespread expression of each gene in neuronal and non-neuronal cell types. In HD for example, the caudate and putamen (which collectively make up the striatum) are the primary sight of neuron loss and atrophy. This raises the following question: how does widespread expression of a common underlying mutation result in selective neuronal cell loss for each disease? Firstly, all of the polyglutamine diseases are believed to exert their pathogenic effects via a toxic gain of function of the respective protein, with glutamine playing a key role (Ross, 1997). Evidence to support a gain, rather than loss, of function for HD is that mice containing a homozygous targeted disruption of the murine HD gene develop lethal phenotype at embryonic day 7.5 (Nasir et al., 1995; Zeitlin et al., 1995; Duyao et al., 1995). Since human HD homozygotes are viable, it is unlikely that the polyglutamine expansion results in loss of function (Wexler et al., 1987). Additionally, heterozygous Hdh mice do not develop an HD like phenotype, again showing that loss of function of 1 allele does not result in HD. Thus expression of an
Table 1.1: The CAG trinucleotide repeat disorders and their features.

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<thead>
<tr>
<th>Disease</th>
<th>Gene Locus</th>
<th>Primary site of Pathology</th>
<th>CAG Size Range In Patients</th>
<th>Protein</th>
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<tr>
<td>HD</td>
<td>4p16.3</td>
<td>caudate/putamen</td>
<td>36-121</td>
<td>Huntingtin</td>
<td>(HDCRG, 1993)</td>
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<td>SBMA</td>
<td>Xq11-q12</td>
<td>anterior horn cranial motor nuclei</td>
<td>38-75</td>
<td>Androgen receptor</td>
<td>(La Spada et al., 1991)</td>
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<td>SCA1</td>
<td>6p23</td>
<td>cerebellum (Purkinje cells) inferior olive</td>
<td>39-81</td>
<td>Ataxin-1</td>
<td>(Orr et al., 1993)</td>
</tr>
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<td>SCA2</td>
<td>12q24</td>
<td>cerebellum (Purkinje cells) inferior olive substantia nigra</td>
<td>34-64</td>
<td>Ataxin-2</td>
<td>(Pulst et al., 1996) (Imbert et al., 1996) (Sanpei et al., 1996)</td>
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<td>SCA3/MJD</td>
<td>14q24.3-q31</td>
<td>dentate nucleus pontine nucleus anterior horn cells spinocerebellar tracts subthalamic nucleus</td>
<td>61-84</td>
<td>Ataxin-3</td>
<td>(Kawaguchi et al., 1994)</td>
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<td>19p13</td>
<td>cerebellum (Purkinje cells) inferior olive</td>
<td>21-27</td>
<td>α-1a-voltage dependent Ca(^{2+}) channel</td>
<td>(Zhuchenko et al., 1997)</td>
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<td>SCA7</td>
<td>3p14-21.1</td>
<td>unknown</td>
<td>38-130</td>
<td>unknown</td>
<td>(Del-Favero et al., 1998) (David et al., 1997)</td>
</tr>
<tr>
<td>DRPLA</td>
<td>12p13.31</td>
<td>dentate nucleus globus pallidus</td>
<td>52-88</td>
<td>atrophin</td>
<td>(Koide et al., 1994) (Nagafuchi et al., 1994)</td>
</tr>
</tbody>
</table>
expanded polyglutamine containing protein somehow disrupts normal neuronal function, ultimately leading to cell death.

To account for the selective neurodegeneration, sequences surrounding the polyglutamine tract may be responsible. Interactions of these sequences in the polyglutamine containing proteins with other selectively expressed proteins may account for this selectivity of neuron loss. However, it should be noted that there is some overlap amongst the areas affected in each disease, suggesting that certain brain regions are more sensitive to polyglutamine based neurotoxicity than others. These include the striatum, globus pallidus, subthalamic nucleus, cerebellar Purkinje cells and dentate nucleus, and brainstem motor nuclei.

1.5 Interacting Proteins

Due to the widespread protein expression yet selective neurodegeneration in HD, much work has recently been focused on identifying proteins that interact with htt in an effort to shed light on this problem. By doing so, one would gain insight into the potential pathways and cellular processes in which huntingtin plays a role, and also may identify selectively expressed proteins, thus accounting for the selective neuropathology. The polyglutamine stretch itself is involved in protein-protein interactions and has been shown to aggregate together to form polar zippers and β-sheets via hydrogen bonding (Perutz et al., 1994). Further studies, using the yeast 2-hybrid system and affinity chromatography, have identified a number of proteins; HAP-1 (Li et al., 1996), HIP-2 (Kalchman et al., 1996), HIP-1 (Wanker et al., 1997; Kalchman et al., 1997), and GAPDH (Burke et al., 1996). HAP-1 is a cytoplasmic protein of unknown function expressed highly in the brain and spinal cord. HIP-2 is the ubiquitously expressed ubiquitin-conjugating enzyme, a critical component of the ubiquitin mediated protein degradation pathway. HIP-1 shows strong homology to the yeast Sla2p protein, known to have involvement in cytoskeletal and membrane related functions (Kalchman et al., 1997; Wanker et al., 1997). It is highly expressed in all brain regions, and
lower in peripheral tissues. Interestingly, interactions of htt with HAP-1 and HIP-1 are modulated by CAG length, albeit in an opposite manner. Interaction strength with HAP-1 increases with increasing CAG size (Li et al., 1996) whereas decreased interaction is observed with increased CAG size for HIP-1 (Kalchman et al., 1997). GAPDH is also ubiquitously expressed and is known to play a role in glycolysis and DNA repair and replication (Burke et al., 1996). While identification of these interacting proteins is of considerable interest and provides valuable clues into huntingtin function, none fully account for the selective neuropathology. Given the large size of the htt (348kD), it should come as no surprise that it could interact with a wide variety of proteins and thus there is much potential for identification of further, as yet unidentified interacting proteins.

1.6 Apoptosis

Although not conclusively proven, there is evidence to suggest that the ultimate mechanism of neuronal cell death in HD is apoptosis rather than necrosis. TUNEL analyses of HD brains have shown increased levels of DNA strand breaks typical of apoptotic cells (Portera-Cailliau et al., 1995; Thomas et al., 1995). Additionally, it has been suggested that the neuron cell loss observed in exon 5 HD knockout heterozygous mice occurs via apoptosis since characteristic apoptotic cells were observed by EM (Nasir et al., 1995). It is also interesting to note that htt interacts with the pro-apoptotic enzyme caspase-3 (Goldberg et al., 1996). Thus most cell death data produced regarding HD consistently leans toward apoptotic rather than necrotic mechanisms.

1.7 HD Gene in Other Species

The homologue of the HD gene has been cloned and characterized in 3 other organisms; the mouse (Lin et al., 1994), rat (Schmitt et al., 1995), and pufferfish (Bäxendale et
A high degree of conservation to human sequences is observed in the coding region of each homologue with 85%, 86%, and 69% identity observed at the nucleotide level respectively. Conservation is higher at the protein level, with 90% identity observed for both rat and mouse compared to human sequences. Each gene contains a polyglutamine repeat tract, although the length of the repeat is considerably lower when compared to human. The mean repeat length in normal human populations is 18, whereas it is 8 for the rat, 7 for the mouse, and 4 for the pufferfish. This then suggests that the ancestral HD gene had a small polyglutamine repeat.

The fact that a homologous HD gene has been found in other organisms offers the potential for the development of an animal model for HD. The mouse has become an important organism for studying mammalian biology and human disease in vivo for a number of reasons. Firstly, it acts as an excellent model system for studying mammalian physiological and cellular function/malfunction since it is more closely related to humans than organisms such as Drosophila and C. elegans. Secondly, its small size and rapid generation time allow for production of large numbers of offspring with which one can perform desired experiments. The use of more closely related organisms such as primates is limiting in that their size and longer generation times provides fewer organisms for study, and ethical issues also play a role. Thirdly, transgenic technology has been largely established in mice, allowing one to manipulate the mouse genome in a variety of genetically defined ways to study certain aspects of cellular and molecular biology. It is no surprise then that a number of mouse models have been generated for neurological/neurodegenerative disorders such as Alzheimer’s disease (Greenberg et al., 1996) and amyotrophic lateral sclerosis (ALS) (Reaume et al., 1996; Kondo et al., 1997). Thus much attention has turned towards the development of a polyglutamine (polyQ) based neurotoxic mouse model in recent years.
1.8 CAG Repeat Mouse Models

In an effort to learn more about the molecular basis of polyQ based neurotoxicity, a number of transgenic animals expressing proteins with expanded polyglutamines have recently been generated. These include transgenic models of SCA-1 (Burright et al., 1995), SCA-3 (Ikeda et al., 1996), and HD (Mangiarini et al., 1996). Of primary relevance to this thesis are the HD exon 1 transgenics that developed a neurological phenotype without any obvious signs of neurodegeneration in any brain region (Mangiarini et al., 1996). Also, expanded polyglutamines have been placed in the context of the hypoxanthine phosphoribosyltransferase (HPRT) gene in knock-in mice to study the effects of polyQ expansion in a non-classical polyQ related gene (Ordway et al., 1997). The findings from these studies suggest that in order to induce an aggressive polyglutamine mediated neurological phenotype in mice, it is necessary to express mutant protein at sufficiently high levels (greater than endogenous) with large stretches of polyglutamines (>70Q) (Burright et al., 1995; Goldberg et al., 1996). Alternatively, expression of truncated forms of polyQ containing proteins enhances disease progression (Mangiarini et al., 1996; Ikeda et al., 1996).

While each model has provided important insight into potential mechanisms of disease progression, each is limited in that expression of the transgene is not under control of the endogenous promoter and/or does not express the polyglutamine within the context of the full-length protein. This raises questions if one wishes to create an animal model that replicates the human condition as closely as possible.

The development of transgenic technologies using YACs offers important advantages for generating transgenic mice expressing human huntingtin. The inclusion of endogenous regulatory elements within the transgene allows for the normal temporal and tissue specific expression of the human gene under study (Schedl et al., 1993; Lamb et al., 1993). Furthermore, expression of transgenes from YACs reach levels comparable to that of the endogenous gene in a copy number dependent and position independent manner (Gaensler et
al., 1993; Peterson et al., 1993). Often, cDNA based transgenes are poorly expressed and inappropriately regulated (Jaenisch, 1988). In addition, genes contained within YACs can be manipulated using a homologous recombination based strategy in yeast (Duff et al., 1994). This allows one to introduce a wide variety of mutations into the gene of interest. In the case of HD, we can substitute normal sized CAG repeats with a number of mutant repeat sizes ranging from adult to juvenile onset. Thus I have adopted a YAC transgenic approach in the interests of developing a truly representative animal model for HD. This would greatly facilitate our understanding of the cellular and molecular mechanisms involved \textit{in vivo} and provide a substrate on which testing of potential drugs aimed at therapy for HD can be performed.

1.9 Hypotheses

The following hypotheses will be addressed in this thesis:

1) The human HD gene expressed from YAC transgenes will be expressed in a temporal and spatial manner that parallels that of the endogenous mouse HD gene (Hdh).

2) Expression of YAC derived mutant human HD protein (huntingtin) will result in the development of behavioral, neuropathological, and neurophysiological phenotypes in the YAC transgenic mice.

3) The CAG repeat within the YAC transgene will be unstable upon transmission through the maternal and paternal germline in HD YAC transgenic mice.

1.10 Thesis Objectives

To address the above hypotheses, these are the objectives of this thesis.
1) To generate anti-huntingtin antibodies using expressed GST-HD fusion proteins, allowing us to assess htt expression in mice.

2) To characterize and introduce expanded CAG repeats into YACs YGA2 and 353G6.

3) To generate YAC transgenic mice using YGA2 and 353G6 and to characterize these mice at the DNA, RNA, and protein levels for evidence of YAC integration and to assess human HD gene expression in a murine background.

4) To perform detailed behavioral analyses on mice expressing YAC derived normal (18 CAG repeats) or mutant (46 or 72 CAG repeats) huntingtin for evidence of a neurological phenotype.

5) To perform a pathological assessment of YAC transgenic mouse brains at light microscopy and EM levels for evidence of neurotoxicity.

6) To perform neurophysiological analyses of YAC transgenic mouse brains for evidence of impairment of neuron function.

7) To assess CAG trinucleotide instability of the YAC transgene in normal and MSH2 knock-out mouse backgrounds.

Note: Unless otherwise stated, I have generated all figures and data contained within this thesis.
1.11 Reference List


CHAPTER 2 – MATERIALS AND METHODS
2.1 Chapter 3: Anti-HD antibody production

2.1.1 Fusion protein production

The pATH expression vectors were generated as follows: E/C 968 was derived by digesting HD clone cD70-2 with EcoRV and ClaI producing a 968 bp fragment stretching from nucleotides 1905 (exon 12) to 2873 (exon 19). This fragment was cloned into pATH 2 digested with Smal and ClaI to maintain the reading frame. E/B912 was produced by digesting HD clone cD149 with EcoRI and BamH1 producing a 912bp fragment extending from nucleotide 5345 (exon 39) to 6257 (exon 44). This fragment was cloned into pATH 1 digested with the same restriction enzymes.

Expression of the fusion proteins was achieved as described (Koerner et al 1991). Briefly, RR1 cells were transformed with 1 of the 2 pATH-HD plasmids transformants selected for on LB-AMP plates. Colonies were picked and grown overnight in M9 media supplemented with tryptophan. Fusion protein expression was induced by diluting overnight cultures 1/10 in M9 media lacking tryptophan and addition of indolacrylic acid (IAA) for a four hour period. Cells were then spun down, lysed in urea-SDS cracking buffer (0.01M sodium phosphate buffer, pH 7.2, 1% β-mercaptoethanol, 1% SDS, 6M urea), and protein extracts fractionated on 10% polyacrylamide gels. Fusion protein expression in each colony was checked by western blotting using an anti-trpE polyclonal antibody (gift from Ivan Sadowski). Colonies showing the highest expression levels were chosen for large scale expression of the fusion protein.

The fusion protein rich insoluble inclusion bodies were isolated by pelleting a 1L (4 X 250 ml) culture of induced cells (5', 3300g) and washed once with 25ml TEN (50mM Tris, pH 7.5, 1mM EDTA, 0.3M NaCl). Cells were treated on ice with 2.5ml, 10mg/ml lysozyme for 15', 0.5ml, 10% NP-40 for 10', and 37ml NaCl-Mg (1.5M NaCl, 12mM MgCl₂) and 125μl, 1mg/ml DNase1 for 1hr with mixing. Inclusion bodies were obtained by spinning the lysate (10', 9000g), washed 2 times with 25ml TEN, and resuspended in a solubilizing urea-SDS cracking buffer.
The fusion protein was further purified by running aliquots of this sample on 10% polyacrylamide gels and cutting the desired band from the gel. This was achieved by cutting the sides of the gel away, staining with coomassie blue for 10’, and then realigning the stained sections with the unstained portion of the gel. The protein was removed from the gel by electroelution for 2hrs, at 150mV, in 1X gel running buffer lacking SDS (1.9M glycine, 250mM Tris base). The protein solution was then dialyzed against 1X PBS and the protein concentration determined, which was usually around 0.1 mg/ml.

2.1.2 Polyclonal antibody production.

Injection of rabbits with antigen was performed by Dr. Gregory Lee, Canadian Genetic Diseases Network antibody core production facility, UBC, Canada.

Two New Zealand white rabbits were used for both E/C968 and E/B912 and were given a 0.5ml primary injection of 200 μg of fusion protein emulsified with Freund’s complete adjuvant (50/50, v/v). This was followed by 4, 0.5ml boosts of 50 μg each with Freund’s incomplete adjuvant (50/50, v/v), each, 2 weeks apart. Seven-ten days following the last immunization, the rabbits were bled to completion and the antibody titer of each rabbit was tested by ELISA using the original trpE-HD antigen and trpE alone as substrates.

2.1.3 Purification of polyclonal antibody

The E/C968 cDNA fragment was cloned in frame into the GST expression vector pGEX-4T-2 (Pharmacia Biotech). GST-HD fusion protein was expressed and purified as described by the manufacturer (Pharmacia Biotech). Purified GST-HD fusion proteins attached to glutathione beads were loaded onto a column, over which crude serum was run, allowing for binding of anti-HD specific antibodies. Any attached antibodies were eluted at low pH (2.5) with 0.1M glycine, and neutralized
with Tris base until pH of the solution was around 7.4. Antibody preps were stored in aliquots at -70°C until needed.

2.1.4 Monoclonal antibody production

Injection of mice with antigen, production of hybridoma cell lines, and screening of hybrid cells was performed by Dr Gregori Lee, in the Hybridoma Core Facility of the Canadian Genetic Diseases Network, UBC, Canada.

The purified fusion proteins were used as the immunogens to produce monoclonal antibodies. Three male Balb/c mice were each subcutaneously injected with 20 μg of E/C968 or E/B912 emulsified in the presence of Freund’s complete adjuvant (50/50, v/v). Injections of 20 μg of fusion protein emulsified in Freund’s incomplete adjuvant (50/50, v/v) were done at 2 and 4 weeks. Following the third injection, the serum antibody titers to the original immunogens and trpE alone (negative control) were determined by ELISA. One mouse from each group which showed the highest specific antibody titers was selected and boosted through the tail vein with 40 μg of antigen 3 days prior to cell fusion experiments.

To generate antibody-secreting hybridomas, cell fusion experiments were performed. Spleen cells of immunized mice were removed and fused with NS-1 myeloma cells in the presence of 50% PEG. Following cell fusion, the hybrid cells were cultured in a semi-solid medium containing 25% fetal calf serum, IMDM, HAT, and LPS. Six to eight days after cell fusion, the hybrid cells that survived selection and formed colonies were removed and cultured in 96 well microplates. The culture supernatants were removed after 2-3 days for antibody screening by ELISA. TrpE alone was used as a negative control. Only those hybridomas that secreted antibodies specific to E/C968 or E/B912 but not to trpE were kept for further culturing. The hybrid clones were frozen separately and stored at -135°C.
2.2 Chapter 4: YAC mutagenesis

2.2.1 Yeast growth

Yeast clones were grown in liquid culture at 30°C in a standard selective dropout medium with selection determined by the markers present on the YAC. For IL the following amounts were added (with the appropriate amino acid removed for selection purposes: 20 mg adenine (hemisulfate salt), 20mg arginine-HCl, 100mg aspartic acid, 100mg glutamic acid, 20mg histidine, 30mg leucine, 30mg isoleucine, 30mg lysine-HCl, 20mg methionine, 50mg phenylalanine, 375mg serine, 200mg threonine, 40mg, tryptophan, 30 mg tyrosine, 150mg valine, 20mg uracil, 1.7g yeast N₂ base without amino acids and ammonium sulfate (DIFCO), 5g ammonium sulfate, 20g glucose. Typically a 10X stock was made by adding the above amounts to 100ml H₂O, and the diluted to 1X as needed. When selective plates were needed for growth, 20g (2%) bactoagar was added.

When selection was not a concern standard YPD (1% yeast extract, 2% peptone, 2% glucose) was used.

2.2.2 Yeast transformations

All yeast transformations were carried out as described (Burgers and Percival, 1987). Briefly, yeast spheroplasts were made by growing a 50ml culture to about 3x10⁷ cells/ml, spun down, resuspended in 20ml SCEM (1M sorbitol, 0.1M Na citrate, pH 5.8, 10mM EDTA, 30mM β-mercaptoethanol), and incubated with 1000U lyticase at 30°C until spheroplasting reached around 50-70%. Cells were then spun down, washed with 1M sorbitol, and resuspended in 2ml STC (1M sorbitol, 10mM Tris, pH7.5, 10mM CaCl₂). Spheroplasts (100μl) were then mixed with plasmid DNA (1-2μg) and carrier DNA (10mg/ml salmon sperm DNA) up to a total of 5μg, 1ml PEG (10mM Tris, pH 7.5, 10mM CaCl₂, 20% polyethylene glycol 8000; filter sterilized), gently resuspended, and left for 10' at room temperature. STC (150μl) and 8ml TOP agar (1M sorbitol, 2.5% bactoagar, 1X selective amino acid mix, 2ml YPD) were added, mixed, and poured onto selective SORB plates (0.9M sorbitol, 3%
glucose, 2% bactoagar, 1X selective amino acid mix). Plates were then placed at 30°C and colonies allowed to grow for 24-48 hours.

2.2.3 YAC mutagenesis

The pRS406 vector used for the pop-in phase of the mutagenesis was transformed into spheroplasted yeast. Pop-in transformants were selected for on ura' trp' lys' plates and grown to confluency in liquid media (ura' trp' lys'). Pop-out clones were selected for by plating pop-in clones (100μl of liquid culture) on trp' lys' plates containing 1mg/ml 5-fluoroorotic acid.

2.2.4 Yeast plugs

High-density yeast plugs were prepared by growing yeast cultures to saturation at 30°C in a 500ml culture of ura' trp' dropout media. After washing, cells were resuspended in 4ml SCE (1M sorbitol, 0.1 M Na citrate, 5 mM EDTA) mixed with 6 ml of 3% InCert agarose (FMC) at 42°C and dispensed into plug molds (Biorad). Once solidified, plugs were incubated overnight at room temperature in 10ml SCE with 10 mg of lyticase (Sigma). This solution was replaced the next day with ES (0.5M EDTA, 1% sarkosyl) with proteinase K (1mg/ml) and placed at 50°C overnight. Plugs were then washed 5 times with T10E50 (10mM Tris pH 8.0, 50 mM EDTA) for 1/2 hour incubations and stored at 4°C.

2.2.5 Pulse field gel electrophoresis

Pulse Field Gel Electrophoresis (PFGE) was performed using the CHEF-DR™ II system (Bio-Rad). High-density yeast DNA plugs were bisected and loaded into wells in a 1% agarose gel (made with 0.5X TBE). Once loaded, the plugs were held in place by adding molten 1% agarose to the well and allowed to solidify. The gel was placed in the running chamber and allowed to equilibrate with the
running buffer (0.5X TBE) for 30 min, which was kept at a constant 14°C for the entire run.

Separation conditions for the YACs were as follows: YGA2 was separated at a 120° reorientation angle at 6.0 V/cm with a 60s switch time for 15 hrs followed by a 120s switch time for an additional 12 hrs. 353G6 was separated at a 120° reorientation angle at 4.0 V/cm with a 50s switch time for 30 hrs.

2.2.6 Southern blotting

Enzyme digested DNA fragments were first separated in agarose gels, and DNA transferred and immobilized on Hybond™-N+ nucleic acid transfer membrane (Amersham) overnight in 0.4M NaOH. Probe DNA was radioactively labeled with α-32P-dCTP (Amersham) using the rediprime random primer labeling system (Amersham) and purified on a Sephadex® G-50 NICK™ column (Pharmacia). Hybridization was carried out as per manufacturer recommendations (Amersham). Autoradiography was done by exposing Kodak X-OMAT scientific imaging film to the washed membrane at −70°C for periods ranging from 1hr-1week, depending on signal intensity.

2.2.7 PCR screening of 5' end of mutant YAC clones

PCR reactions (named GH4.1) were run in a final reaction volume of 25μl using the following final concentrations: 1mM MgCl₂, 0.8μM each primer, 0.1mM dNTP, 0.5U Taq DNA polymerase, 1X PCR buffer (Gibco BRL). Cycling conditions were 94°C for 1min; 34 cycles of 94.5°C, 63°C, 72°C, each 45s; 72°C 10min.

Primer 1: GH4.1F 5'CAAGGCCACCTCGGCTCAGAG 3'
Primer 2: GH4.1R 5'GGTAGGGCGTGGCGAGGC 3'.
2.3 Chapter 5 Production and characterization of YAC transgenic mice

2.3.1 YAC purification for microinjection

YAC DNA was prepared for microinjection essentially as described (Schedl et al., 1992) with a few modifications (Smith et al., 1995). High-density yeast plugs were prepared as described (section 2.2.4) and loaded into a preparative well to run high molecular weight YAC DNA on pulse field gels (section 2.2.5). The outer segments of the gel were stained with ethidium bromide (1µg/ml) for 15-30 min. and used to locate the YAC DNA. The relevant part of the gel was excised and the agarose containing the YAC DNA was cut into 2.5 cm long pieces, aligned side to side, and embedded in 4% Nusieve agarose. The DNA was subjected to pulse field gel electrophoresis to concentrate the YAC DNA using a reorientation angle of 90°, 4V/cm gradient, a 50s switch time, and a 12 hr run time. After the YAC had been concentrated, a small strip was cut from the side of the gel, stained to locate the YAC, and the unstained portion of the YAC excised from the gel. This gel slice was equilibrated in 5 volumes of β-agarase buffer (100mM NaCl, 10mM Tris-Cl, pH 7.5, 1mM EDTA, pH 8.0) for two 30 min incubations. The agarose was melted by incubation at 68°C for 12 min and digested using 4U β-agarase (New England Biolabs) per 100 mg of gel with incubation at 42°C for 2-3 h. Following agarose digestion, YAC solution was dialyzed overnight at room temperature against microinjection buffer (100mM NaCl, 10mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0). Dialysis was accomplished by placing YAC DNA solution on Millipore Minitan-S polysulfone membrane (100,000 NMWL) and floating the membrane on 50 ml of microinjection buffer. Following YAC purification, an aliquot was checked on a pulse field gel to ensure that the YAC was intact and degradation was minimal. Typically, YAC DNA was obtained at a concentration of 1ng/µl.

Microinjections of YAC DNA was performed by Dr. Mary Stevens, Lawrence Berkeley Labs, Berkeley, CA.
2.3.2 PCR analysis of transgenic founders

DNA was extracted from mouse-tails for PCR analysis as described (Smith et al., 1995). Primer pairs were used to identify YAC sequences from the left and right YAC arms (Smith et al., 1995), the CAG repeat in exon 1 (Goldberg et al., 1993), the CA repeat in intron 1 (this thesis), and the ΔG in exon 59 of the HD gene (Ambrose et al., 1994). Conditions for each PCR are listed below if the protocol was modified from the original reference. All PCRs were run using 1mM MgCl₂, 0.8μM each primer, 0.1mM dNTP, 0.5U Taq DNA polymerase, 1X PCR buffer (Gibco BRL) in a final reaction volume of 25μl unless stated otherwise. Typically PCRs were initiated with a 94°C denaturation step for 1min, and finished with a 10min extension at 72°C. Exceptions are noted.

RYA
As described (Smith et al., 1995).

LYA
As described (Smith et al., 1995).

CAG
Primer 1: HD344 5' CCTTCGAGTCCCTCAAGTCCTTC 3'
Primer 2: HD482 5' CCGACTCCTTCGACTCCTC 3'
Final concentrations: 2mM MgCl₂, 3.5% formamide, 15% glycerol, 0.2mM dNTPs, 0.5μM each primer.
Cycling conditions: Initial denaturation 94°C 3 min; 94°C, 61°C, 72°C each 1 min, 28 cycles

CA
Primer 1: In1-1 5' TGAGGCTGAGTGTAGCTATGA 3'
Primer 2: In1-2 5' CTCACCAGCATGTGTATGATG 3'
Final concentrations: 2mM MgCl₂, 0.1mM dNTP mix, 7%DMSO, 0.8μM each primer.
Cycling conditions: Touchdown PCR; 94°C 40s, 63°C 40s, 72°C 50s, annealing decreasing by 1°C per cycle, 7 cycles. Followed by 94.5°C 40s, 59°C 40s, 72°C 50s, 30 cycles.

ΔG

Primer 1: HD8302 5’ CCTGGAGTTGACTGGAGACGTG 3’
Primer 2: HD8191 5’ GCTGGGGAACAGCATCACCC 3’

Final concentrations: 2mM MgCl\textsubscript{2}, 4% formamide, 14% glycerol, 0.15mM dNTP mix, 0.8μM each primer.

Cycling conditions: 94°C, 65°C, 72°C each 1 min, 35 cycles.

2.3.3 RNA analysis

RNA was extracted from mouse-tails using the guanidium isothyocyanate method (Chomczynski and Sacchi, 1987). A total of 5 μg of random hexamer primed RNA was reverse transcribed using the Superscript preamplification system following manufacturer’s instructions (BRL). PCR on reverse transcribed DNA was done using the following reaction. PCR HD1201 was run using 2mM MgCl\textsubscript{2}, 0.8μM each primer, 0.2mM dNTP, 0.5U Taq DNA polymerase, 1X PCR buffer (Gibco BRL) in a 25μl volume with the following cycling conditions: 94°C 30s, 72°C 30s, 35 cycles

Primer 1: HD1201 5’GTGCTC TTAGGCTTACTCGTTCCTTG 3’
Primer 2: HD1481 5’GGCGGTCTGAAGAGCTGCTGCAAC 3’

2.3.4 Fluorescence in situ hybridization (FISH)

FISH analysis was performed by Mary-Beth Dinulos in the laboratory of Christine Distesch, University of Washington, Seattle, WA.
A cosmid probe with a 35 kb insert containing the human HD gene was labeled with biotin-11-dATP by nick translation (BRL). The size of the product was determined to be between 200 and 400 bp. Metaphase chromosome preparations from 4 transgenic FVB mice were obtained using 0.075 M KCl as a hypotonic buffer and methanol:acetic acid (3:1, v/v) as fixative. The hybridization was carried out as previously described by Chance et al. (Chance et al., 1993). Hybridization signals were detected using a detection system from Vector. After blocking with goat serum and an incubation with fluorescein avidin DCS, slides were rinsed in modified 4xSSC (4xSSC, 0.03% Triton). A second incubation with biotinylated anti-avidin D and a rinse in modified 4xSSC followed. A final incubation with fluorescein avidin DCS and a rinse in modified 4xSSC completed the detection. The chromosomes were banded using Hoechst 33258-actinomycin D staining and counterstained with propidium iodide. The chromosomes and hybridization signals were visualized by fluorescence microscopy, using a dual band by-pass filter (Omega).

2.3.5 Immunocytochemistry

Immunocytochemistry was performed by Dr. Kazutoshi Nishiyama in the laboratory of Dr. Michael Hayden, UBC, Vancouver, Canada

Brain tissues were obtained from two YAC transgenic mice (lines B60 and D212) and two normal control mice, which were perfusion-fixed with 4% v/v paraformaldehyde/0.01 M phosphate buffer (4% PFA) under deep anesthesia with halothane. The brain tissues including spinal cord were removed, immersion-fixed in 4% PFA for 1 day, washed in 0.01 M phosphate buffered saline (PBS) for two days, and then equilibrated in 30% w/v sucrose/0.01 M phosphate buffer for two days. The samples were then snap-frozen in Tissue Tek molds by liquid nitrogen. After warming to −20°C, frozen blocks derived from frontal cortex, caudate/putamen, globus pallidus, parietal cortex, hippocampus, thalamus, cerebellum, brainstem, and spinal cord were cut into 20-μm-thick sections for immunohistochemistry. Following soaking in 10 mM PBS (pH 7.2), the tissue sections were blocked using 2.5% v/v normal goat serum for 60 min to eliminate nonspecific binding. Primary antibodies
diluted with PBS (pH 7.2) were applied to the sections overnight at 4°C. Optimal dilutions were determined to be of 1:50 for BKP1 and 1:500 for MAB 2168 (Chemicon). This incubation was followed by sequential incubation of samples with biotinylated secondary antibody and then an avidin-biotin complex reagent (Vectastain Elite ABC Kit: Vector) at room temperature for 45 min each, and the color was developed using 3-3’-diaminobenzidine tetrahydrochloride and ammonium nickel sulphate.

To confirm the specificity of the observed immunoreactivity, sections were treated as mentioned above except that the primary antibodies were replaced with normal rabbit serum or normal mouse serum. As an additional negative control, BKP1, was preabsorbed with excess peptide prior to incubation with the tissue sections.

2.3.6 Subcellular fractionation of brain tissue

Cortical matter from frozen mouse brains was fractionated. Cortical matter was separated from brainstem, cerebellar and nuclear regions of whole brains, immediately frozen in isopentane, cooled in liquid nitrogen and stored frozen at -70 °C. Brain tissue was thawed and homogenized on ice in 10 volumes of sucrose buffer (0.303 M sucrose, 20 mM Tris-HCl (pH 7.2), 0.5 mM EDTA, 1 mM MgCl2, 1 mM PMSF, 1 mM benzamidine, 5 μg/ ml leupeptin and 10 μg/ ml soybean trypsin inhibitor) using a glass-teflon IKA-RW 15 homogenizer (Tekmar Company, Cincinnati, Ohio) at maximum speed. The homogenate was fractionated by a differential centrifugation protocol at 4°C as described (Wood et al., 1996). Centrifugation of the homogenate for 5 minutes at 1300 x g gave rise to the nuclear and cellular debris pellet (P1). The supernatant (S1) was saved and pooled with a second S1 fraction derived from re-homogenizing the P1 pellet. The S1 supernatants were centrifuged for 20 min at 11,750 x g to give a mitochondrial/synaptosomal P2 pellet. The cream-coloured upper portion of P2 (P2S), enriched in synaptosomes, was crudely removed from the lower brown-coloured portion containing mitochondria (P2). The S2 supernatants were centrifuged at 142,000 x g for 35 min to pellet microsomal (ER and Golgi) and plasma membranes (P3). The final supernatant from this step
(S3) contained cytosolic protein. The P1, P2, P2S membrane fractions were washed twice in sucrose buffer and then solubilized by heating for 20 minutes in 3M urea, 1% SDS, 1mM DTT, TBS, pH 8. Protein was determined by a modified Lowry protein assay (Bio-Rad DC Protein Assay, Bio-Rad) and 80 µg of each fraction was analyzed by Western blotting.

2.3.7 Tissue distribution of huntingtin

Frozen tissue samples were homogenized in sucrose homogenization buffer (see protocol above). Brain tissue was homogenized with a glass-teflon homogenizer and peripheral tissues were homogenized using a Polytron. Homogenates were sonicated 3 x 5 seconds at maximum % output on a Microson ultrasonic cell disrupter (Heat Systems Ultrasoncis, Farmingdale, N.Y.) and then centrifuged for 5 min at 1300 x g to precipitate cellular debris. Total protein was assayed and 200 µg of each tissue fraction was mixed 4:1 in sample loading buffer and analyzed by Western blotting.

2.3.8 Determination of protein expression levels in 353G6-72 founders

Bone marrow fibroblast cultures were established from YAC72 founders 2498 and 2511 and an age matched FVB mouse in DMEM, 10% FCS, Penicillin/streptomycin, 20mM L-Glutamine (Canadian Life Technologies). Cells were maintained at a humidified atmosphere of 37°C, 6% CO₂. For the isolation of proteins, cells were scraped in culture media and washed twice with cold PBS. 40µl of cold cell lysis buffer (1.0% NP40, 0.15M NaCl, 50mM Tris (pH 8.0), 0.01% Azide, ½ tablet of complete protease inhibitors (Boehringer Mannheim) was added to each cell pellet, and left on ice for 30 minutes. 100µg of total protein was resolved in 7.5% SDS-PAGE mini gels and subject to Western analysis.
2.3.9 Western blotting

Western blotting was done using standard procedures. Proteins were diluted 4:1 in sample loading buffer (250mM Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.02% bromophenol blue and 7% β-mercaptoethanol) and resolved by SDS-PAGE using 7.5% mini gels (Bio-Rad Mini-PROTEAN II Cell system). For the analysis of huntingtin proteins, gels were electroblotted overnight in transfer buffer containing 10% methanol at 30V onto PVDF membranes (Immobilon-P, Millipore). Membranes were blocked with 5% skim milk in 1xTBS and probed with primary antibody at varying dilutions depending on antibody being used and batch of that antibody.

2.3.10 Rescue of embryonic lethality

FVB/N mice expressing the YAC transgene were mated to mice heterozygous for the murine HD gene disruption. Tail DNA was extracted from offspring and screened using the RYA PCR (see above) and genotyped at the murine HD locus for the null allele using primers P8 and P9 (Nasir et al., 1995). 1X PCR buffer (GIBCO BRL), 2mM MgCl2, 800 mM of each primer, and 0.5 U taq polymerase were mixed in a 25 µl reaction and subject to PCR: denaturing at 95°C for 1 min, 35 cycles at 94°C, 62°C, and 72°C for 60, 45, and 45 sec respectively followed by 10 min extension at 72°C. Heterozygous mice containing the YAC were bred to heterozygous littermates. Offspring from this second breeding were screened again with the RYA PCR and positive mice were genotyped by southern analysis as described using an α32P radiolabelled 540 bp Xba/HindIII fragment from mouse intron 5 as a probe on EcoR1 digested mouse tail DNA. Filters were exposed to Kodak X-OMAT overnight at −70°C. Alternatively, offspring were screened by PCR using 3 primers (P8, P9, P586) that detect both normal and null mouse alleles (Nasir et al., 1995).
2.4 Chapter 6 Behavioral assessment

Behavioral analyses were performed by Fred LePiane in the laboratory of Dr. Anthony Phillips, Department of Psychology, UBC, Vancouver, Canada

2.4.1 General assessment

2.4.1.1 Spontaneous Behavior in home cages

Observation of activity and exploratory behavior in home cages was recorded on videotape and categorized over 2 hr sessions. Each mouse was observed for a period of two hours in its home cage. For a 10sec interval out of every minute of the session all of the behaviors of the subject were recorded. This provided a means of quantifying the occurrence of normal and abnormal behaviors. The behaviors recorded include inactivity, grooming, scratching, sniffing, gnawing, burying, rearing, locomotion, climbing, circling, jumping and mounting. The frequency of each behavior was tallied over the length of the session.

2.4.1.2 Response to sensory stimuli

Auditory response in mice was tested using a clicking sound, repeated 10 times around all sides of the animal. Olfactory response was tested by placing peppermint and acetic acid solutions near the mice in their cage. Responsiveness to touch was assessed by touching the mice on 4 sides using a cotton applicator.

2.4.2 Activity and motor control

2.4.2.1 Activity

Activity was measured in a photocell box over a 4hr session (2hrs light, 2hrs dark) as previously described (Nasir et al., 1995). Total number of photobeam breaks were recorded over the course of the session.
2.4.2.2 Mesh-Climbing

A ramp made of 6mm wire mesh (10 cm wide x 35 cm high) was secured against the wall at 60° angle. Mice were placed on the center of the ramp and observed until they reached the top or bottom.

2.4.2.3 Crossing a Balance Beam

A wooden dowel (12 cm diameter) was suspended over a chasm (45 x 40 x 25 cm deep) with platforms at both ends. Each mouse was placed on the center of the beam and observed to see how long it took to initiate a movement, how many times it fell off the dowel, and how long it took to reach safety, given up to 10 min.

2.4.2.4 Block Test

Each mouse was lowered onto a bench top and was observed for its ability to orient itself properly and place all 4 feet on the surface.

2.4.2.5 Footprint Pattern

Each mouse had all four feet painted with tempera, placed on a white sheet of paper and allowed to walk until the paint had worn off. Footprint patterns were checked for both hind and forepaws for evidence of abnormal gait.

2.4.3 Spontaneous alternation in a Y-maze

Analysis was carried out as described (Hsiao et al., 1995). Following a 5min period of habituation, each mouse was observed for exploratory behavior into different arms of the
maze for a period of 8 min. Each arm was 39 cm long x 8 cm wide and the perimeter of the maze was enclosed by walls 15 cm high. Arms were arbitrarily designated as A, B, and C. The number of arms entered and sequence of arm entries were recorded. A triad was defined as entry into 3 arms. Percent alternation was calculated by dividing the number of triads containing entry into 3 different arms (A, B, and C) by the total number of alternation opportunities (the number of arms entered - 2). Between each trial, the Y maze was washed extensively with a disinfecting deodorizing spray and allowed to dry.

2.5 Chapter 7 Pathology and Immunocytochemistry

Pathological assessment of YAC transgenic mouse brains was performed by Dr. Claire-Anne Gutekunst in the laboratory of Dr. Steven Hersch, Emory University School of Medicine, Atlanta, GA.

2.5.1 Brain perfusion and sectioning

Each mouse was deeply anesthetized with 100 μl sodium pentobarbital (50 mg/ml), injected intraperitoneally with 300 U of heparin, and then perfused intracardially with 200 ml of 3% paraformaldehyde and 0.15% gluteraldehyde in 0.1 M phosphate buffer (PB) at pH 7.2. Brains were removed and post-fixed overnight in 2% paraformaldehyde. Brains were sectioned in ten series of 40 μm coronal sections using a vibratome (Pelco, Redding, CA), collected in PB, and rinsed in 0.05 M phosphate buffered saline (PBS, pH 7.2) and stored at 4°C.

2.5.2 Light level immunocytochemistry

Four antibodies against various regions of htt were used: a rabbit polyclonal antibody (EM48) to the N-terminal portion of htt (amino acids 1-256 excluding polylglutamine and polyproline repeat stretches) which specifically immunoreacts with htt aggregates in human tissue, (Li and Li, 1998), a
rabbit polyclonal antibody (HD-N) to the first 17 amino acids of human htt (generously provided by Peter Detloff), a rat monoclonal antibody (mHD549) to the internal region of htt (amino acids 549-679) (Gutekunst et al., 1995) and a monoclonal antibody previously described (GHMI) directed to the internal region of htt (Hodgson et al., 1996). Other antibodies used in this study included: antibodies against the vesicular acetylcholine transporter (VAT), which labels cholinergic interneurons (Gilmor et al., 1998), against nitric oxide synthase (NOSI, Chemicon International Inc., Temecula), which labels neurons containing somatostatin, neuropeptide Y, and NADPH-diaphorase activity, and parvalbumin (PARV, Sigma, St. Louis, MO), which labels another distinct population of medium-sized interneurons.

Free-floating sections were pre-blocked in 4% normal goat serum (NGS) in PBS, and avidin (10μg/ml) for 1 hr at 4°C. Sections were rinsed in PBS and incubated in biotin (50μg/ml) for 1 hr at 4°C. Following PBS rinses, sections were then incubated in primary antibodies in PBS containing 2% NGS. Sections were then rinsed in PBS for a total of one hour, and incubated overnight in biotinylated goat anti-rabbit antibody or goat anti-rat antibody (Vector ABC Elite, Burlingame, CA) in PBS containing 2% NGS. Following several rinses in PBS, the sections were incubated in avidin–biotin complex (Vector ABC Elite, Burlingame, CA) for 1 hr and rinsed several times in PBS. Immunoreactivity was visualized by incubation in 0.05% 3–3’–diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) and 0.01% hydrogen peroxide in 0.05M Tris buffer pH 7 until a dark brown reaction product was evident (5 to 10 min.). For double immunolabelling experiments, EM48 antibody was combined with either VAT, NOSI or Parvalbumin antibody in the primary incubation. Controls included the omission of primary antisera and single labeling for each antibody. Following DAB visualization, sections were osmicated (1% OsO4 in 0.1M cacodylate buffer), rinsed, and stained overnight in 2% aqueous uranyl acetate.
2.5.3 Electron microscopy immunocytochemistry

For more precise spatial resolution, we also used pre-embedding immunogold labeling of EM48. Vibratome sections were pre–blocked in 5 % NGS and 0.05% Triton-X in PBS for 30 minutes and incubated at 4°C on a shaker platform in primary antibodies in 2% NGS–PBS for 60hr. Sections were then rinsed in 6 changes of PBS for a total of 60 minutes, and were incubated overnight in Fab fragments of goat anti-rabbit secondary antibodies (1:50) conjugated to 1.4nm gold particles (Nanoprobes Inc., Stony Brook, NY) in PBS with 2% NGS. After rinsing in PBS and then PB, sections were fixed with 2% gluteraldehyde in 0.1M PB. Following several washes in PB, sections were silver intensified following a modification (Yi and Hersch, 1998) of the Burry method (Burry et al., 1992), post-fixed for 10 minutes in 0.5% OsO4 in PB, and processed for electron microscopy as described below.

All the sections used for electron microscopy were dehydrated in ascending concentrations of ethanol and propylene oxide (1:1) and flat embedded in Eponate 12 (Ted Pella, Redding, CA). Ultrathin sections (90nm) were cut using a Leica Ultracut S ultramicrotome, counterstained with 5% aqueous uranyl acetate for 5 minutes followed by Reynolds lead citrate for 5 minutes. Thin sections were examined using a HITACHI H-7500 electron microscope. From each case, following DAB staining, some sections were mounted for light microscopy.

2.5.4 Assessment of Neurodegeneration

Neurodegeneration was assessed in sections from hippocampus, striatum, cortex, and cerebellum in all the lines at 9, 12 and 20 months of age. Semi-thin sections (1.5 μm) were cut using a Leica Ultracut S ultramicrotome, counterstained with Toludine Blue or Cresyl Violet, differentiated in 95% alcohol and coverslipped. Sections were visualized using a Nikon Microphot FXA equipped with a 60x oil immersion lens. Neuronal degeneration was
quantified using the following method. EM48 immunoreacted sections (1.5 μm) were visualized on a Nikon Labophot-2 microscope using a 40x lens equipped with a 1 mm² ocular grid. A starting frame was systematically selected so that one side of the ocular grid was touching the most lateral portion of the corpus callosum. From this starting point, counts were made using three 240 μm² frames obtained by moving the grid towards the medial portion of the striatum, one grid length at a time. This was repeated in each section. Within each frame, all neurons with visible nuclei were counted and categorized as normal or degenerating and the presence or absence of EM48 staining was noted. In each counting frame, the top and right frame lines were excluded from analysis.

2.6 Chapter 8 Neurophysiology

Neurophysiological analyses were performed by Dr. Nadia Agopyan in the laboratory of Dr. John Roder, Faculty of Pharmacy, University of Toronto, Toronto, Canada.

2.6.1 Electrophysiological analysis

400 μm thick hippocampal slices from 6 or 10 month old sex-matched mice were obtained as described previously (Agopyan and Avoli, 1988; Agopyan and Agopyan, 1991) and perfused in an interface chamber at 32°C with oxygenated ACSF containing (in mM): 124 NaCl; 3.0 KCl; 1.25 KH₂PO₄; 4.0 CaCl₂; 26.0 NAHCO₃; 10 D-glucose and 10 μm bicuculline methiodide (GMI; Sigma Chemical Co.). The connections between CA3 and CA1 were cut to prevent spread of epileptiform activity in the absence of GABA-ergic inhibition. Field excitatory post-synaptic potentials (fEPSPs) were acquired as described previously. Stable slices were tetanized to evoke saturated LTP by delivering 5 high frequency stimulations (HFS) at a 5 min interval. Each HFS consisted of a 100 Hz train delivered for 200 ms and repeated 5 times every 10 sec. A control pathway, which did not receive pattern stimulation, was also monitored to determine the general state of the slice. Slices which
demonstrated either increase or decrease in the field EPSP slope evoked by the stimulation of the control pathway, following HFS of the test pathway were omitted from data analysis. Data are expressed as mean ± s.e.m. Significance was tested using the Student's t test.

2.7 Chapter 9: CAG Instability

2.7.1 Assessment of CAG trinucleotide size

CAG trinucleotide instability was assessed using the CAG PCR (see above). PCR products were run out on 6% polyacrylamide gels and sized relative to previously characterized standards. PCR products were detected 1 of 2 ways: 1) by addition of $^{32}$P radioactively end labelled HD344 primer to the PCR mix or 2) transferring the PCR products onto hybond-N+ membrane and probing with alkaline phosphatase conjugated (GTC)$n$ probe (Lifecodes) followed by detection using the Quick-Light detection system (Lifecodes). Gels/membranes were exposed to X-omat film (Kodak). The 6% gels were made by diluting a 20% acrylamide stock (19.3% acrylamide, 0.7% methylene bis-acrylamide, 7.8M urea, in 1X TBE) in 7.8M urea (in 1X TBE) to a final volume of 80ml, and polymerized with the addition of 500µl ammonium persulfate (0.1%) and 60ul Temed (BRL).

2.7.2 Screening mice at the MSH2 locus

PCR analysis at the MSH2 locus was done as described (Reitmair et al., 1995). A 3 primer PCR assay was used to distinguish wildtype (+/+), heterozygous, and homozygous(-/-) mutant MSH2 animals. Primers U771 and L926 amplify a 174bp fragment from the untargeted MSH2 allele, whereas primers U771 and L1211 (neo) amplify a 460bp fragment identifying the neo gene. PCR reactions were run in a 25µl volume with the following final concentrations: 1X BRL PCR buffer, 1.5mM MgCl$_2$, 0.2mM dNTPs, 0.4µM C771, 0.8µM
L926, 0.4μM L1211, 0.5U Taq polymerase. Conditions were 94°C denaturing 1min, 40 cycles of 94°C 1min, 62°C 30sec, 72°C 2min, and a 72°C extension for 10min. Primer sequences:

U771 5' GCT CAC TTA GAC GCC ATT GT 3'
L926 5' AAA GTG CAC GTC ATT TGG A 3'
L1211 5' GCC TTC TTG ACG AGT TCT TC 3'
2.8 Reference List


CHAPTER 3 - ANTI-HD ANTIBODY PRODUCTION
3.1 Hypothesis

Expression of the mutant HD protein plays a central role in HD pathogenesis.

3.2 Objective

To develop antibodies directed against the HD protein as an aid for studying protein localization and function.

3.3 Introduction

Despite the cloning of the HD gene and identification of the underlying mutation (HDCRG, 1993), few clues were provided regarding the function of the HD protein (huntingtin) due to lack of homology with other known genes. The widespread expression of the HD mRNA in normal and affected individuals (Strong et al., 1993; Li et al., 1993) suggests that the HD protein may be involved in the selective pattern of neuronal loss. To further assess the cellular localization and functions of both the normal and mutant forms of the HD protein, we sought to develop anti-huntingtin specific antibodies.

The use of fusion proteins as antigens for the production of antibodies has been well established. Such proteins are commonly produced using bacterial expression vectors that allow for high levels of protein expression in E. coli. One such system is the pATH expression system in which cloned DNA sequences are fused in-frame to the trpE gene of E. coli, which encodes for anthranilate synthase (Koerner et al., 1991). Therefore, hybrid proteins contain the amino terminal of the trpE gene product followed by the sequence specified by the cloned DNA. One of the key advantages of using pATH vectors is that they are maintained at a high copy number within E. coli and thus produce large amounts of hybrid protein under inducing conditions. Additionally the anthranilate synthase amino terminus provides stability to the fusion protein, making it less susceptible to degradation post translation. Finally, hybrid proteins are usually stored in E. coli as insoluble inclusion bodies, which greatly facilitates the purification process. It is no surprise then that TrpE fusion proteins have been
extensively used to generate antibodies to a wide variety of proteins which include transcription factors (Zhang et al., 1993), neurotransmitter receptors (Shigemoto et al., 1993), and viral proteins (Szilvay et al., 1992). We therefore chose the pATH expression system in an effort to generate antibodies against the HD protein.

3.4 Results

3.4.1 Antigen Production

The basic outline used for generation of trpE-HD hybrid proteins is illustrated in figure 3.1. Two regions were chosen from the HD cDNA (E/C968 and E/B912) since they could be cloned in frame into one of the pATH vectors and were of large enough size to offer multiple antigenic epitopes to which antibodies could be raised. E. coli cells were transfected with pATH-HD clones and colonies were checked for expression of a fusion protein by coomassie staining and western blotting (Figure 3.2A and B). Most colonies that survived antibiotic selection did express a fusion protein although not at the same levels (e.g. clones 5 and 15 in figure 3.2 expressed the highest levels). This is most likely a reflection of the copy number of the plasmid in the cells. As with most trpE fusion proteins, the trpE-HD hybrids remained in the insoluble pellet fraction and could be separated from most other E. coli proteins by centrifugation (Figure 3.2C). This step enriched for the fusion protein, however, many other bacterial proteins were still present, and therefore the hybrid was further purified by SDS-PAGE and electroelution of the protein from the gel prior to injection.

3.4.2 Polyclonal Antibody Production

Serum antibody titers were checked from each rabbit using ELISA showing that 3/4 rabbits failed to produce titers significantly above background. In other words, the sera were as reactive to the trpE portion of the fusion as the trpE-HD fusion itself, indicating that the vast
Figure 3.1 TrpE-HD fusion protein expression

A schematic diagram of the general strategy used to produce trpE-HD fusion proteins. Two segments of the HD cDNA were separately cloned into the appropriate pATH vector to ensure that the correct reading frame was maintained. Clones were transfected E. coli RR1 cells and removal of tryptophan (trp) from the media and addition of the inducing molecule indoleacrylic acid (IAA) induced expression of the fusion protein. The binding of a trp-repressor complex to the promoter down regulates protein expression. In the absence of trp, the repressor is unable to bind to the promoter expression is induced. Addition of IAA, a trp analogue, further induces expression since it competes for binding to the repressor with any residual trp present in the cell. The IAA-repressor complex is unable to bind to the promoter and decrease expression.
HD cDNA (10kb)

5'  CAG  E/C968  E/B912  3'

Clone fragments

Inducible Promoter  →  pATH  ←  Trp E  ←  Cloning Site

amp

Express in RR1

Bacterial fusion protein

Trp E  →  HD segment
Figure 3.2 Screening clones for TrpE-HD fusion protein expression

A) A coomassie stain of whole cell lysate from 7 clones (1, 2, 5, 15, 16, 17, and 3c) transformed with the E/B912 fragment. The final lane labeled c is a control cell transformed with a pATH plasmid containing no insert that would thus express the 37 kDa trpE protein alone. Fusion protein expression is highest in clones 5 and 15 and can be detected as a darker staining band at approximately 70 kDa.

B) Western blot of a similar gel probed with an anti-trpE polyclonal antibody. The 37 kDa trpE band is detected in the control lane c, but is not present in the E/B912 clones where the presence of a new 70 kDa band is observed. The exception is clone 3c. It is likely that this clone contains plasmids with and without inserts since both trpE and the fusion protein are detected.

C) A coomassie stain of the same clones following separation of the insoluble inclusion bodies from the rest of the cell lysate. The fusion protein is highly enriched in the sample following this step.
CLONE

1  2  5  15  16  17  3c  C

A

E/B912

B

E/B912

trpE

C

E/B912
majority of the antibodies produced were directed against the bacterial portion of the fusion. One rabbit did however show a higher titer with the fusion protein rather than trpE alone and was derived from the amino terminus E/C968 fusion protein. Crude serum tested for anti-huntingtin specificity by western blot analysis detected a band around 350 kDa in mouse kidney and brain tissues, correlating well with the expected size of the HD protein. However, a poor signal to background ratio was present on these blots and steps were taken to further purify the HD specific antibodies.

To enrich for anti-HD specific antibodies in the antiserum, the HD E/C968 insert was cloned in frame and expressed from the PGEX-4T-2 expression vector which uses the 5' region of bacterial glutathione S-transferase (GST) gene as its amino terminus. Crude serum was run over a column containing the GST-E/C968 fusion, and any bound antibodies eluted at low pH. Purified antibodies were again used for Western blotting, showing immunoreactivity to a high molecular weight protein consistent with the expected size of huntingtin (350kDa). However, the signal to noise ratio was not improved following this purification step suggesting that these antibodies had a low affinity for huntingtin.

### 3.4.3 Monoclonal Antibody Production

After 3 immunizations with the trpE-HD hybrids E/C968 and E/B912 as separate immunogens, antisera titers were determined by the ELISA method. The antisera of all immunized mice reached titers of 3200 or higher. One mouse from each immunized group was used for cell fusion. Initial antibody screening of the cell hybrids derived from the E/C968 fusion protein injections revealed that 2/600 clones produced antibodies that recognized the trpE-E/C968 antigen. A further ELISA screening of these 2 putative positive clones by using trpE coated plates showed that the secreted antibodies recognized the bacterial portion of the fusion protein, and were not specific to HD. In the case of the trpE-E/B912 fusion protein, among 600 hybrid colonies that were removed, 35 were shown to secrete antibodies reacting with the fusion protein. A total of 28 of these clones were reactive to the trpE portion of the fusion, whereas the remaining 7 showed reactivity to the HD portion, as
determined by ELISA. These antibody producing hybrid cell lines (termed THB1, THB5, THB17, THB18, THB29, THB32, and THB33) were further cultured and frozen to establish cell lines. All hybrid cell lines were determined to secrete antibodies of the IgG<sub>2b</sub> subclass except THB29, which was IgG<sub>2a</sub>.

Culture supernatants were taken from each cell line and further tested for HD specific immunoreactivity on Western blots. The first round of screening was done using bacterial cell lysates from RR1 cells expressing trpE, trpE-E/C968, or E/B912. Clones THB5 and THB29 produced antibodies that reacted with all 3 proteins suggesting that these antibodies did in fact recognize the trpE portion of the fusion protein and were not HD specific. The remaining 5 clones were tested on Western blots with human and mouse brain tissue. Only 1 clone, THB-17, produced an antibody that recognized a high molecular weight band in the human samples. The remaining clones, despite not recognizing the trpE portion of the fusion, failed to detect a high molecular weight protein, suggesting that they had very weak immunoreactivity to huntingtin.

Clone THB17 was used to generate ascites fluid, from which concentrated antibody (termed GHM1) was extracted on a proteinA-sepharose column and used for subsequent analyses. Western blots using GHM1 consistently recognized a high molecular weight band in human samples but failed to detect anything in mouse protein preparations (Figure 3.3). Immunodetection of this human specific band could be eliminated by preabsorbing the GHM1 primary antibody with the trpE-E/B912 fusion protein but not trpE alone, suggesting that the observed 350kDa band was in fact HD specific. GHM1 has proven invaluable in protein expression studies in YAC transgenic mice expressing human huntingtin (see chapter 5 for details). GHM1 was also able to immunoprecipitate human huntingtin and has been successfully used to confirm an interaction between huntingtin and the ubiquitin conjugating enzyme, originally identified using the yeast 2-hybrid screen (Kalchman et al., 1996).
Figure 3.3 Immunoreactivity of monoclonal antibody GHM1.

An immunoreactive high molecular weight band (huntingtin) is observed in the human 293 cell lysate lane but no signal is seen with mouse brain tissue. Equal amounts of protein were loaded in each lane.
3.5 Discussion

The pATH expression system proved useful in that we were able to produce a monoclonal antibody (GHM1) that specifically recognized the human form of huntingtin on Western blots. The success rate at which anti-HD antibodies were produced was however low. Only 1 out of 4 rabbits used for polyclonal antibody produced a titer above trpE alone (as determined by ELISA), and the HD specific portion of the antiserum from that rabbit failed to recognize huntingtin strongly on Western blots. Production of monoclonal antibodies fared better, but again only 1/37 antibody-producing hybrid clones generated an antibody with high affinity for huntingtin on Westerns (30/37 clones produced antibodies that recognized trpE). It appears that the bacterial trpE portion of the fusion proteins was relatively more antigenic than either of the HD derived portions. It would therefore be preferable to remove the bacterial portion of the fusion prior to injection. This is not possible for the pATH expression system since no protease cleavage sites are present between trpE and the cloned DNA segments. Other systems are however available to circumvent this problem. The GST gene fusion system (Pharmacia), ThioFusion™ Expression System (Invitrogen), and ProEX™ HT Prokaryotic Expression System (Life Technologies) all offer the advantage of proteolytic removal of the bacterial portion of the fusion prior to injection. This would provide a large, native protein product with multiple epitopes against which an immune reaction can be raised. Another advantage these other systems offer is the ease with which the fusion protein can be purified following expression in bacteria. The bacterial portion of the fusions has very high, specific affinity for certain compounds. Thus after applying a bacterial cell lysate to a column containing these compounds, the fusion protein will vigorously stick to the column while all other contaminating proteins run through. No such compounds exist for trpE, thus making purification via SDS-PAGE and electroelution a relatively long and tedious procedure.

Another option for producing antigens to generate antibodies is to synthesize peptides (18-22 amino acids long) from the gene of interest. This method offers the advantage that it is quicker and
does not require the use of elaborate bacterial expression and purification systems. One has to be careful, however, to ensure that the region(s) chosen for synthesis are exposed on the surface of the protein in its native form and not internalized. Looking at hydrophilicity plots of the protein sequence can help identify epitopes that may be exposed. The main disadvantage of using peptides as antigens is that they provide only 1 or 2 epitopes to which antibodies can be raised. Fusion proteins, however, provide a multitude of epitopes, thus allowing for greater potential to elicit a strong immune response.

While the pATH expression system has been used successfully to generate antibodies to a wide variety of proteins, including HD (in this study), there are many other systems now available, which make antigen production easier and more efficient. Also, given the apparent antigenic nature of trpE, alternatives to the pATH system should be considered, prior to future attempts at generating anti-HD antibodies.

3.6 Conclusion

Expression of trp-E-HD fusion proteins in bacteria is a feasible method of producing antigenic epitopes for generating anti-HD antibodies.
3.7 Reference List


CHAPTER 4 - YACs AND YAC MUTAGENESIS
4.1 Hypothesis

Expanded/mutant sized CAG repeats can be introduced at the HD locus on YACs using homologous recombination in yeast.

4.2 Objective

To replace the normal sized CAG repeat (18 CAGs) on YGA2 and 353G6 with mutant sized repeats (46 CAG and 72 CAG) and to characterize these mutated YACs to ensure appropriate insertion of the mutation.

4.3 Introduction

An animal model for HD would greatly facilitate our understanding of the cellular and molecular mechanisms of pathogenesis. An extremely important consideration if one wishes to develop and study a model system that closely mimics the human condition is to ensure that transgene expression occurs in an appropriate temporal and spatial manner.

The development of transgenic technologies using YACs offers important advantages for generating transgenic mice expressing human huntingtin. Studies on YAC transgenic mice have demonstrated appropriate developmental regulation of the human β-globin gene cluster in a mouse background (Peterson et al., 1993). Additionally, human β-amyloid precursor protein (APP) and β-globin YAC transgenic mice demonstrated that expression of the human protein occurred in the same tissues as the corresponding mouse protein (Lamb et al., 1993; Gaensler et al., 1993). Other studies on YAC transgenic mice have demonstrated that the levels of transgene expression is largely copy number dependent and not influenced by site of integration into the mouse genome (Schedl et al., 1993; Gaensler et al., 1993; Lamb et al., 1993). Expression levels similar to endogenous levels were typically obtained by integration of 1-2 YAC copies (Schedl et al., 1993; Gaensler et al., 1993). These studies illustrate the advantages of YACs for transgenic experiments since cDNA based transgenes are
often poorly expressed and inappropriately regulated (Jaenisch, 1988). An additional attractive feature of YACs is that site directed mutations can be introduced into any gene of interest using a homologous recombination based strategy in yeast (Duff et al., 1994). This allows one to introduce a wide variety of mutations into the gene of interest. In the case of HD, we can substitute normal sized CAG repeats with a number of mutant repeat sizes ranging from adult to juvenile onset.

This chapter deals with a description of the 2 YACs used in this study and the strategy used to introduce expanded CAG repeats into each YAC. The following chapter addresses the production and preliminary characterization of the transgenic lines generated with both YACs.

4.4 HD YACs

To generate transgenic mice expressing the human HD gene, we used 2 previously characterized YACs, YGA2 and 353G6 (Baxendale et al., 1993). Both YACs show no obvious rearrangements based on extensive mapping analysis and both contain the entire HD gene but differ with respect to size and flanking DNA sequences (figure 4.1). Detailed studies of this genomic region have identified other genes, cDNAs, and ESTs that map to this region (Hadano et al., 1998). The genomic organization for YGA2 and 353G6 are presented in figures 4.2A and 4.2B respectively. Genes found on these YACs and their functions (if known) are presented table 4.1.

4.5 Generation of YACs Containing Expanded CAG repeats

Both YGA2 and 353G6 contain 18 CAG repeats, well within the normal range seen in the general population. It is unlikely that mice expressing the human protein from these YACs would manifest an HD like phenotype since SCA1 cDNA transgenics expressing the normal cDNA showed no signs of neuropathology (Burright et al., 1995). Therefore, a previously described strategy was adopted as a way to introduce expanded CAG repeats into both YACs (Duff et al., 1994). The general idea behind this approach is that one can introduce any desired mutation into YAC DNA by use of the
Diagram showing the relationship between the HD gene and the two YACs, YGA2 and 353G6. YGA2 is 600kb in size and extends approximately 350kb 5' and 50kb 3' of the gene (Baxendale et al., 1993). 353G6 is 350kb in size and extends approximately 25kb 5' and 120kb 3' of the gene. Arrows show the five PCR markers used for screening the transgenic mice. The left YAC arm (LYA), denoted as a black box, contains the TRP1 gene, ARS, and CEN4. The right YAC arm (RYA), denoted as a white box, contains the URA3 gene.
Figure 4.2 Genomic organization of YAC YGA2 and 353G6.

Genomic organization of YAC YGA2 (A) and 353G6 (B). Genomic markers are listed along the top. Genes and direction of transcription (where known) are indicated by arrows. For reference, the HD gene reads 5'-3' from telomere to centromere. Function and expression information for each gene is listed in table 3.1.
A) YGA2

RYA  ΔG  CA  CAG  OT17  D4S95  OS14  D4S182  LYA

HUMY16EST  HD  IT11  IT10C3  ADD1  CD1-R2

50 Kb

RES4-24  RES4-25  SH3BP2
Table 4.1 Detailed list of the genes and their functions found on YGA2 and 353G6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipase A2-like</td>
<td>cDNA clone with high similarity to phospholipase A2</td>
<td>(Rommens et al., 1993)</td>
</tr>
<tr>
<td>HUMY16EST</td>
<td>cDNA fragment showing 93% aa identity with rat 40S ribosomal protein S21</td>
<td>(Jou et al., 1994)</td>
</tr>
<tr>
<td>IT11</td>
<td>G protein-coupled receptor kinase</td>
<td>(Ambrose et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Expression levels low in brain, high in testes</td>
<td></td>
</tr>
<tr>
<td>RES4-24</td>
<td>Unknown function</td>
<td>(Hadano et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Expression levels low in brain</td>
<td></td>
</tr>
<tr>
<td>RES4-25</td>
<td>Unknown function</td>
<td>(Hadano et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Ubiquitous expression, highest in skeletal muscle</td>
<td></td>
</tr>
<tr>
<td>IT10C3</td>
<td>Similarity to superfamily of transporter proteins</td>
<td>(Duyao et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Ubiquitous expression</td>
<td></td>
</tr>
<tr>
<td>ADD1</td>
<td>α-adducin; membrane associated cytoskeletal protein</td>
<td>(Goldberg et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Expression levels high in brain</td>
<td></td>
</tr>
<tr>
<td>SH3BP2</td>
<td>Putative adapter protein involved in signal transduction</td>
<td>(Bell et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Ubiquitous expression</td>
<td></td>
</tr>
<tr>
<td>CD1-R2</td>
<td>High similarity to gene for MHC-related antigen</td>
<td>(John et al., 1994)</td>
</tr>
</tbody>
</table>
highly efficient homologous recombination observed in yeast. The general strategy is outlined in figure 4.3.

The first stage of this strategy involves disruption of the URA3 gene present in the right arm of the YAC vector, PYAC4 since the mutagenesis event involves a positive-negative selection strategy using URA3 as marker. This retrofitting phase can be accomplished using the retrofitting vector pRV1 which, when transformed into yeast cells with the desired YAC (YGA2 or 353G6), disrupts the URA3 gene by insertion of the lysine gene (lys).

Retrofitted YACs can then be used for the replacement of the normal sequences with the desired mutation, in this case, an expanded CAG repeat. The mutation plus homologous flanking DNA sequences is cloned into the shuttle vector pRS406 (containing the URA3 marker), linearized to promote homologous recombination, and transformed into the yeast containing the YAC, where insertion of the vector is selected for by plating transformants on media lacking uracil. The resulting 'pop-in' clone will contain both normal and mutant sequences plus the shuttle vector sequences (figure 4.3). Selected clones can be checked for appropriate insertion by Southern blotting and PCR. An intrachromosomal 'pop-out' recombination event can then be induced by plating desired clones on agar containing the compound 5-fluoroorotic acid (FOA) since yeast expressing the URA3 gene cannot grow on agar containing FOA. This results in either excision of wild type or mutant sequences. Due to the multiple recombination events involved in this strategy and the inherent unstable nature of YACs due to their large size, all YACs containing the desired mutation must be extensively checked for rearrangements by PFGE and Southern blotting.
Figure 4.3 The YAC mutagenesis procedure

A schematic representation of the YAC mutagenesis procedure used to introduce expanded CAG repeats into 353G6 and YGA2. The whole procedure takes advantage of the highly efficient process of homologous recombination in yeast. Pop-in clones are generated by transformation of the shuttle vector (pRS406) containing insert sequences with the desired mutation (in this case 48 or 72 CAG repeats) plus flanking homologous DNA. The vector is first linearized using a restriction enzyme that cuts the vector once within the cloned DNA segment (in this case BstEII) to promote homologous recombination. Pop-in clones therefore contain a duplicated segment of DNA in the YAC represented by the region of homology containing normal (18 CAG) or mutant (48 CAG) sequences, flanking pRS406 vector sequences with the URA3 gene marker. Pop-in clones are selected for on ura⁻ plates.

An intrachromosomal pop-out event is induced in the pop-in clones by plating these cells on media containing the compound 5-flourooorotic acid (FOA). Only those cells having undergone intrachromosomal recombination and elimination of URA3 will grow on the plates, since yeast expressing the URA3 gene product fail to grow in the presence of FOA. Recombination at this point will result in the production of a pop-out clone containing either normal or mutant DNA sequences, depending where the recombination event takes place (a or b).
pRS406

URAG3

X

18

POP-IN

F.O.A

Intrachromosomal recombination

X

18

a

POP-OUT

b

48

or

18
4.6 Results

4.6.1 Characterization of 5' End of 353G6

Following cloning of the HD gene, it became evident that the 5' end of 353G6 was very close to the 5' end of the HD gene. Thus it became important to accurately map the distance of the HD promoter to the end of the YAC to ensure that the majority of regulatory sequences were present. This was accomplished by Southern blotting using a probe lying approximately 4kb 5' of exon 1 (EP581). YAC DNA from 353G6 and YGA2 was digested with a multitude of enzymes, and banding patterns between the 2 YACs was compared. Since YGA2 is known to map ~250kb 5' of the HD gene, similarities and/or differences between the 2 YACs would provide an estimate of the distance. The enzymes BamHI and EagI were of particular interest. A band of ~ 17kb was seen in a BamHI digest of both YGA2 and 353G6, demonstrating that at least 17kb of 5' HD sequence was present in 353G6 (figure 4.4). An EagI digest however, revealed a different banding pattern, producing a band of ~35kb in YGA2 and ~25kb in 353G6. The difference between the 2 YACs could be attributed to 1) identification of the 5' end of the 353G6 YAC or 2) a DNA polymorphism producing an RFLP. Regardless, these results demonstrate that at least 25kb of 5' genomic DNA is present in 353G6 and would probably contain most of the regulatory sequences necessary to allow for appropriate regulation of the HD gene in a transgenic mouse model.

4.6.2 YAC Mutagenesis

The region of DNA chosen for the mutagenesis strategy was an EcoRI-EagI fragment (EE640) that provides 160bp of 5' and 410bp of 3' sequence flanking the CAG repeat. This region provides the necessary amount of flanking homologous DNA needed for efficient homologous recombination, in addition to providing a unique restriction enzyme site (BstEII) necessary for vector linearization prior to transformation. The EE640 fragment containing 48 CAG repeats was cloned
Figure 4.4 Characterization of the 5' end of YAC 353G6

Characterization of the 5' end of YAC 353G6 by Southern analysis. EP581 was used as a probe and lies ~4kb 5' of IT15 exon 1. The BamHI fragment is ~17kb in size. The EagI fragment in 353G6 is ~25kb in size, showing that this YAC contains at least that much DNA 5' of the HD gene.
from the L191F1 cosmid containing an expanded CAG repeat. This same fragment containing 72 repeats was shotguncloned from patient DNA. Colonies containing the desired insert were screened by colony hybridization using EE640 with 18 repeats as a probe. These clones were used independently to mutagenize YGA2 and 353G6 using the pop in-pop out (PIPO) procedure discussed above.

The screening strategy used to identify pop-out clones with intact YACs and expanded CAG repeats was as follows. Firstly, pop-in and pop-out clones were screened by using the CAG PCR for evidence of appropriate sized expanded alleles. Subsequent analysis of the YAC DNA integrity was done on all pop out clones containing expanded alleles using a combination of Southern blotting and Pulse Field Gel Electrophoresis (PFGE).

The results of the PIPO are summarized in table 4.2 and details are discussed below.

### 4.6.2.1 353G6-48

YAC 353G6 was chosen initially for mutagenesis since it is the smaller of the 2 YACs and easier to handle when prepping YAC DNA for microinjection. A total of 4 pop-in clones were identified as having incorporated an expanded allele, 1 of which was chosen for the pop-out procedure. A low percentage of pop-out clones (3%) were determined to have retained the expanded allele. Subsequent analysis by PFGE revealed that all 3 clones had lost a significant portion of DNA sequences since the YACs migrated roughly 25-35kb lower on PFGE (fig. 4.5A, B). Additionally, Southern analysis using the probe KRP7 (beginning 20bp 5’ of the CAG repeat), revealed an unexpected banding pattern when compared to the original 353G6 YAC (fig 4.6). Subsequent analysis using a further 5’ probe pGB354 (beginning 100bp 5’ of the CAG repeat) showed no hybridization to the mutant YACs at all, suggesting loss of these 5’ sequences.

To further characterize the deletion breakpoint, the aberrant 3.5kb PstI fragment (fig 4.6) was cloned from YAC pop-out clone R2J into pBluescript and sequenced from both ends using the T3 and
### Table 4.2 Summary of PIPO mutagenesis.

<table>
<thead>
<tr>
<th>YAC</th>
<th>Pop-In Clone</th>
<th>Pop-out Clones with expanded allele</th>
<th>Intact/stable YAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>353G6-48</td>
<td>353R1</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>353R2</td>
<td>3/100</td>
<td>0/3</td>
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<td></td>
<td>353R3</td>
<td>ND</td>
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<td>353R4</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td><em>ND</em></td>
<td><em>3/100</em></td>
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<td>YGA2-46</td>
<td>Y17-12</td>
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<tr>
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<td>Y17-13</td>
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<tr>
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<td>Y17-21</td>
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<td>0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>8/167</em></td>
<td></td>
</tr>
<tr>
<td>353G6-72</td>
<td>353-18</td>
<td>1/36</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>353-19</td>
<td>6/36</td>
<td>4/6</td>
</tr>
<tr>
<td></td>
<td>353-34</td>
<td>1/30</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>8/102</em></td>
<td></td>
</tr>
</tbody>
</table>

*ND: not determined*
Figure 4.5 Pulse field gel electrophoresis analyses of mutagenized YACs.

A and C represent ethidium bromide stained gels. B and D represent these same gels, transferred to Hybond-N+ membrane, and probed with an HD specific cDNA probe, cD70-2. Yeast DNA preps containing normal YACs are shown in A and B, lane 1 (353G6-18), and C and D, lane 5 (YGA2-18). Arrows indicate the location of the normal YAC. YAC 353G6-48, A and B, lanes 2 and 4, migrates at a distance ~25kb lower than expected, suggesting deletion of a large part of the YAC. 353G6-72, A and B, lane 3, migrates at the expected size (350kb). YGA2-46, C and D, lane 6, also migrates at the expected size (600kb) but probing with cD70-2 reveals the presence of an additional band at ~450kb.
Figure 4.6 Southern analysis of 353G6-48.

Southern blot of 353G6 and 3 pop-out clones containing 48 CAG repeats (R2J, 88, 2814) using the 350bp probe KRP7 that lies 20bp 5' of the CAG. The expected 4.1kb EcoRI and 1.6kb PstI fragments are observed in 353G6 but different sized bands are seen in the pop-out clones suggesting rearrangement.
T7 primers. As expected, sequence from one end of the clone was from the HD gene exon 1 since a PstI site lies 15bp 3' of the exon 1-intron 1 boundary of the HD gene. Sequence analysis from the other end of the clone revealed a high homology to the original pop-in vector, pRS406. These results, in conjunction with the Southern and PFGE analyses clearly show that these pop-out YACs severely rearranged during the mutagenesis procedure, resulting in loss of the HD gene regulatory region, and likely the entire 25kb of 5' sequence in the YAC. The most likely 5' end of HD sequence in the 353G6-48 pop-out clones is the EagI site used to initially clone the CAG repeat into pRS406 (lying 170bp 5' of the CAG) since sequences from this region overlap with probe KRP7 (which hybridized with the 353G6-48 YACs), but not with probe pGB354 (which failed to hybridize with the pop-out YACs).

4.6.2.2 YGA2-48

Only ~ 5% (8/167) of the pop-out clones screened contained expanded CAG alleles. To confirm the presence and stability of any identified expanded alleles, pop-out clones were grown and restreaked under selective pressure, and representative colonies were picked for analysis. Pop-in clone Y17-18 produced 5 pop-out clones with expanded repeats but showed clear evidence of unstable maintenance of the CAG repeat. A total of 4/5 of these pop-out clones had expanded alleles less than 48 repeats in addition to the presence of a lower allele of 18 repeats. One clone did show faint amplification of an allele in the mid 40 range, however upon restreaking and subsequent screening of 10 colonies from this clone, none contained an expanded allele. Clone Y17-21 showed 2 potential clones with expanded repeat sizes, however subsequent screening of daughter colonies from this clone failed to show any amplification of higher allele sizes. Pop-out clone Y17-14 did show stable maintenance of a CAG repeat expansion. Restreaking of this clone and subsequent screening of 10 colonies showed that all had retained a single expanded allele of 46 CAG repeats. This repeat size is 2 CAG repeats less than the original DNA fragment used for the pop-in procedure (48), thus a loss of 2 CAG repeat units was observed in this particular yeast clone, probably during the pop-out phase.
of the procedure. Since Y17-14 was the only pop-out clone that demonstrated stable transmission of
the CAG repeat in yeast, I chose this clone for further characterization of YAC integrity.

PFGE of this YAC showed that it migrated at the expected size (fig 4.5 C, D). However, when
these gels were hybridized with an HD specific probe (cD70-2), an additional band at ~450kb was
observed. It is possible that this yeast cell contains 2 mutagenized YACs, one of which is normal, the
other being rearranged. Subsequent Southern analysis was therefore done on the YAC running at the
expected size (600kb) by isolating the DNA as would be done when prepping it for microinjection (see
methods). YGA2-18 and YGA2-46 DNA was digested with a variety of restriction enzymes and
probed with the full-length human HD cDNA to generate a fingerprint of both YACs (Figure 4.7).
Most bands were identical between both YACs, with the exception of those containing the CAG repeat
which differed by approximately 100bp, indicative of the difference in CAG size. The 4.1kb EcoRI
fragment was observed in YGA2-46, indicating that, unlike 353G6-48, the first 4kb of regulatory gene
sequence was present. Taken together, the data strongly suggested that this mutant YAC contained an
intact HD gene and was therefore used to generate a series of YAC transgenic mice.

4.6.2.3 353G6-72

Initial attempts at identifying an intact 353G6 YAC with 72 repeats using the original
screening strategy proved unfruitful. A total of 305 pop-out colonies were screened, revealing
that 21(6.8%) had retained the higher CAG allele, none of which had retained 5' flanking
DNA sequences of the HD gene (as revealed by Southern blot). In an attempt to address this
problem in more detail, another series of 353G6-72 pop-in clones was generated and screened
by Southern blotting using a 5'HD probe (Eco4.1), in addition to CAG PCR screening.
Interestingly, only 3/18 pop-in clones showed the expected banding pattern via Southern
blotting and CAG allele sizes of 18 and 72. These 3 clones were used for the pop-out phase of
the mutagenesis procedure. Again, the vast majority of pop-out clones that were screened
retained the lower allele (92%), consistent with what was observed with the other YAC
Figure 4.7 Southern analysis of mutagenized YAC, YGA2-46.

Lanes are run in pairs, representing normal YGA2-18 (18) and mutant YGA2-46 (46) YACs, digested with 1 of 5 restriction enzymes (listed above). The blot was probed with the full length HD cDNA, generating the observed fingerprint of the HD gene within the YAC. Most bands are identical between the 2 YACs, except those containing the CAG repeat (indicated by arrows). The 1.6kb PstI fragment runs at 1.7kb in the mutant YAC, and the 600bp TaqI fragment runs at 700bp. The 4.1kb EcoRI fragment also shows a subtle shift in band migration. This fragment is of particular interest since it is known to contain the first 4kb of the HD gene regulatory sequences. No band shifts are observed in the BamHI lanes and HindIII lanes since the CAG containing fragments are too large to resolve a 100bp difference.
<table>
<thead>
<tr>
<th>EcoRI</th>
<th>BamHI</th>
<th>PstI</th>
<th>TaqI</th>
<th>HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>46</td>
<td>18</td>
<td>46</td>
<td>18</td>
</tr>
</tbody>
</table>
clones. To test rapidly whether the pop-out clones with higher CAG repeats had retained the promoter sequences of the HD gene, they were screened using a PCR protocol that amplifies a region of the HD promoter approximately 600bp 5' of the CAG repeat. This region was consistently deleted in the 353G6-48 YACs. Of the 8 clones containing 72 CAG repeats, 4 amplified this region, suggesting that the HD promoter was present in these clones. These clones were further analyzed by PFGE (Fig 4.5 A, B) and Southern blotting (Fig 4.8), confirming that the 5' region of the HD gene was intact and that no major rearrangements had occurred within the YAC. YAC 353G6 was therefore prepped for micro-injection.

4.7 Discussion

Analysis of the pop-out clones from all 3 attempts to produce mutant YACs consistently revealed that retention of the lower allele (18 CAG) was favored over the higher allele (46 or 73 CAG repeats). Typically pop-out clones with expanded CAG repeats were retrieved 3-8% of the time. This figure is well below what might expected prior to beginning the procedure. If the chance of getting a recombination event during the pop-out phase (see figure 4.3) was simply dictated by length of the DNA strand, then we would expect that ~30% of the pop-out clones would retain the higher allele. This is because the length of DNA in region a (5' of the CAG) (fig. 4.3), which would result in a YAC clone with expanded repeats, represents 30% of the total homologous region cloned into the pRS406 vector (160bp/570bp). It is likely that other factors are playing a role in determining which allele will be retained. One possibility maybe that the site of recombination favored by the yeast during the pop-out phase is the same, or very close to, the site used during pop-in. The BstEII site used to linearize the vector to promote homologous recombination during pop-in lies 3’ of the CAG (fig 4.3). Perhaps some kind of ‘recombination imprint’ remains in the DNA sequence at original site of recombination thus promoting recombination at that site during pop-out. Another possibility could be that yeast favor smaller sized CAG repeats and usually retain the lower sized allele over the
### Figure 4.8 Southern analysis of 353G6-72.

Southern characterization of the 5’ end of 353G6-72 pop-out clone 19-30 using the 4.1kb EcoRI fragment as a probe. Note the shift in band size of the 4.1kb Eco and 1.6kb PstI fragments due to the presence of the expanded CAG repeat.
higher. Regardless, screening large numbers of pop-out clones produced sufficient numbers on which further screening could be performed. Integrity of the YAC transgene was also a critical issue during the PIPO procedure. Of the YAC clones that had retained the higher CAG repeats, ~30% (5/18) were intact. This phenomenon is not without precedent as 60% (3/5) of pop-out clones from a similar study on the APP gene were intact, with the remainder containing deletions around the site of mutation (Duff et al., 1994). Additionally, a detailed analysis of our pop-in clones on YAC 353G6 with 72 repeats revealed that only 17% (3/18) contained the correct CAG allele sizes and desired banding pattern on Southerns. Clearly this illustrates the importance of assessing YAC integrity at all stages of this procedure.

4.8 Conclusion

Using homologous recombination in yeast, it is possible to introduce expanded CAGs up to 72 consecutive repeats into YACs at the HD locus.
4.9 Reference List


CHAPTER 5 - CHARACTERIZATION OF YAC TRANSGENIC MICE
5.1 Hypothesis

YAC transgenic mice containing the normal and mutant HD gene will express the HD protein in tissue and cell specific manner at the appropriate time during development as the corresponding endogenous mouse gene.

5.2 Objective

To generate and characterize YAC transgenic mice at the DNA, RNA, and protein expression levels for evidence of YAC integration and protein expression patterns.

5.3 Introduction

As mentioned, previous studies on YAC transgenic mice demonstrated that the human genes contained within the YACs were regulated such that appropriate temporal and spatial expression of the transgene was achieved (discussed in chapter 4). This is an extremely important consideration if one wishes to develop and study a model system that is as close a representation of the human condition as possible. The initial experiments in this study were therefore designed to establish how the human HD gene was regulated from both YGA2 and 353G6. This chapter describes the production and detailed characterization of YAC transgenic mouse lines with respect to the temporal and spatial regulation of the human HD gene in a murine background.

5.4 Results

5.4.1 Generation and Identification of YAC Transgenic mice

Production of YAC transgenic mice by pronuclear microinjection of purified YAC DNA was originally published using a small 35kb YAC containing a tyrosinase mini-gene (Schedl et al., 1992). Numerous other reports have since been described, all showing germline transmission of the YAC and appropriate transgene expression in identified founders (Schedl et al., 1993; Peterson et al., 1993;
Gaensler et al., 1993; Frazer et al., 1995). We therefore chose to purify YAC DNA for microinjection rather than attempting the more labor intensive route of generating ES cells carrying integrated YAC DNA, followed by injection into blastocysts (Lamb et al., 1993; Strauss et al., 1993; Lamb et al., 1993). YAC DNA was purified away from the rest of the yeast chromosomes by separation via pulse field gel electrophoresis and subsequent cutting of the YAC from the gel (see materials and methods). Since YGA2 runs at 600kb in size, it is impossible to separate it from yeast chromosomes 5 and 6 which run at 565kb and 610kb respectively. Thus these 2 yeast chromosomes were co-purified along with YGA2 during preparation. YAC 353G6 could however be cleanly separated away from all other yeast chromosomes. Purified YACs were used for microinjection into FVB/N fertilized egg pronuclei.

As a preliminary assessment to identify positive transgenic founder mice, we screened mouse tail DNA with 5 PCR markers that span the entire length of the YACs for evidence of successful YAC integration (see figure 4.1). Primer pairs from the left (LYA) and right (RYA) YAC arms (22) and intragenic primer pairs from exon 1 (CAG), intron 1 (CA repeat), and exon 59 (ΔG) were used. Following this preliminary screen, transgenic mice were further analyzed by using additional markers within the YACs, and an estimate of copy number was done by Southern blotting using either the human HD specific cDNA cD70-2 or the 4.1 kb EcoR1 fragment encompassing the HD gene exon 1 and promoter. Tables 5.1 and 5.2 summarize the data obtained for YGA2 and 353G6 respectively and discussed in detail below.

5.4.2 YGA2 Transgenic Lines

Injections of YGA2 yielded mice with evidence of YAC integration of 15% (8/54) and 30% (7/22) for YGA2-18 and YGA2-46 respectively. Extensive analysis of most of these lines revealed that only 1/15 total positive transgenic founders appeared to have integrated the full YAC intact (line 29). This is likely due to the fact that this YAC is more prone to shearing during preparation of the
Tables 5.1 and 5.2 Summary of the screening of the YAC transgenic founders for YGA2 (table 5.1) and 353G6 (table 5.2).

Number of the transgenic line is represented in the Founder column. The DNA markers used to screen the transgenic lines (LYA, OS14, D4S95, OT17, 4.1, CAG, CA, ΔG, RYA) are represented along the top. + represents presence of the marker, – represents absence of the marker, ND = not determined. Size of the CAG repeat (e.g. 18) is indicated in the CAG column. Presence (+) or absence (-) of protein expression is indicated in the protein column. +, ++, +++ represents higher levels of protein expression in the respective line, as determined by Western blot analyses.
### Table 5.1: YGA2 YAC Transgenic Screening Summary

<table>
<thead>
<tr>
<th>Founder</th>
<th>LYA</th>
<th>OS14</th>
<th>D4S95</th>
<th>OT17</th>
<th>4.1</th>
<th>CAG</th>
<th>CA</th>
<th>ΔG</th>
<th>RYA</th>
<th>copy #</th>
<th>protein</th>
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<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>-</td>
<td>ND</td>
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<td>ND</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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Table 5.2: 353G6 YAC Transgenic Screening Summary

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DNA for injection because of its large size (600kb). Lines were established from most founders (except 1, 40, and 664 that failed to breed) with a frequency of transmission from the founders usually between 20-30%, suggestive of mosaicism of the germline (Smith et al., 1995). Breedings of subsequent progeny, however, resulted in a 50% frequency of transmission, consistent with a single integration event and Mendelian transmission of the YAC. Southern analysis comparing signal intensity to human genomic DNA demonstrated that all YGA2 transgenic lines had integrated a low number of YACs, roughly 1 to 2 copies.

5.4.3 353G6 Transgenic Lines

Injections of 353G6-18, 353G6-48, and 35G6-72 produced YAC transgenic founders with frequencies of 40% (11/28), 27% (5/18), and 18% (7/40) respectively, consistent with what was observed with YGA2. However, the frequency with which full length integrants were observed for 353G6 (16/23, 70%) was higher than that of YGA2 (7%), and is probably attributable to the smaller size of 353G6 and its easier handling during YAC preparation. However, detailed analyses of the 180kb region 3’ of the HD gene in 353G6 was not done and may reveal the presence of small deletions or rearrangements between the end of the HD gene and the most distal 3’ LYA marker. Again, as was observed with YGA2 transgenics, the frequency of transmission from founder mice was lower than expected, around 20-30%. Southern analysis on these transgenic lines revealed that 3 (B60, D212, F2498) had integrated a higher copy number relative to the other lines, which had integrated roughly 1-2 copies (Figure 5.1A, 5.2). Densitometry analysis revealed that 363G6-18 line B60 had integrated approximately 3 copies whereas D212 (353G6-18) and F2498 (353G6-72) had each integrated 4-5 copies of the YAC.

5.4.4 Chromosomal Location of the HD Transgene

As a further assessment of YAC integration, the HD transgene was mapped to metaphase chromosomes of most 353G6-18 and YGA2-18 transgenic lines by FISH using a 35 kb human
Figure 5.1 Relationship between YAC copy number and protein expression levels.

A) Southern blot analysis showing hybridization pattern of human HD specific probe cD70-2 on 5 YAC transgenic lines. Each line was compared with the signal obtained from human genomic DNA. 353G6-18 line D212 shows a stronger signal intensity, indicating integration of a higher number of YAC copies. Equal amounts (5μg) of DNA were loaded per lane.

B) Western blot of protein extracted from mouse cortex probed with the human specific antibody GHM1. Three low copy number (1-2) YAC lines (D206, B57, 29) are shown to express human huntingtin at low levels whereas high copy number line D212 expresses at much higher levels. A non-transgenic mouse (WT) and human cortex are included as negative and positive controls respectively.

C) Western blot using anti-huntingtin antibody BKPl comparing the expression levels of line D212 with endogenous mouse protein. Expression levels in this line are approximately 2 times that of endogenous levels.
Figure 5.2 353G6-72 YAC copy number.

Southern blot of 353G6-72 YAC transgenic founder mice using human HD specific probe cD70-2.

The copy number in founder F2498 is higher than that of 2 other full-length founders (2510 and 2511) and human DNA. Founders 2494, 2712, and 2787 are missing this portion of the YAC. Equal amounts (10μg) of DNA were loaded per lane.
Human (x 0.5)

Human (x 1.0)

353G6-72 FVB
Copy Number

2787
2712
2511
2510
2498
2494
Figure 5.3 Fluorescence in situ Hybridization Analysis.

Metaphase spread showing FISH signal on both chromatids of mouse chromosome 2 band C3-D for 353G6-18 line B60: a) fluorescein signals; b) actinomycin D staining of mouse chromosomes for identification. (Data and figure provided by Dr M. Dinulos).
cosmid probe from the 5' end of the gene (L83d3) (Baxendale et al., 1993). Between 15-63 cells were scored for each line. A single significant hybridization signals was noted in each line (Figure 5.3), indicating that the YACs had integrated into one site in the mouse genome, consistent with ratios observed in the breedings. Additionally, a double hybridization signal was observed on each chromatid for mouse lines D212 and B60 confirming a higher copy number, consistent with the Southern blot data. This demonstrates that integration of multiple YAC copies into the mouse genome in these lines occurs at a single site and likely results in a tandem array of YACs at that site.

5.4.5 RNA Expression Analysis

Since our primary goal was to create transgenic mice expressing the human HD gene, we checked for human mRNA expression using reverse transcription polymerase chain reaction (RT-PCR) on the YGA2-18 and 353G6-18 lines. RNA was extracted from mouse tails and following the initial RT reaction, human specific PCR primers from exon 7 and exon 9 were used to amplify human transcript derived from the YAC. All founders that had tested positive for 5' HD sequences (CAG and/or 4.1) by PCR and Southern screens were shown to express the human HD transcript (Figure 5.4 A and B). 353G6 founder D211 showed no evidence of expression but may express a shorter transcript that would not be detected by the primers used here. All transgenic founders that resulted from injections of the 353G6-48 YAC failed to express any human HD transcript (Figure 5.4C) which is likely due to the fact that the HD promoter was deleted from this YAC during the mutagenesis procedure (see chapter 4).

5.4.6 Protein Expression Analyses

5.4.6.1 YAC Derived Human Huntingtin Expression

For all protein studies performed on the YAC transgenic mice, we used two primary antibodies: GHM1 and BKP1. GHM1 is a monoclonal antibody generated from amino acid residues
Figure 5.4 RNA Expression Analysis.

RT-PCR analysis from A) 353G6-18, B) YGA2-18, and C) 353G6-48 founders using human specific primers derived from exon 7 and exon 9. The expected 281bp product is observed in all founders positive for the 5' portion of the HD gene. Non transgenic YGA2 founder number 4 and 353G6 founder D211 are not expressing human HD transcript that can be detected with the primers used here. None of the 353G6-48 founders express human transcript since this YAC is missing the HD promoter.
1678-1981 that detects human but not murine huntingtin on Western blots. BKPI is a polyclonal amino terminal antibody, generated from amino acid residues 3-16, which detects both mouse and human huntingtin on Western blots with equal proficiency (Kalchman et al., 1996). The 350kDa band detected by both antibodies was shown to be competed out upon incubation with the appropriate antigen.

To detect the presence of human huntingtin in the transgenic lines, protein from cortical tissue of transgenic mice was analyzed by Western blotting using GHM1. Human cortex and non-transgenic mouse cortical tissue were used as positive and negative controls, respectively. Human huntingtin expression was detected in all lines known to be expressing human mRNA (Figure 5.1B) with line D212 showing the highest levels of expression. Western blots from mice at a wide variety of ages, up to and including 1 year of age, have determined that protein expression is maintained postnatally.

To compare the levels of expression between the human huntingtin in line D212 and endogenous murine HD, equal amounts of protein were probed on Western blots with BKPI as the primary antibody, and densitometry scanning was performed (Figure 5.1C). Human huntingtin expression levels in line D212 were estimated to be 1-2 times that of mouse endogenous protein. The high level of transgene expression in this line can be attributed to the increased transgene copy number, which we estimate to be 4-5 copies. All other lines, having integrated 1-2 copies of the YAC, express human huntingtin at levels estimated to be around \( \frac{1}{4} \) to \( \frac{1}{2} \) that of the endogenous protein.

Similar analyses on all mutant lines having integrated the HD gene intact from YGA2-46 (668, 1746, 1747, 1749) and 353G6-72 (2511) YACs demonstrated that all expressed equivalent levels of full-length protein, again, at levels \( \frac{1}{4} \) to \( \frac{1}{2} \) that of the endogenous protein. Assessment of mutant htt expression in different fibroblast cell lines from YAC72 founders revealed htt expression in founder 2498 to be 2-3x that seen in founder 2511 (Figure 5.5), consistent with the YAC copy number estimated by Southern analysis.

To assess whether N-terminal truncated protein products were expressed from transgenic lines having integrated fragments of YACs containing 5' HD gene sequences (lines 44, D211, and
Figure 5.5 353G6-72 founder protein expression levels.

YAC transgenic mice expressing human huntingtin with 72 glutamines have varying levels of expression depending on the copy number of the gene. YAC72 founder 2498, expresses human huntingtin at higher levels than founder 2511 using lysates from fibroblast lines assessed using GHMI. Equal loading was confirmed by equivalent actin levels in each lane (42kDa).
Western blots using BKP1 were done. No smaller length protein products were detected. Since each line tested positive for the 4.1kb EcoRI fragment (which contains the HD gene regulatory sequences), it is unlikely that this lack of expression is due to loss of promoter sequences. Since RT-PCR analysis detected transcript expression from line 44, failure to detect huntingtin in these lines probably results from aberrant processing of the mRNA transcript, errant translation, and/or rapid proteolytic processing of the protein product. Alternatively, the truncated protein may acquire an unusual conformation such that the antibody can no longer detect it on Western blots. Although it remains to be seen whether 353G6-72 founder F2494 expresses a truncated version of huntingtin, the above analyses suggest that detection of such a product using this method is unlikely.

5.4.6.2 Tissue distribution of YAC and murine derived huntingtin

It has been shown that the pattern of gene expression from human YACs in a mouse background closely mimics that of the corresponding endogenous murine gene for genes such as the amyloid precursor protein (Lamb et al., 1993) and the β-globin genes (Gaensler et al., 1993). To test whether this was true for the YGA2 and 353G6 YACs, human and murine derived huntingtin expression were assessed in brain tissues which included cortex, cerebellum, brainstem, subcortical brain region (primarily comprised of the basal ganglia, thalamus, and hippocampus), and various peripheral tissues including heart, lung, liver, spleen, kidney, testes, and skeletal muscle. Equal amounts of total protein from each tissue were compared on Western blots using GHM1 for all YAC transgenic lines and BKP1 for normal mouse tissues.

As seen in figure 5.6, tissue expression patterns of human (both normal and mutant) and mouse huntingtin are identical, with the highest expression levels detected in the cortex, cerebellum and testes. A longer exposure showed that huntingtin was also expressed in all other peripheral tissues tested, albeit at much lower levels.
Figure 5.6 Tissue distribution of murine and human huntingtin.

The tissue expression pattern of endogenous mouse (wt) and human huntingtin with 18 (tg18), 46 (tg46), and 72 (tg72) polyglutamines. Normal and YAC transgenic protein (100 μg of total protein from each tissue) were probed with BKP1 and GHM1, respectively, showing that huntingtin expression levels are highest in cortex, cerebellum, and testes. A longer exposure of the blots did show that murine and human huntingtin is expressed at low levels in the other tested tissues.
Figure 5.7 Subcellular fractionation of human and murine huntingtin.

An identical subcellular fractionation pattern is observed between endogenous mouse full-length huntingtin (wt) and YAC derived human huntingtin with 18 (tg18), 46 (tg46), and 72 (tg72) polyglutamines. The tissue used was cortex. Non-transgenic mouse blots were probed with BKP1, whereas YAC transgenic blots were probed with GHM1. The majority of immunoreactivity is observed in the soluble cytosolic and membrane associated fractions.
5.4.6.3 Subcellular Fractionation

To assess the subcellular localization of huntingtin in the YAC transgenic mice, cortical tissue from a wild type mouse and each of the different YAC transgenics (353G6-18, YGA2-46, 353G6-72) was fractionated into nuclear, mitochondrial, membrane enriched, and soluble fractions and subjected to Western blotting (Figure 5.7). The results indicate that mouse and YAC-derived human huntingtin are predominantly localized to the soluble cytosolic and membrane-associated fractions, with lower detected levels of immunoreactivity in the nuclear and mitochondrial fractions. Similar results were obtained using cerebellum and testes.

5.4.6.4 Immunohistochemistry

The localization of huntingtin was compared in multiple brain regions from both normal and YAC transgenic mice (353G6-18 and YGA2-18). Frozen sections including frontal cortex, caudate/putamen, globus pallidus, parietal cortex, hippocampus, thalamus, cerebellum, brainstem, and spinal cord were examined using BKP1. In each of these regions immunostaining with BKP1 revealed cytosolic immunoreactivity strongest in neuronal cells, with a lower intensity of staining seen in the cytosol of glial cells (Figure 5.8). No immunoreactivity was seen in the nuclei. The specificity of the demonstrated immunostaining was confirmed by the ineffectiveness of this antibody on sections from both normal and YAC transgenic mice after preincubation with excess BKP1 antigen (Figure 5.8D).

The distribution of immunostaining was identical between normal and YAC transgenic mice. However, more intense staining was observed in the YAC transgenics, indicative of expression of human huntingtin. This data shows that the human and mouse proteins are localised similarly in the cytosol predominantly in neurons in all brain regions observed.
Figure 5.8 Immunohistochemical analysis of human and murine huntingtin.

Immunohistochemical analysis using BKP1 (1/50 dilution) on sections derived from normal and YAC transgenic mice (353G6-18 shown here) from A) cortex, B) striatum, C) brainstem. The YAC transgenic mice show a greater intensity of staining in each region (A-C), attributable to the expression of the human protein. Both endogenous and human protein are localised to the cytoplasm with no nuclear staining observed. Immunoreactivity observed in a normal mouse cerebellar section (D; left) can be competed out with excess peptide (D; right) showing that the observed immunostaining is specific to huntingtin. A) and D) 400X magnification; B) and C) 1000X magnification. (Data and figure provided by Dr. K. Nishiyama).
5.4.7 Rescue of the HD Knock-out Lethal Phenotype

Since the data showed that the HD gene within all YACs was regulated in a manner that was the same as that of the mouse endogenous gene, I tested whether YAC derived human huntingtin was functional and expressed appropriately during development by crossing the YAC onto the mouse HD knock-out background. Mice homozygous for a targeted disruption of the HD gene usually die at embryonic day 8.5 (Nasir et al., 1995; Duyao et al., 1995; Zeitlin et al., 1995). Therefore if human huntingtin is expressed appropriately during development and functional in a murine background, we would expect rescue of the embryonic lethality. YAC transgenic mice from YGA2-18 (lines 29 and 30), 353G6-18 (lines B50 and B60), and YGA2-46 (line 668) were bred to mice heterozygous for the targeted disruption in the mouse HD gene (on the C57Bl/6 mouse background). Live-born mice were genotyped for the presence of the YAC by PCR and at the murine HD locus by Southern blotting using a mouse specific probe from intron 5 that detects the normal and disrupted alleles (Figure 5.9). All five lines demonstrated efficient rescue of the embryonic lethal phenotype. Genotyping the offspring for homozygous normal: heterozygous: homozygous null alleles produce the expected 1:2:1 Mendelian ratio (Table 5.3). A total of 52 non-transgenic offspring were genotyped at the mouse HD locus, none of which were homozygous for the targeted disruption. This rescue demonstrates that human huntingtin was appropriately expressed in the correct cells prior to day 7.5 of gestation from all YACs and that the human protein functions similarly to the mouse protein during development. Additionally, since YGA2-46 rescued the embryonic lethality, expansion of the trinucleotide repeat does not disrupt normal function of the HD protein, adding further support to the gain (rather than loss) of function hypothesis for HD.

5.5 Discussion

A series of YAC transgenic lines have been created using 2 YACs spanning the HD gene. YGA2 (600kb) is one of the largest YACs used for transgenic studies that has been retained intact. However
Figure 5.9 Rescue of embryonic lethality by YAC derived human huntingtin.

Southern blot analysis showing the genotype of 7 offspring generated from the knock-out rescue breedings using YGA2 YAC transgenic line 29. The mouse intron 5 probe used (see materials and methods) detects two bands; a 7.5 kb band which indicates a normal mouse HD gene (from the FVB/N mouse line) and a 6.4 kb band which represents a knocked-out mouse HD gene containing the neo gene in exon 5. +/+, +/-, -/- represent homozygous normal, heterozygous, and homozygous null for the mouse endogenous gene respectively. Two of the 7 mice shown here are homozygous null at the murine HD locus but are viable due to human huntingtin expression derived from the YAC in line 29 (YGA2).
Table 5.3 Summary of Embryonic Lethality Rescue

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integration of a full-length YAC was obtained in only 1/15 YGA2 lines showing that the size of this YAC is approaching the upper limit from which one can obtain full-length integrants. This is likely due to technical limitations while preparing the YAC for microinjection since DNA of this size is highly prone to shearing. Greater success of full-length integrants was obtained from the smaller (350kb) YAC 353G6 (16/23). Furthermore, transgenics lines having integrated a higher number of YAC copies were only seen for YAC 353G6 (lines B60, D212, F2498). Again, this is likely due to the fact that 353G6 is approximately half the size of YGA2. Since there is a limit to how much DNA can be injected into a mouse pronucleus, more copies of 353G6 could be injected at any one time.

Transgenic lines B60 and D212 (353G6-18), which demonstrated a higher hybridization signal by FISH and a greater signal intensity than human genomic DNA by Southern blotting indicative of an increased copy number, had the highest levels of huntingtin protein expression. In most other transgenic lines with lower copy numbers (1-2), human protein levels were estimated to be around 1/3 to 1/2 that of mouse endogenous protein. Indeed, studies whereby mouse huntingtin expression was reduced to below 50% endogenous levels (~1/3) demonstrated a perinatal lethal phenotype caused by abnormal brain development (White et al., 1997). The fore- and midbrain regions were grossly misshapen, and architectural anomalies were observed in multiple structures including the thalamus, cortex, and striatum. Since this phenotype was never observed in the YAC rescue experiments and all breedings (including the low expressing lines) produced the expected 1:2:1 Mendelian ratio, it is likely that the minimum expression levels seen in the YAC transgenics is greater than 1/3 endogenous levels. Thus as copy number increases in the YAC transgenics to 4-5 copies, one would expect to obtain mutant protein expression levels between 1-2X that of endogenous. These data are consistent with the notion that YAC transgene expression levels are largely determined by copy number (Schedl et al., 1993; Lamb et al., 1993).

Human huntingtin expressed from the YAC transgenes demonstrated identical tissue distribution as well as cellular and subcellular localization as endogenous mouse huntingtin. Expression patterns of human and mouse huntingtin revealed highest levels of expression in the brain and testes with lower levels in peripheral tissues.
Immunohistochemical analysis revealed human and murine huntingtin both localized to the cytosol. More intense staining was observed in the YAC transgenic mice, indicative of expression of both murine and human huntingtin. In the brain, huntingtin was predominantly expressed in neurons. Furthermore, analysis of the subcellular localization of human and murine huntingtin revealed that both mouse and YAC derived huntingtin are primarily localized to the soluble cytosolic fraction. These data closely parallel what has been observed in human brain tissue (DiFiglia et al., 1995; Sharp et al., 1995).

The embryonic lethality rescue experiments clearly demonstrate that human huntingtin derived from both YAC transgenes (353G6 and YGA2) is appropriately expressed prior to day 7.5 of gestation and can compensate for loss of the mouse protein in development. Additionally, expansion of the CAG repeat (46 CAG) in YAC YGA2 did not prevent rescue of the phenotype and demonstrates that polyglutamine expansion does not disrupt the normal function of the HD protein. This adds support to the hypothesis that HD is caused by a toxic gain of function rather than loss of protein function. Finally, the data suggest that the human huntingtin undergoes appropriate post-translational modification and can interact with other murine proteins which might be crucial for normal functions. Thus despite a 10% difference in amino acid residues (Lin et al., 1994), this does not interfere with the ability of human huntingtin to substitute for the murine protein early in development.

The results presented here clearly show the YAC derived transgene expression of the human HD protein is appropriately expressed during prenatal and postnatal development and the human protein is localized to similar subcellular compartments as murine huntingtin. This tightly regulated pattern of expression is of considerable importance for studies on HD since transgene based expression of expanded polyglutamines can result in selective neuron loss dependent upon the promoter used. For example, an ataxic phenotype was developed in mice expressing the mutant SCA-1 protein from a cDNA under control of a Purkinje cell specific promoter PCP-1 (Burright et al., 1995). Neuropathological studies revealed severe loss of Purkinje cells in the cerebellum. A similar phenotype is seen when the expanded polyglutamine tract of the gene causing SCA-3 (Machado-
Joseph disease) is expressed under control of the same PCP-1 promoter (Ikeda et al., 1996). In this instance, only a few residues besides the polyglutamine tract were included in the construct, suggesting that it is the polyglutamine itself, and not the surrounding gene sequences that play a significant role in mediating the neurotoxicity. Of interest is the observation that Purkinje cells are only mildly affected in SCA-3 patients (Ikeda et al., 1996). Furthermore, a late onset neurological disorder was observed in mice expressing full length hypoxanthine phosphoribosyltransferase (HPRT) containing 146 polyglutamines, demonstrating that expression of polyglutamine tracts does not need to be in the context of 1 of the classic repeat disorder genes to have a neurotoxic effect. Taken together, these results suggest that polyglutamines can exert neurotoxic effects irrespective of protein context and expression pattern, underscoring the importance of the YAC transgene-based expression of huntingtin in developing a truly representative animal model for HD.

5.6 Conclusion

YAC transgenic mice express normal and mutant human huntingtin in a temporal and spatial manner that closely parallels that of the corresponding mouse HD gene.
5.7 Reference List


CHAPTER 6 - BEHAVIORAL ANALYSES
6.1 Hypothesis

Expression of mutant human huntingtin in transgenic mice produces a phenotype that can be observed by behavioral changes in those mice.

6.2 Objective

To characterize the behavioral phenotype of wild type and YAC transgenic mice expressing normal and mutant huntingtin.

6.3 Introduction

The first stage of analysis for detecting phenotypic differences between mutant expressing transgenic lines and control mice was behavioral analysis. A comprehensive study was set up that would detect any obvious changes in mouse behavior or locomotor performance. Additionally, this investigation would provide a starting point for further study should any consistent or progressive changes be noted amongst the groups. Two control groups (wild type and tg18) and 2 mutant groups (different tg46 lines) were included for 2 reasons. Firstly, if any changes were detected between mutants and both controls, this would establish that the differences are attributable to mutant but not normal htt expression. Secondly, any differences noted in 1 mutant transgenic line should also be observed in the second mutant line to eliminate the possibility that these differences are not a line related effect. This could occur if the YAC transgene had integrated into an important locus in the mouse genome causing a dominant phenotype unrelated to transgene expression.

The tests were arranged into 3 main groups: 1) general assessment designed to check overall mouse behavior, 2) tests for motor control and movement, and 3) tests of cognition. Each test was repeated at an interval of two months (unless otherwise noted) in order to determine whether there might be a progression of deficits in any of these areas between 8 and 20 months of age. There were four populations of FVB/N mice in the study: Wild type (wt, n=7), YGA2 YAC transgenic line (30) with 18 CAG repeats (tg18, n=7), YGA2 YAC transgenic lines with 46 repeats, line 668 (tg46 668,
n=7) and line 1747 (tg46 1747, n=3). Analyses were also done on high copy number 353G6-72 founder F2498 and age matched wild type controls. All mice used in this study were pure FVB/N, eliminating confounding effects of differences in mouse background.

6.4 Results

6.4.1 General Assessment

6.4.1.2 YGA2-46

General observation of mouse behavior was performed on a weekly basis as mice were transferred to clean cages. No obvious differences in behavior were noted between all 4 groups over the course of the study. Weights of each mouse were recorded over this time period showing no significant differences in actual weight or weight gain in each group (figure 6.1A). All groups showed healthy growth curves from 8 to 20 months of age. Although the tgl8 group appeared lighter than the other groups, these results were not significant. Additionally, both tg46 groups showed no differences in growth compared to controls, showing that mutant huntingtin expression did not disrupt overall eating and drinking behavior.

In order to assess the behavior of the FVB/N mice in their home cages and to establish a baseline for future studies, mice were videotaped at 8 and 20 months of age for a period of two hours in their home cages. For a 10sec interval out of every minute of the session, all of the behaviors of the subject were recorded. This provided a means of quantifying the occurrence of normal and abnormal behaviors. The behaviors recorded include inactivity, grooming, scratching, sniffing, gnawing, burying, rearing, locomotion, climbing, circling, jumping and mounting. The frequency of each behavior was tallied over the length of the session. All mice were active over the course of the 2hr session, showing high frequencies of gnawing on objects within the cage, burying in the bedding, rearing on the cage walls, and general movement around the cage. Sniffing and grooming were also commonly observed.
Figure 6.1 Growth curves of YAC transgenic mice

A) Growth curves of wild type and YAC transgenic (tg18, tg46) FVB/N mice from 8 to 20 months of age. All groups show similar growth rates over this same time period, with continued weight gain until month 17, at which point they begin to lose weight gradually. Although tg18 mice (line 30) generally appear to be lighter than the other groups, this difference is not significant. Points represent means +/- SD.

B) Growth curve of 353G6-72 high copy number founder F2498, low copy number founder 2511, and a non transgenic littermate (wt). Note that despite the smaller weight of F2498 relative to her siblings, her weight was maintained up until the time of sacrifice (52 weeks). *: pregnant or lactating.

(Data provided in collaboration with F. LePiane)
Other behaviors such as sudden jerky movements and jumping were seen in all groups, albeit infrequently (figure 6.2). No obvious differences were noted amongst all groups.

Mice were also tested for their responsiveness to sensory stimuli including touch, auditory, and olfactory cues. Since FVB/N mice are blind, all mice failed to respond to movement of objects waved in front of them until the object touched its whiskers. Additionally, each mouse was touched with a cotton applicator on the sides, back, rear and tail, all showing a reaction to each touch indicating no sensory deficit. Auditory responsiveness to a sharp clicking sound (repeated ten times) was no different between groups with all mice showing a startle (freezing and twitching of the ear with each click) that did not habituate with repetitions. Finally, two different odor cues (peppermint and acetic acid) were presented to see if the mice could detect and approach the smell. All mice detected the smell and showed interest as they approached and sniffed for a period of 1-3sec.

6.4.1.3 353G6-72

Assessment of founder mice generated from 353G6-72 injections showed that the high copy number female founder F2498 was dramatically reduced in body weight and size. Weight was first recorded at 9 weeks of age, showing that this mouse weighed approximately half that of her female siblings, which included low copy number founder F2511 (Figure 6.1B). This weight was maintained throughout her lifetime (up to 1yr) but never exceeded 20g, showing that despite her hyperactive phenotype (see below) she ate and drank enough during this period to sustain her weight.

Observations of spontaneous behavior were carried out on this YAC72 founder with high copy number (figure 6.3B) and an age and sex matched control (figure 6.3A) at 3, 5, and 9 months of age. While control mice showed a behavioral profile consistent with that observed in the studies on the YGA2-46 mice, F2498 showed dramatic circling throughout each of the sessions. The circling behaviour was persistent over time and by 9 months was associated with obvious choreoathetoid movements of the head and neck. The circling was tight and rapid with a full turn being
Figure 6.2 Behavioral profile of YGA2-46 YAC transgenic mice.

Behavioral profile of wild type FVB/N and YAC transgenic mice at 8 months of age. All groups show a similar profile over the 2hr-observation period. IN: inactivity; SN: sniffing; GR: grooming; SC: scratching; GN: gnawing; BU: burying; RE: rearing; LO: locomotion; CL: climbing; FL: head flicks; JE: sudden jerky movements; DA: darting; JU: jumping.

(Data provided in collaboration with F. LePiane).
Figure 6.3 Behavioral profile of 353G6-72 YAC transgenic mouse

Behavioral profile of a wild type FVB/N mouse (A) and 353G6-72 founder F2498 (B) at 3, 6, and 9 months of age. The profile of the wild type mouse is consistent with that observed in all FVB/N mice. FVB/N mice are quite active showing high frequencies of gnawing, rearing, burying, and general movement around the cage. Circling behavior is not a common feature of this strain. However, F2498 spends the vast majority of her time circling in the cage at the expense of other normal behaviors at each time point. IN: inactivity; SN: sniffing; GR: grooming; SC: scratching; GN: gnawing; BU: burying; RE: rearing; LO: locomotion; CI: circling; CL: climbing; MO: mounting; JU: jumping.

(Data provided in collaboration with F. LePiane)
completed in approximately 1/3 of a second (figure 6.4). When held by the tail, F2498 seemed disoriented and showed a foot clasping posture reminiscent of the HD exon 1 transgenic mice (Mangiarini et al., 1996). Additionally, as she was lowered to the bench-top, unlike normal FVB/N mice that place both forepaws on the bench immediately upon sensing it, she failed to orient herself properly and usually ended up on her side before correctly orienting herself. In contrast to this founder, the F2511 founder and its offspring have not demonstrated any obvious behavioural changes up to 1 year of age. In general, founder F2498 was highly responsive to external cues, showing responses seen at the high end of the spectrum in normal FVB/N mice. She was extremely reactive to touch, demonstrating a hyperactive flicking reaction followed by an immediate attempt to evade the touch. Her response to the sharp clicking sound was to stop circling and try to orient herself to the sound. Once clicking stopped, circling immediately resumed. Finally, she demonstrated no olfactory deficits, as she approached and sniffed the peppermint and acetic acid odors.

6.4.2 Activity and motor capabilities

6.4.2.1 YGA2-46

Since mice exhibiting neuron loss in nuclei of the basal ganglia also demonstrated a hyperactive phenotype (Nasir et al., 1995), we tested whether the YGA2-46 YAC transgenics exhibited a similar phenotype. Activity was measured by recording movement in a photocell box during two hours of light and two hours of darkness. No significant differences in total activity were noted amongst the groups at each timepoint (figure 6.5A). All began the session actively exploring the box, which was followed by a gradual reduction in activity over the 4hr session (figure 6.5B). Normally in mice and rats, the period of switching from light to dark is associated with an increase in activity. However no such increase occurred in these mice, likely attributable to their blindness (figure 6.5B). Motor control and coordination were tested in 4 ways: (1) climbing a wire screen, which tested strength and ability to perform a relatively
Figure 6.4 Circling behavior in 353G6-72 founder 2498.

Composite image derived from video footage of F2498’s circling behavior. Four frames were selected from the video and combined to produce a full circle. The time taken to complete the circle is ~1/3 of a second (11 frames from start to finish of the circle at 30 frames/second). A non-transgenic age matched control is shown in the top right hand corner.
Figure 6.5 Activity assessment of YGA2-46 YAC transgenic mice

Activity assessment of YAC transgenic mice. A) Total number of photocell breaks over the 4 hour testing period (2 hours light, 2 hours dark). Although it appears there are differences at certain time points (e.g. tg46 1747 appears more active than most other groups at 8 months), no significant differences were noted. B) Activity trace for all 4 groups over the 4hr period at 8 months of age. Traces are similar, with activity beginning high and tapering off over time. The dashed line indicates the switch from the light to dark phase. (Data provided in collaboration with F. LePiane).
complex motor task (2) beam-crossing to test balance and coordination (3) footprint test to evaluate gait, and (4) movement from an awkward posture.

All mice were able to climb the wire screen and cross the beam successfully without any noticeable differences in ability to maneuver on the mesh or maintain balance on the beam. The footprint test showed a consistently normal gait (no tiptoe walk or circling) throughout and all mice were proficient in reorienting themselves once placed in an awkward posture. Thus all mice maintained their ability to perform simple and complex motor tasks during the 12 months of observations up to and including 20 months of age.

6.4.2.2 353G6-72

F2498's performance on the wire mesh was not obviously different to controls. Her grip strength seemed normal and she was able to maneuver on the mesh without much difficulty. However, she failed to complete the beam-crossing task successfully. Her first 6 attempts at moving down the beam resulted in falls. When she was placed on the beam for the seventh time, she froze and stayed in 1 position for 7 minutes until she was removed. These data suggest that her grip strength was comparable to controls but her coordination was severely diminished. Analysis of her gait showed a normal footprint, despite circling throughout the experiment.

6.4.3 Cognition

As a quick check for deficits in cognition, the mice were assessed for performance in a Y maze. The advantage of this assay is that it is a relatively quick and simple test (relative to other more complex assays such as the Morris Water Maze and Fear conditioning) and provides insight into the exploratory behavior, curiosity and working memory in rodents (Douglas, 1990). The main test in the Y-maze is to assess spontaneous alternation behavior (SAB). Spontaneous alternation is a general phenomenon in rodents, typically interpreted as a manifestation of curiosity (Gerlai et al., 1994).
However, other factors such as fear will decrease SAB, which can be intuitively interpreted as a terrified animal preferentially wanting to visit the most familiar location rather than the most novel (Douglas, 1990). Additionally, dysfunction of the hippocampus results in decreased SAB and is important in that the hippocampus plays a critical role in regulation of exploratory activity and in incorporating spatial information (Douglas, 1990). Additionally deficits in SAB have been observed in FVB/N transgenic mice overexpressing human or mouse Alzheimer amyloid precursor protein (Hsiao et al., 1995).

The wt, tg18, and tg46 cohorts of transgenic mice were tested in the maze at 8, 12, 16, and 20 months of age. The arms of the maze were arbitrarily designated as A, B, and C. Following a 5 minute habituation period, mice were scored for each arm that was entered and the sequence in which the entries took place. Alternation was scored if all three arms (A, B, C) were entered in any 1 triad. No significant deficits in percent alternation (total number of alternations/total number of triads) were noted between all groups of YAC transgenic mice at any time point (figure 6.6A). Additionally, no significant differences in the number of arms visited (# of triads) were noted amongst the groups at all time points (figure 6.6B), demonstrating that all mice were equally motivated to explore the environment and no differences between locomotor activity exist.

6.5 Discussion

6.5.1 Behavior in HD YAC transgenic mice

No differences were noted amongst all 4 groups of wild type and YGA2 (18 and 46) YAC transgenic lines in each test up to 20 months of age. Each group showed comparable performance in locomotor and coordination tests and spontaneous behavior in home cages. This strongly suggests that the levels of protein expression in the 2 mutant lines (1/3 - 1/2 endogenous) in conjunction with 46 CAG repeats are not sufficient to induce a
Figure 6.6 Performance of YGA2-46 YAC transgenic mice in a Y maze.

Performance of YAC transgenic in a Y maze at 8, 12, 16, and 20 months. Percent alternation (A) and # of triads (B) is similar amongst all groups at each time point, with no significant differences noted. (Data provided in collaboration with F. LePiane).
neurological/behavioral phenotype during the life span of these mice. However, the possibility remains that observable neurodegeneration may be present in these mice, which is not great enough to manifest as an observable neurological phenotype in the mice. This question is addressed in chapter 8.

Attempts pick up a more subtle neurological phenotype were done by monitoring spontaneous alternation behavior (SAB) in the Y maze. Impaired spatial alternation is associated with hippocampal dysfunction and thought to reflect a diminished motivation to explore a novel environment, inattention to regions previously explored, or failure to recall recently acquired information (Gerlai et al., 1994). Again, no significant deficits in SAB were noted between the various groups of YAC transgenic mice at 8, 12, 16, and 20 months of age. The observation that these mice show abnormal neurophysiological properties in the CA1 region of the hippocampus beginning as early as 6 months of age (see chapter 8), yet perform equally well in the hippocampal dependent SAB task, is interesting. This is likely a reflection of the complexity of the system involved in the hippocampus. Bilateral lesions of the hippocampus in rats completely abolish SAB, demonstrating the importance of this brain structure in SAB (Douglas, 1990). However, massive unilateral lesions in the hippocampus do not affect SAB. Additionally, small or partial lesions do not completely abolish SAB, and full recovery occurs if the rat is given sufficient time (~2 weeks) to recover after surgery (Douglas, 1990). These data suggest that subtle (or even large unilateral) changes in hippocampal physiology may not result in altered SAB. Thus, despite hippocampal malfunction in the YAC transgenic mice (chapter 8) this change is only 1 part of a very complex system and may not disrupt the system as a whole enough to manifest as a deficit in SAB in the Y maze.

6.5.2 Circling behavior and the basal ganglia

The observation of dramatic circling behavior in transgenic founder 2498 is of considerable interest. Circling behavior in rodents is associated with perturbations in basal
ganglia function (see below), and nuclei in the basal ganglia (caudate and putamen) are the regions most effected in HD. To help facilitate this discussion, I will briefly describe the anatomy of the basal ganglia and how malfunction of this complex network may result in some clinical aspects of HD.

6.5.2.1 The basal ganglia and HD

The general anatomy and connections of the basal ganglia is outlined in figure 6.7. The 2 main pathways within the basal ganglia, the direct (DP) and indirect (IP) pathways, have opposite effects on output to the presupplementary motor area. Excitation of the indirect path (mediated by the neurotransmitters GABA/enk) has the net result of decreasing neuronal excitation to the presupplementary motor area, thus inhibiting movement. Excitation of the direct pathway (mediated by the neurotransmitters GABA/SP) results in enhanced excitation to the presupplementary motor area, thus helping facilitate movement. Striatal GABA/enk projections (IP) degenerate earlier than the GABA/SP projections (DP) in HD patients (Hayden and Kremer, 1995). Degeneration of the GABA/enk projections would result in over-excitation of the presupplementary motor area and may account for choreoform movements observed in the early stages of HD. Later degeneration of the GABA/SP projections would have the opposite effect causing over-inhibition of the presupplementary motor area. This may account for the rigidity seen in the latter stages of HD.

To further add to the complexity of basal ganglia circuitry, the dopaminergic (DA) nigrostriatal pathway acts to inhibit the indirect pathway and excite the direct pathway, thus facilitating movement. In a feedback loop, the direct pathway (GABA/SP) acts to inhibit dopaminergic input to the striatum, resulting in inhibition of movement. Loss of these GABA/SP cells
The 5 nuclei of the basal ganglia and their anatomical connections (Cote and Crutcher, 1991). These nuclei found in this subcortical brain region are the caudate and putamen (which together comprise the striatum), the globus pallidus (made up of an internal (GPi) and an external (GPe) component), the substantia nigra (made up of the pars compacta (SNc) and the pars reticulata (SNr)), and the subthalamic nucleus (STN). The thalamus is also anatomically connected to, but not considered a part of, the basal ganglia. There are 2 major pathways in the basal ganglia: the indirect and the direct pathways, which act to inhibit or excite output to the presupplementary motor area, respectively. Not shown in this diagram are the interneurons found in the striatum that contain the neurotransmitters acetylcholine, somatostatin, and neuropeptide Y. Black arrows denote an inhibitory synaptic connection; white arrows denote an excitatory synaptic connection.
in HD would lead to hyperactivity of the nigrostriatal DA pathway and enhanced movements in HD patients. Indeed, drugs that increase DA transmission exacerbate HD choreiform movements, whereas decreases in DA transmission reduce the magnitude of involuntary movements (Hayden and Kremer, 1995). Thus, the circuitry of the basal ganglia is complex, and detailed knowledge of the timing and neuron cell types involved in neurodegeneration would greatly facilitate our understanding of disease progression.

6.5.2.2 Circling behavior in rodents

Circling behavior in rodents has long been associated with perturbations in the nigrostriatal dopaminergic (DA) pathway in the basal ganglia. To date, the genes for 5 dopamine receptors (DAR) have been cloned and characterized. All are G-protein coupled receptors, but they are divided into 2 main subfamilies based on pharmacological properties (Lachowicz and Sibley, 1997). The D1 subfamily (D1 and D5) acts to elevate intracellular levels of cAMP by activating adenyl cyclase, whereas the D2 subfamily (D2, D3, and D4) has the opposite effect, acting to reduce cAMP levels. Of particular interest are the D1 and D2 receptor subtypes, since these are highly expressed in the striatum, whereas the others are not. Unilateral lesions of this pathway with the neurotoxin 6-hydroxydopamine (6-OHDA selectively destroys dopaminergic neurons in the substantia nigra) results in a functional imbalance between the 2 sides of the brain, inducing circling behavior in which the animal turns away from the side with the higher dopaminergic activity (Loscher et al., 1996).

Recently, 2 genetic models of rodent circling behavior have been observed, caused by asymmetric imbalances in the nigrostriatal DA pathway. The first is a transgenic insertional mutant showing autosomal recessive inheritance and no transgene expression, suggesting disruption of an endogenous mouse locus (Fitzgerald et al., 1992). Despite normal DA and D1 receptor levels in the striatum of these mice, asymmetric bilateral elevations of D2 receptors were observed in the lateral portions of the striatum. The direction of rotation was contralateral to the side showing higher striatal D2 levels.
The second model is a spontaneously occurring rat mutant named circling (ci) that shows an autosomal recessive pattern of inheritance (Loscher et al., 1996). Individual ci rats demonstrated a rotational preference in circling behavior, but could be grouped into 2 classes: right-sided and left-sided rotational preferences. Neurochemical studies revealed that dopamine (DA) levels in the striatum were significantly lower in the brain side ipsilateral to the preferred turning direction. No differences were seen in the nucleus accumbens or the frontal cortex. These 2 rodent models of circling behavior demonstrate that neurochemical imbalances in the brain can be produced via endogenous processes, particularly by genetic means, without the need for an external and unilateral introduction of neurotoxins.

Recent evidence has implicated the nigrostriatal DA system in HD. Reduced D1 and D2 mRNA expression levels have been observed in the striatum of HD patients, even those showing no overt striatal pathology (Augood et al., 1997). PET analysis has also demonstrated reduced D1 and D2 receptor binding in both choreic and rigid patients, although the reduction was more pronounced in patients with rigidity (Turjanski et al., 1995). Finally, transgenic mice expressing exon 1 of the HD gene with ~130 CAG repeats show significant reductions in D1 and D2 receptor binding even prior to onset of symptoms (Cha et al., 1998). Taken together, these results strongly suggest that DA/DAR imbalances may play a role in manifestation of some HD symptoms. It is tempting to speculate that imbalances in this pathway account for the observed circling behavior in the YAC transgenic founder mouse F2498, especially in light of the observed neuropathology in the lateral striatum (see chapter 7).

It should be noted however, that circling behavior has been described in a number of other mouse models, due to defects in morphology of the inner ear (Lyon et al., 1996). Although most are inherited in a recessive fashion, 10 mutants have been described in which the pattern of inheritance is dominant or semidominant. This possibility cannot be excluded in founder 2498 at this point. Production of further high copy number 353G6-72 transgenic lines will certainly help to address these issues.
6.6 Conclusion

Expression of low (less than endogenous) levels of mutant huntingtin with 46 polyglutamines is not sufficient to induce an obvious behavioral phenotype in FVB/N mice. However, expression of higher levels of protein with larger polyglutamine repeats (72) produces an obvious neurological impairment with similarities to human HD in FVB/N mice.
6.7 Reference List


CHAPTER 7 - PATHOLOGY
7.1 Hypothesis

Expression of mutant human huntingtin will result in neuropathological changes and neurodegeneration as seen in human HD patients.

7.2 Objective

To perform detailed pathological and immunohistochemical analyses on HD YAC transgenic mouse brains.

7.3 Introduction

The hallmark neuropathological feature of HD is atrophy of the caudate and putamen, (collectively known as the striatum), and is characterized at the cellular level by neuron loss and gliosis (Hayden and Kremer, 1995). The striatum is part of a larger subcortical brain structure known as the basal ganglia, which is an interconnected collection of 5 nuclei involved in processing of motor information (McGeer et al., 1987). The striatum is the principal-input nucleus in the basal ganglia, receiving excitatory glutamatergic input from all regions of the cortex. The main neuronal cell types found in the striatum can be divided into 2 classes; spiny and aspiny neurons. Medium sized spiny neurons (MSN) are the most abundant making up approximately 80% of the neurons in the striatum, and are further divided into TypeI (containing the neurotransmitters GABA/enk) and TypeII (containing the neurotransmitters GABA/SP) cells. The spiny neurons project to neurons outside of the striatum. Aspiny cells function as interneurons and use somatostatin and neuropeptide Y (medium sized neurons) or acetylcholine (large aspiny neurons) as neurotransmitters. Within the striatum, the GABA/enk and GABA/SP-containing medium spiny neurons are particularly susceptible to neurodegeneration in HD, whereas aspiny neurons are relatively spared. The cortex also shows generalized atrophy in many HD patients, whereas other brain regions such as the cerebellum and brainstem appear relatively unaffected.
To assess the neuropathological consequences of mutant human huntingtin expression in YAC transgenic mice, we performed detailed histological and immunohistochemical analyses on mice brains. The results are presented below.

7.4 Results

Immunohistochemical and morphological analyses were done on wild type (wt), and YAC transgenic mice expressing normal (YAC18), or mutant (YAC46, YAC72) huntingtin. These studies were done on a number of different transgenic lines at time points ranging from 5 to 20 months, summarized in table 7.1.

7.4.1 General brain appearance

The overall morphology of the brains from wt, YAC18, YAC46, YAC72 mice at 5, 9, 12 and 20 months of age was similar. The cortex and cerebellum were well developed in each mouse and no significant differences in brain weight were noted (Table 7.1, Figure 7.1). Although the brain weight from high copy number YAC72 founder F2498 was lower compared to other age and sex-matched mice, this was likely a reflection of an overall reduction in body size, since her body weight/brain weight ratio was comparable to controls.

7.4.2 Nuclear Translocation of N-terminal Huntingtin

For preliminary immunohistochemical analysis of the YAC transgenic mouse brains, anti HD antibody EM48 was used. EM48 is a rabbit polyclonal antibody generated against the N-terminal portion of htt (amino acids 1-256 excluding polyglutamine and polyproline repeat stretches) which primarily immunoreacts with htt aggregates in human tissue (Li and Li, 1998).
Table 7.1 Summary of pathological assessment of HD YAC mouse brains.

<table>
<thead>
<tr>
<th>Line</th>
<th>YAC</th>
<th>Age (month)</th>
<th>Sex</th>
<th>CAG Size</th>
<th>Brain weight (g)</th>
<th>Nils</th>
<th>Striatal pathology</th>
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Nils: Neuronal Intranuclear Inclusions
Figure 7.1 YAC transgenic mice brain weight.

Brain weights of non transgenic (wt), and YAC transgenic mice expressing normal (YAC18) and mutant (YAC46) human huntingtin at 12 and 20 months of age. No differences in brain weight were noted. Shown for comparison at 12 months are 353G6-72 founders F2498 (circling phenotype) and F2511 (no obvious behavioral phenotype). The reduced brain weight of F2498 is likely a result of her overall reduced body size since her body weight/brain weight ratio was similar to all other mice.
A) 12 months

![Graph showing brain weight (g) for different YAC mouse groups at 12 months.](image)

B) 20 months

![Graph showing brain weight (g) for different YAC mouse groups at 20 months.](image)
In the wild-type controls at all time points examined and in the YAC46 and in YAC72 mice (line 2511) at 6, 9 and 12 months of age, a faint EM48 staining was present in neurons throughout the brain. In labeled neurons, light immunoreactivity was present in the cytoplasm and sometimes also found in the nuclei. In contrast, in the 12 month old YAC72 founder (2498), EM48 intensely stained many nuclei within neurons in the striatum (Figure 7.2A), olfactory tubercle, nucleus accumbens (Figure 7.2B) and fundus striati. Small numbers of neurons in the septum and the granule cell layer of the cerebellar cortex (Figure 7.2E) also had nuclear EM48 immunoreactivity. This intense nuclear staining was not seen in any other brain regions including cerebral cortex (7.2C), CA1 region of the hippocampus (7.2D), and thalamus (7.2F), nor was it present in the other transgenic mice, wild-type controls of the same age (figure 7.2G) or when the primary antibody was omitted. EM48 nuclear staining was heterogeneous in intensity between neurons as well as more intense in the lateral portion of the striatum compared to the more medial portion. In the YAC46 at 20 months of age, which had no evidence of a clinical phenotype, EM48 stained the nuclei of a few neurons in the lateral striatum.

It has been proposed that mutant htt is cleaved in the cytoplasm and that only the N-terminal portion of the protein is translocated into the nuclei where it may form aggregates (Goldberg et al., 1996; Li and Li, 1998; Cooper et al., 1998). To investigate whether full-length or N-terminal fragments of htt were present in the nuclei of YAC72 mouse striatal neurons, immunocytochemistry was performed using mHD549, a rat monoclonal antibody that reacts with an internal region of huntingtin (amino acids 549-679). Adjacent sections containing the striatum from YAC72 mice from both 2498 and 2511 lines at 12 months of age and wild-type controls were used. Sections stained with mHD549 alone exhibited intense staining of neuronal perikarya and dendrites as described previously (Gutekunst et al., 1995). HD549 immunoreactivity was not detected in neuronal nuclei (Figure 7.2I), unlike EM48, which intensely stained neuronal nuclei (Figure 7.2H), indicating that only N-terminal fragments of mutant huntingtin (<549 amino acids) translocate to the nuclei.
Figure 7.2 EM 48 Nuclear staining in YAC72 founder 2498.

Many neuronal nuclei show intense EM 48 staining in the lateral striatum (A) and the nucleus accumbens (B). Pyramidal neurons in cortex (C) and the CA1 region of hippocampus (D), and neurons in the thalmus (F) contained only low levels of EM 48 immunoreactivity in both cytoplasm and nuclei. In the cerebellar cortex (E), EM 48 staining was present in the nuclei of some neurons in the granule cell layer (gr). Nuclei of Purkinje cells (P) had either no labeling or faint staining. Although EM 48 immunoreactivity was also seen in the wild-type controls, it was significantly less intense (G). EM 48 staining was very intense in neuronal nuclei (H), whereas HD 5.9 staining was restricted to the cytoplasm (I). EM48 nuclear staining (arrowheads) did not occur in VAT (J), NOSI (K), or PARV (L) immunoreactive neurons (arrows). Scale bars:

A-F: 10 μm, G-I: 30 nm, J-L: 15 nm

(Data and figure provided in collaboration with Dr. CA Gutekunst)
7.4.3 Striatal Nuclear Staining is Specific to Medium Spiny Neurons

Because they are so abundant, many of the striatal neurons with EM48 immunoreactive nuclei must be medium spiny neurons. To determine whether nuclear staining was also found in interneuron populations, we performed double-immunolabeling using EM48 and antibodies specific for each interneuron population. These included the vesicular acetylcholine transporter (VAT), which labels cholinergic interneurons (Gilmor et al., 1998), nitric oxide synthase (NOSI), which labels neurons containing somatostatin, neuropeptide Y, and NADPH-diaphorase, and parvalbumin (PARV), which labels a distinct population of medium sized interneurons. None of these interneurons had nuclear staining by EM48 (Figures 7.2J-L). These findings strongly suggest that in the striatum, translocation of the N-terminal portion of htt to the nucleus selectively occurs in medium spiny neurons.

7.4.4 Identification of Micro and Macroaggregates

In YAC72 founder 2498, many striatal nuclei were diffusely labeled with EM48 and also contained intensely labeled puncta (Figure 7.3A) that were 1 to 2 μm in diameter. In the accumbens, some labeled nuclei also contained one or two larger labeled puncta (Figures 7.2B-C) that ranged from 2 to 5 μm in diameter and were found in both intensely and lightly stained nuclei. In all stained nuclei there was a non-stained region corresponding to the nucleoli by EM (Figure 7.3D). Similar results were obtained using another N-terminal anti-huntingtin antibody raised against the first 17 amino acids of human htt (a gift from Peter Detloff).

To examine these potential htt aggregates, immunogold electron microscopy was performed using EM48 on sections containing striatum from YAC72 (2498 and 2511), YAC18 and FVB wild-type mice at 12 months of age. In YAC72 founder 2498, immunogold particles were abundant in the nuclei but were also evident at lower frequency in the cytoplasm (Figure 7.3E). Within nuclei, immunogold particles were found singly or in clusters (Figure 7.3E). Individual immunogold particles
Figure 7.3: Micro and macroaggregates in YAC72 founder 2498.

Nuclear staining was diffuse and also included small (A: arrows) and large puncta (B, C: arrowheads). Nucleoli were visible in relief as unlabeled regions of the nucleus (D: arrows). By electron microscopy, immunogold particles were seen both in the cytoplasm (c) and nucleus (n). Microaggregates containing more than five particles were found in the nuclei (E: arrows). Particles were also found in nuclear pores (F: arrowheads). Nuclear pores not containing immunogold particles are indicated by the arrows (F). At higher magnification, microaggregates appear to be associated with amorphous electron-dense material (G, H). Scale bars: A-D: 5 μm, E: 250 nm, F-G: 120 nm, H: 50 nm.

(Data and figure provided in collaboration with Dr. CA Gutekunst)
could also be identified within nuclear pores (Figure 7.3F). Most immunogold particles were present singly or in groups of two or three. Larger collections of immunogold particles (more than 5) were visualized in the nucleus but not in the cytoplasm (Figures 7.3E, and G-H). Most of these were too small to have been visualized by light microscopy and were thus termed microaggregates. These data strongly suggest that cleavage of htt occurs in the cytoplasm, and that N-terminal fragments are translocated through nuclear pores to the nucleus where they are present diffusely as microaggregates or as macroaggregates visible by light microscopy. In the control and YAC72 mice (line 2511), occasional immunogold particles were seen both in the cytoplasm and nuclei of neurons. Particles were found singly or in clusters of no more than 3 particles.

7.4.5 Neurodegeneration

Neuronal degeneration was assessed by light and electron microscopy in the striatum, the cortex adjacent to the lateral striatum, the hippocampus and the cerebellar cortex from YAC18 (n=8) and YAC46 (n=8) at 5, 9, 12 and 20 months of age, and YAC72 line 2511 (n=3) at 9, and 12 months, and YAC72 2498 founder at 12 months of age. Significant degeneration was only seen in the striatum of the YAC72 mice at 12 months compared to the YAC46, YAC18, and wild-type controls (Figures 7.4A-D). In the striatum of founder 2498, there was extensive neuronal degeneration which was most severe laterally and decreased in a graded fashion more medially (Figures 7.4E-F). By EM, degenerative changes included reduced neuronal size, presence of nuclear membrane irregularities, increased density of the cytoplasm, swelling of some mitochondria, vacuolization of the endoplasmic reticulum or Golgi, nuclear shrinkage, condensation, and margination of heterochromatin (Figures 7.4G and H). Up to 80% neurons in the lateral portions of the striatum were in various stages of degeneration. There were no obvious signs of reactive gliosis by glial fibrillary acidic protein staining (data not shown).
Figure 7.4 Neurodegeneration in YAC-72 founders 2498 and 2511.

Micrographs of EM48-stained striatal semi-thin sections showing degenerated neurons (arrowheads) in YAC72 founders 2498 and 2511 (A and C respectively: arrowheads) as compared to YAC18 (B) and wiltype control (D) at 12 months of age. In founder 2498, many neurons were degenerated in the most lateral part of the striatum (E: stars) as compared to the more medial part (F). Intense EM48 staining is seen in a neuronal nucleus (E) in the lateral striatum and in the cytoplasm of neurons in the medial portion of the striatum (F). Some degenerated neurons contained immunogold particles in their nuclei (n) and cytoplasm (c) (G). Evidence of degeneration included darkened cytoplasm, swelling of mitochondria (m), vacuolization of endoplasmic Golgi (g), and margination of the heterochromatin (G and H).

In founder 2511, many neurons which did not show degenerative signs had abnormal wavy nuclear membrane (I). Scale bars: A-F: 20 μm, G: 250 nm, H: 200, I: 250 nm.

(Data and figure provided in collaboration with Dr. CA Gutekunst)
In the striatum of founder 2511, up to 50% of neurons demonstrated signs of degeneration and were found in clusters throughout the striatum. By EM many neurons that did not have signs of frank degeneration contained nuclei with subtle morphologic abnormalities which appeared more oval and scalloped in outline than controls (Figure 7.4I). No evidence for neurodegeneration was present in YAC72 mice line 2511 at 9 months of age, nor in YAC18, YAC46, or wild-type controls, at any age examined.

The hippocampus was examined since YAC46 mice had abnormal hippocampal LTP and neuronal loss in the CA1 region of the hippocampus is seen in patients with HD (Spargo et al., 1993). Examination of Nissl stained sections (50 μm) from wild-type, YAC18 and YAC46 mice at ages 5, 9, 12 and 20 months showed no obvious differences in the gross organization and neuronal density of the CA1 pyramidal cell layer. Semi-thin sections were also examined and revealed no increase in the presence of degenerating neurons compared to controls.

7.5 Discussion

7.5.1 Aggregates and toxic fragments in polyglutamine disorders

The observed neuronal nuclear htt aggregate formation in founder F2498 is of considerable interest in light of recent findings in HD and other polyglutamine related diseases. A growing body of evidence has revealed that truncated protein fragments containing expanded polyglutamine tracts are more toxic to cells than when the tract is present within the context of the full-length protein. Furthermore, these polyQ containing fragments tend to aggregate and form insoluble inclusions within the cell, usually associated with the nucleus.

The first body of evidence to suggest the involvement of toxic fragments came from studies on HD knock-out mouse models. Three groups published results demonstrating embryonic lethality in
mice homozygous for a targeted disruption of the murine HD gene (Nasir et al., 1995; Duyao et al., 1995; Zeitlin et al., 1995). However, neurological impairment and subtle neuron loss in the subthalamic nucleus and globus pallidus was reported in heterozygous mice in which the gene had been disrupted in exon 5 (Nasir et al., 1995). No such phenotype was observed in mice that had the gene disrupted in the promoter (Zeitlin et al., 1995) suggesting that a putative toxic protein fragment (the N terminus of murine huntingtin) was produced in the exon 5 mice and could account for the difference. However, exon 4 knock-out mice (Duyao et al., 1995) did not show such a phenotype in heterozygous mice. This apparent discrepancy could be accounted for in a number of ways. Firstly, the mouse background used was different (CD1 vs C57Bl/6). Secondly, detailed morphological and behavioral analysis of the heterozygous exon 4 mice was not done, leaving the possibility open that a subtle phenotype was not detected in these mice. Thirdly, the small difference in protein sequence between the exon 4 and exon 5 knock-outs could be enough to account for the difference.

More compelling evidence for the involvement of toxic polyQ fragments in pathogenesis came from SCA-3/MJD transgenics (Ikeda et al., 1996). A series of mice were created that expressed mutant polyQ either in the context of the full MJD protein or a few residues flanking the tract. Only mice expressing the truncated form of the protein developed an ataxic phenotype and neuron loss as early as 4 weeks of age, suggesting that the shorter fragment is much more potent at inducing neurotoxicity than the full-length protein. Furthermore, transgenic mice expressing only exon 1 of the HD gene with ~115-150 CAG repeats develop a neurological phenotype typically beginning at around 11 weeks of age (Mangiarini et al., 1996). These mice, however, did not show any obvious signs of neuron loss or gliosis in the brain regions studied, including the basal ganglia, cortex, hippocampus and cerebellum. Further detailed investigation of these mice revealed the presence of a single htt-immunoreactive intranuclear inclusion (NI) in a large number of neurons (but not glial cells) in the striatum, cortex, cerebellum, and spinal cord but less so in the hippocampus, thalamus, substantia nigra, and globus pallidus (Davies et al., 1997). Only antibodies generated against the N-terminus of htt contained within transgenic protein recognized the inclusion, suggesting that endogenous mouse htt
does not colocalize with the inclusion. The inclusion was approximately the size of the nucleolus and was never observed in control animals.

The development of a neurological phenotype despite no observed neurodegeneration in the HD exon 1 mice suggests that the neurons are somehow functionally compromised, with the NI perhaps playing a key role. Thus polyglutamine induced neurodegeneration (in mice at least) may not be necessary for neurological impairment. This argument is further supported by mice expressing expanded polyglutamines in the context of the hypoxanthine phosphoribosyltransferase gene which also develop a late-onset neurological phenotype and widespread neuronal nuclear inclusions despite the lack obvious cell loss or gliosis (Ordway et al., 1997). Additionally, mutant SCA1 cDNA transgenic mice develop immunoreactive nuclear inclusions in the Purkinje cells of the cerebellum prior to the onset of an ataxic phenotype (Skinner et al., 1997). Since the Purkinje cell loss observed in these mice occurs after the onset of ataxia, this further argues that neuron dysfunction rather than loss is responsible for obvious neurological impairment in mice. What is less clear is the role of the nuclear inclusions in disease progression. Despite the fact that formation of inclusions is observed prior to symptom onset in HD exon1 (Davies et al., 1997) and SCA-1 (Skinner et al., 1997) transgenic mice, it is unclear at this point whether the inclusions are causative of neuronal dysfunction or simply develop as a byproduct of proteolytic processing of their respective proteins due to unrelated toxic cellular processes.

One obvious question related to aggregate formation is whether or not these inclusions are observed in human patient material. Neuronal, but never glial, intranuclear inclusions have been observed in postmortem material from HD (DiFiglia et al., 1997; Becher et al., 1997), SCA-1 (Skinner et al., 1997), SCA-3 (Paulson et al., 1997), and DRPLA (Becher et al., 1997) patients. What is striking about these studies is the close parallel between the region of the brain containing neuronal aggregates and the sites of pathology in each disease. Inclusions were found most frequently in the regions most affected, less frequently in the regions more mildly affected. In HD studies, N-terminal immunoreactive NIs were primarily observed in all layers of the cortex and medium sized neurons of
the striatum (likely to be medium spiny neurons), and were more frequent in juvenile onset cases (DiFiglia et al., 1997; Becher et al., 1997). Aggregates were also observed in dystrophic neurites in cortical layers of HD brains (DiFiglia et al., 1997). Dystrophic neurites are believed to form due to malfunction of retrograde axonal transport.

How these aggregates form is another question of considerable interest. It has been suggested that polyglutamine stretches have the capacity to form hydrogen bonds with other stretches of polyQ in an antiparallel manner, resulting in a ‘polar zippers’ and β-sheets (Perutz et al., 1994). Circular dichroism and X-ray diffraction studies on Asp2-Gln15-Lys2 peptides (pure Gln peptides are insoluble) produced data consistent with β-sheet formation. Purification of the peptide by filtration revealed 2 fractions, one corresponding the monomer and the other to a broad distribution of aggregates. At physiological pH, the peptide gradually precipitated. Taken together, these data show that polyQ containing proteins have a propensity to aggregate and form stable, insoluble aggregates, likely via β-sheets. Further in vitro studies using GST-HD exon1 fusion proteins support this hypothesis (Scherzinger et al., 1997). Expression of short (20 and 30) and long (51, 83, and 122) polyQ containing GST fusions revealed that the higher polyQ fusions (83 and 122) had a tendency to form high molecular weight aggregates during protein purification. Proteolytic removal of the GST fusion allowed for aggregation of all mutant-sized polyQ fragments (51, 83, 122) but not for the normal-sized peptides (20 and 30). Electron microscopy of these in vitro aggregates and of aggregates formed in HD exon1 transgenic mice demonstrated that they contain fibrous structures around 10nm in diameter, reminiscent of amyloid-like fibrils observed in Alzheimer’s Disease. These data reveal that polyQ-containing proteins have a propensity to aggregate and do so more readily if additional surrounding amino acids are cleaved away. Also, there appears to be a lower polyQ size limit that will allow for aggregate development. PolyQ 21 and 30 failed to produce aggregates whereas 51 and up did. This observation is consistent with the CAG/polyQ size ranges seen in HD patients and controls.
In light of these observations, it appears that proteolytic cleavage of htt and liberation of an N-terminal polyQ containing fragment would facilitate aggregate formation and toxicity. It has been shown that htt is cleaved by the pro-apoptotic enzyme caspase-3, liberating a 50-60 kD N-terminal protein fragment (Goldberg et al., 1996). Additionally, yeast two-hybrid screens have shown that htt interacts with the ubiquitin conjugating enzyme (Kalchman et al., 1996), suggesting that htt is a target for the ubiquitin mediated proteolytic pathway and thus a potential source of polyQ fragment production. Interestingly, ubiquitination is a common feature of the observed aggregates in mouse and humans (Davies et al., 1997; DiFiglia et al., 1997), suggesting that these aggregates or polyQ proteins are being targeted for proteolytic breakdown.

Taking all observations into account, a unifying model has been proposed to account for formation of inclusions in the polyglutamine diseases (Ross, 1997): Expansion of the polyglutamine repeat induces conformational changes in the protein by formation of stable hairpin formation via a polar zipper. This in turn alters protein interactions with htt (HAP-1, HIP-1), possibly resulting in pathological events within the cell. Additionally, the conformational change may make htt more susceptible to proteolytic cleavage by caspase-3 and other, as yet unidentified, proteins, thus liberating a small polyQ fragment that then forms aggregates via β-sheets both within the nucleus and cytoplasm. These aggregates are then ubiquitinated in an effort to target them for proteolytic removal from the cell. This model is preliminary, and the sequence of events needs to be further clarified. For example, is aggregate formation necessary for neurotoxicity or merely a marker for other cytotoxic events that cause htt cleavage? The observed hippocampal neurophysiological dysfunction at 6 months of age in the YGA2-46 YAC mice (chapter 8) despite no evidence of aggregate formation or neurodegeneration up to 20 months of age demonstrates that cellular dysfunction can occur in the absence of aggregate formation. This suggests that cellular toxicity is independent of aggregate formation and that aggregates are simply a marker of toxic cellular processes already underway within that cell.
7.5.2 Neurodegeneration in CAG mouse models

Neurodegeneration was observed in the YAC transgenic mice expressing htt with 72 glutamines at 1 year of age but was not seen in wild type, YAC18, or YAC46 mice up to 20 months of age. Neurodegeneration at 12 months of age was more obvious and widespread in the high copy number founder 2498, showing that higher levels of mutant protein expression induce a more aggressive neuropathological phenotype. Additionally, the lack of observed neurodegeneration in YGA2-46 mice but neurodegeneration in 353G6-72 founder F2511 at 12 months with similar levels of protein expression (1/3 to ½ endogenous) demonstrates that increased CAG size enhances the neurodegenerative process in mice. Since no neurodegeneration was observed in YGA2-46 mice up to 20 months of age, expression levels 1/3 to ½ endogenous with a CAG size in this range are not sufficient to induce a neurodegenerative phenotype in the FVB/N mouse strain. Additionally, since marked neurodegeneration was observed in 353G6-72 founder 2511 at 1 year of age without the development of neuronal nuclear aggregates, htt aggregate formation is not required for induction of neurodegeneration.

Assessment of neurodegeneration in other CAG repeat animal models has revealed an interesting association of cell loss with mouse strain. The 2 models demonstrating clear behavioral changes and aggregate formation despite lack of cell loss were produced and maintained in a C57BL/6 (or C57BL/6xCBA hybrid) background (Mangiarini et al., 1996; Ordway et al., 1997). SCA-1 transgenic mice showing clear evidence of neurodegeneration were produced and bred in the FVB/N mouse strain (Burright et al., 1995). The YAC transgenic mice produced in this study are consistent with these results. What makes this observation interesting is that C57BL/6 mice are relatively resistant to excitotoxic cell death mediated by kainic acid (KA), a glutamate analogue that preferentially binds to and activates the NMDA receptor (Schauwecker and Steward, 1997). FVB/N mice, however, exhibited excitotoxic cell death similar to that seen in rats. The implication of these results has direct relevance to HD since the excitotoxic hypothesis has been proposed as a potential
cause of cell death in HD (discussed in detail in chapter 8). These results argue that the C57BL/6 strain carries gene(s) that convey protection from glutamate-induced excitotoxicity and perhaps accounts for the lack of neurodegeneration in the HD exon 1 (Mangiarini et al., 1996) and HPRT (Ordway et al., 1997) mutant transgenic mice.

7.6 Conclusion

High levels of expression (1-2X that of endogenous) of mutant human huntingtin with large CAG sizes (>70) is sufficient to induce a selective neurodegenerative and neuropathological phenotype in FVB/N mice consistent with that observed in HD patients.
7.7 Reference List


CHAPTER 8 - NEUROPHYSIOLOGY
8.1 Hypothesis

Expression of mutant human huntingtin in YAC transgenic mice will disrupt neuron physiology.

8.2 Objective

To assess hippocampal synaptic function and synaptic plasticity in the YAC transgenic mice.

8.3 Introduction

8.3.1 HD learning and memory

In addition to the obvious clinical presentation of motor disturbances such as chorea in the vast majority of HD patients, mental disturbance is present in some form in all affected individuals (Hayden, 1981). One of the key categories of mental disturbance is dementia, which has been described as cognitive impairment, usually reflected in the decline of intellect, the disturbance of memory, and reduced capacity for conceptual thought (Hayden, 1981). Disturbance in memory is usually one of the first signs of mental changes. Memory tests on HD patients have revealed that memories are stored but cannot be retrieved at will (Folstein, 1989).

The underlying cause of dementia in HD patients remains unknown. It has been suggested that the malfunction in the caudate may play an important role in cognitive impairment since the cognitive functions involved in HD are those served by the prefrontal cortex, which provides the major cortical input to the caudate (Folstein, 1989). However it is unclear whether the dementia is solely attributable to frontal cortex-caudate connections, thus other brain areas such as the hippocampus, which is also involved in primary dementias and learning and memory, have been studied (Spargo et al., 1993). Neuron counts in the CA1, CA3, and CA4 regions of the hippocampus were compared between HD patients and controls, revealing normal cell density in the CA3 and CA4 regions but a 35% cell loss in the CA1 region. This study then provides support that the hippocampus may play a role in the
dementia observed in HD patients. In light of this, we wished to study neurophysiological consequences of mutant huntingtin expression in the hippocampus of the YAC transgenic mice. To facilitate discussion in this chapter, I will provide a background of the anatomy and some of the functional aspects of the hippocampus.

8.3.2 Anatomy of the rodent hippocampus

The rodent hippocampus occupies most of the ventroposterior and ventrolateral walls of the cerebral cortex. Studies involving human and rats show that the hippocampus plays a key role learning and memory, and much research in recent years has been focused on understanding the molecular and biological basis of memory formation.

The general cytoarchitecture of the rodent hippocampus is illustrated in figure 8.1A and the neuronal connections between each region are illustrated in figure 8.1B (Bayer, 1985). The most obvious features observed are Ammon’s horn (Amn) and the dentate gyrus (DG). The DG is divided into 3 layers; the molecular layer (Mol) comprised largely of dendrites from granule cells, the sharply folded granule cell layer (gc) containing densely packed granule cells, and the hilus (h) containing mossy cells and interneurons. The extensive dendritic network of the granule cells receives the vast majority of its input from the entorhinal cortex via the perforant fiber pathway (PP). Granule cell axons exit via the mossy fiber pathway (MF) and synapse with mossy cells of the hilus and pyramidal cells of the ipsilateral (same hemisphere) CA3 region of Ammon’s horn.

Ammon’s horn is a folded U-shaped structure comprised of 4 layers; the stratum oriens (Or), the densely packed pyramidal cell layer (Pyr), the stratum radiatum (Rad), and the lacunosum moleculare layer (Lmol). The pyramidal cell layer is divided into 3 distinct areas based on cytoarchitectural differences, the CA1, CA2, and CA3. Apical dendrites from these pyramidal cells extend into the Rad and Lmol layers, whereas basal dendrites extend into the Or layer. Axons from the CA1 cells extend through the Or layer and bifurcate, heading to the subicular complex and the
Figure 8.1 Neuroanatomy of the rodent hippocampus

A) General cytoarchitecture of the rodent hippocampus in coronal sections. The key neuronal cell types found in the hippocampus are the granule cells of the dentate gyrus and the pyramidal cells of Ammon’s horn. For details, see text. Abbreviations: Amn, Ammon’s horn; CA1, CA2, CA3, subfields of Ammon’s horn; DG, dentate gyrus; Fi, fimbria; gc, granule cell layer of dentate gyrus; h, hilus; Lmol, lacunosum moleculare layer; mol, molecular layer of dentate gyrus; Or, stratum oriens layer; Pyr, pyramidal cell layer of Ammon’s horn; Rad, stratum radiatum layer.

B) Neuronal connections in the rodent hippocampus. Each subfield of the hippocampus is intimately connected via key neuronal pathways. Refer to text for details on these pathways. Abbreviations: C, contralateral efferent neurons from CA3; C/A, commissural/associational pathway; MF, mossy fiber pathway; PP, perforant pathway; S, Schaffer collateral pathway. Arrows indicate the direction of travel in the neurons.
fimbria (fi). Of primary interest for this study are the axons that emanate from the CA3 cells. They form an extensive network of interactions with CA1 cells in Ammon’s horn in both the Or and Rad layers via the Schaffer collateral pathway (S). These connections occur both ipsilaterally (same hemisphere) and contralaterally (opposite hemisphere). The contralateral efferents (c) to the CA1 from the CA3 enter via the fimbria and are closely associated with Schaffer collaterals. These 2 pathways together are often referred to as the Schaffer/Commissural pathway (SC). Axonal fibers from the CA3 region also connect with other pyramidal cells within the CA3 region both ipsi- and contralaterally via the commissural/associational (C/A) pathways (commissural pathways connect opposite hemispheres whereas associational pathways connect regions within a hemisphere).

The aforementioned cells and connections primarily involve excitatory transmission. Interlaced within this complex excitatory network is a GABAergic inhibitory network that is mediated primarily through interneurons.

### 8.3.3 Synaptic plasticity

It has long been postulated that one potential molecular mechanism that may account for learning and memory is that of activity-dependent synaptic potentiation, in which frequent stimulation of a particular excitatory neuronal pathway increases the efficiency of synaptic transmission within that pathway (Bliss and Collingridge, 1993). Activity-dependent synaptic potentiation involves a number of mechanistically and temporally distinct components. These are paired-pulse facilitation (PPF), post-tetanic potentiation (PTP), short-term potentiation (STP), and long-term potentiation (LTP). PPF and PTP are features of normal synaptic transmission and are caused by the increase in pre and postsynaptic intracellular Ca$^{2+}$ levels, respectively. If a second pulse follows the original pulse within a very short period of time (up to 200ms), the second pulse is always potentiated.

STP and LTP can be distinguished from one another both mechanistically and temporally. STP and LTP are observed as an increase in synaptic strength, typically induced by a tetanus (50-100
stimuli at 100 Hz) to a pathway of interest. Recordings of synaptic strength can be taken from individual cells or populations of neurons. However STP and LTP can be distinguished in terms of time required to decay. STP (the least understood of the forms of synaptic plasticity) typically decays within minutes following a tetanus whereas LTP can last hours to days. They can also be mechanistically differentiated by use of protein kinase inhibitors, which demonstrate that potentiation lasts only minutes in the presence of these inhibitors. Despite the distinction, the relationship between these 2 forms of plasticity are unclear due to the fact that pharmacological treatments have failed to selectively inhibit STP (Stevens et al., 1994). It may be that STP is a necessary step in the induction of LTP.

LTP can be divided into 2 main components. The transient phase (E-LTP) typically lasts about 1 hour in hippocampal slices and is blocked by kinase inhibitors but not by inhibitors of protein synthesis (Bliss and Collingridge, 1993; Abel et al., 1997). Late phase LTP (L-LTP), which lasts for up to 8 hrs in hippocampal slices and days in the intact animal, is blocked by inhibitors of protein synthesis and requires both transcription and translation (Abel et al., 1997). The period in switching from E-LTP to L-LTP is referred to as the consolidation period.

There are 3 basic properties of LTP; cooperativity, associativity and input specificity. Cooperativity demonstrates the need for an intensity threshold to induce LTP. This cooperativity is complex and involves a combination of stimulus intensity and pattern of stimulation. Associativity refers to the fact that LTP can be induced in a weak input provided that a strong input is present at the same time in a separate but convergent pathway. Input specificity states that other inputs not active at the time of tetanus do not become potentiated. By combining all 3 properties of LTP, we provide the following summary: a synapse will become potentiated if, and only if, it is active at a time when the region of a dendrite on which it terminates is sufficiently depolarized.

One additional form of synaptic plasticity is long-term depression (LTD), which results in a decrease in synaptic transmission (Linden, 1994) and is usually induced by low frequency stimulation.
(1 Hz). LTD can occur at the same synapses as LTP, thus whether a synapse fires more or less efficiently is determined by its previous stimulation history.

Given the observed deficits in cognitive function and learning and memory in HD patients, we tested whether the YAC transgenic mice showed any perturbations in synaptic plasticity. We did this using a well-established protocol for measuring synaptic transmission at the CA1-SC synapses of the hippocampus.

8.4 Results

8.4.1 Basal Synaptic Transmission

Field responses measured as field excitatory postsynaptic potentials (fEPSPs) were assessed in the CA1 region elicited by basal stimulation of SC-CA1 synapses in hippocampal slices. Basal synaptic transmission was normal and similar in all mice tested, showing that neurons in the SC-CA1 pathway were able to respond equally to stimulation.

8.4.2 Presynaptic Site

To assess the presynaptic efficacy, we investigated the extent of the paired pulse facilitation (PPF) in Schaeffer collaterals of wild type mice, and transgenics with 18 (YAC18) and 46 (YAC46) CAG repeats. A change in the extent of paired pulse facilitation is manifested by an increase in the slope of the second field EPSP when two pulses are given successively at various intervals up to 200ms. Paired pulse facilitation did not differ between mutant transgenic, wild type transgenic, and non-transgenic hippocampi over an interpulse interval range of 10 to 500 ms in any transgenic line at 6 months of age (Figure 8.2). These data imply that the expression of normal and mutant human huntingtin is not associated with any deficits in presynaptic release mechanisms.
Figure 8.2 Paired Pulse facilitation in YGA2 YAC transgenic mice.

Paired pulse facilitation of non-transgenic mice (wt), and YGA2-18 (line 30), and YGA2-46 (line 668) at 6 months of age. There is no difference between each group suggesting that expression of mutant huntingtin does not impair presynaptic release mechanisms.
8.4.3 Long term potentiation in synaptic efficacy

We next investigated the effect of mutant huntingtin gene on the induction and maintenance of homosynaptic long-term potentiation (LTP defined here as an increase in synaptic strength lasting more than 1 hour). At six months of age we tested the response of SC-CA1 synapses to high frequency stimulation (HFS) on wild type, YGA2-18 (lines 29 and 30), and YGA2-46 (line 668) mice. We observed a decrease in both the induction and maintenance of long term potentiation in mutant transgenic mice hippocampi (Figure 8.3A, 8.4). The fact that response in YGA2-18 control mice (line 29) was different to mutant YGA2-46 mice (line 668) shows that this phenotype is not associated with expression of other genes on the YAC. If this were true, these other genes would also be expressed in YGA2-18 line 29 and result in expression of a phenotype. Thus the deficits in LTP induction and maintenance can be attributed to expression of mutant human huntingtin. By 10 months of age we were not able to induce LTP in the SC-CA1 synapse of mutant transgenic hippocampi (Figure 8.3B). The normalized field EPSP slope 60 min after tetanus was significantly reduced in slices from 10-month old mutant mice compared to wild type controls (Figure 8.4). Three mutant lines were tested (1746 n=8, 1747 n=1, 1749 n=3), and all showed similar decreased responses.

8.4.4 Human huntingtin expression in the hippocampus

To confirm that mutant human huntingtin was expressed in the hippocampus of the YAC mice, protein was extracted from hippocampus which was dissected away from other brain regions. Human protein expression was identified by Western blotting on protein extracts from YGA2-46 mutant lines (668, 1746, 1747, 1749) and YGA2-18 lines (29, 30) using human specific-antibody GHM1 as the primary antibody (fig 8.5).
Figure 8.3 Long term potentiation in YGA2 YAC transgenic mice.

Response of hippocampal slices to HFS from wild type (wt) and YAC transgenic mice (YGA2-18 and YGA2-46) over a 60min period of recording. Normalized fEPSP slope (S2/S1) is reduced in mutant transgenics at both 6 and 10 months of age. The reduction in LTP response at 10 months of age is more dramatic than at 6 months. (Data and figure provided in collaboration with Dr. N Agopyan).
LTP

A) 6 months

B) 10 months
Response to high frequency stimulation (HFS) in wild type (wt), YGA2-18, and YGA2-46 YAC transgenic mice. The normalized fEPSP 60 minutes after HFS is reduced in YGA2-46 mutant mice (line 668) at 6 months. At 10 months normalized fEPSP 60 minutes after HFS is significantly reduced (P=0.0168) in YGA2-46 mutant mice (lines 1746, 1747, 1749).

Figure 8.4 Response to high frequency stimulation in YGA2 YAC transgenic mice.

Response to high frequency stimulation (HFS) in wild type (wt), YGA2-18, and YGA2-46 YAC transgenic mice. The normalized fEPSP 60 minutes after HFS is reduced in YGA2-46 mutant mice (line 668) at 6 months. At 10 months normalized fEPSP 60 minutes after HFS is significantly reduced (P=0.0168) in YGA2-46 mutant mice (lines 1746, 1747, 1749).
Figure 8.5 Human huntingtin expression in the YAC transgenic hippocampus.

Western blot showing htt expression in the hippocampus of 2 YGA2-46 mutant lines (1749 and 1746) and 1 YGA2-18 control line (29). GHM1 was used as a primary antibody thus does not detect expression in non-transgenic mice (wild type).
8.5 Discussion

Our results clearly demonstrate that YGA2-46 YAC transgenic mice are impaired in LTP induction, whereas YGA2-18 (lines 29 and 30) and wild type controls are not. The studies show a progressive decrease in the maintenance and induction of LTP in the mice expressing mutant huntingtin. At 6 months of age tetanic stimulation induced LTP, but the rate of decay was much faster than the control mice, thereby indicating that LTP was not maintained. However, at 10 months of age we could not induce LTP. Such a decline in the maintenance and induction of LTP could be due to faulty NMDA receptor function, perturbations in the levels of intracellular calcium, or disruption of 1 or more of the many secondary messenger cascades involved in maintaining LTP (discussed below).

It is, however, possible that the observed differences in LTP induction and maintenance could be a line-related phenomenon. YGA2-46 line 668 was used for study at 6 months whereas lines 1746, 1747, and 1749 were used at the 10-month time point. However, no obvious differences in protein expression levels were detected amongst the lines which suggests that the differences are attributable to mouse age. However, further experiments testing line 668 at 10 months and/or lines 1747, 1746, and 1749 at 6 months will definitively address this question. This work is currently in progress.

Induction and maintenance of LTP at the molecular level is extremely complex, involving a multitude of proteins and biochemical cascades. To provide a brief background of this immense field and to provide some potential insight into how mutant huntingtin may interfere with this process, I will present what has been learned in recent years from animal models of LTP.

8.5.1 LTP induction involves distinct molecular mechanisms at different synapses

Studies using transgenic mouse models have begun to unravel the complex nature of synaptic plasticity in the rodent hippocampus (as well as other brain regions) and have provided clues into the molecular mechanisms underlying LTP. Most studies on mouse models of LTP have focused on a few
key synaptic junctions. In the following discussion, the following terminology is used: the region receiving the neuronal input is listed first, followed by the neuronal pathway leading to that region. For example, CA1-SC refers to the synapses present in the CA1 region of the hippocampus coming in via the Schaffer/commissural pathway.

One of the underlying principles is that there are distinct ways in which LTP can be induced at any given synapse within the brain, mediated by distinct molecular mechanisms. Of interest are the nNOS/eNOS double knockout mice in which CA1-SC synapses were tested for LTP in 2 layers of the CA1 region (the stratum radiatum and the stratum oriens). Rad synapses showed impaired LTP whereas Or synapses showed normal LTP, thus demonstrating that synaptic input to a particular groups of cells (CA1 pyramidal cells) via the same efferent neuronal pathway (SC) utilizes different molecular mechanisms to enhance synaptic transmission. Additionally, the NMDA receptor NR2A/NR2B knockout mice nicely demonstrate synapse selective LTP within the same CA3 pyramidal cell, which depends on the subunit composition of the NMDA receptor at that synapse and the efferent input pathway (Ito et al., 1997). Additionally, LTP can be induced via NMDA receptor dependent and independent mechanisms. Within the hippocampus, for example, CA3-MF synapses are NMDA receptor independent, whereas CA1-SC and DG-PF synapses require a functional NMDA receptor (Bliss and Collingridge, 1993). Therefore, it must be understood that observed deficiencies in LTP in one brain region do not necessarily imply that other regions will show the same deficits. Thus it is important to understand the components involved in synaptic plasticity at the SC-CA1 synapses since these were studied in the HD YAC transgenic mice. These details are discussed below.

8.5.2 LTP mouse models and SC-CA1 synaptic plasticity

One of the most extensively studied synaptic junctions in the hippocampus is the SC-CA1 pathway (in the stratum radiatum layer) and was the region chosen for study in the YAC transgenic mice. Using transgenic mouse models, a number of molecular components have been identified that
play a key role in modifying synaptic plasticity at these synapses. These results are summarized in table 8.1 and figure 8.6. Genes were selected for study in knockout mice based on pharmacological experiments that demonstrated decreases in hippocampal LTP in rats following administration of molecular inhibitors (Bliss and Collingridge, 1993). The components involved in synaptic plasticity can be subdivided into distinct categories; 1) neurotransmitter receptors, 2) secondary messengers, 3) retrograde messengers, and 4) other miscellaneous components. Of critical importance for LTP at SC-CA1 synapses is the NMDA receptor (NMDAR) since LTP is eliminated in CA1 specific NMDAR knockout mice (Tsien et al., 1996). The general mechanism by which LTP is produced involves influx of Ca\(^{2+}\) through activated NMDAR channels (Bliss and Collingridge, 1993). This increase in intracellular Ca\(^{2+}\) then activates a series of signal transduction mechanisms that activate kinases and other proteins involved in activating gene transcription and translation. Presynaptic mechanisms are also considered necessary to potentiate synaptic activity, and it is believed that retrograde messages produced in the postsynaptic dendritic spine diffuse back to the presynaptic cell to induce molecular changes that enhance transmitter release. LTD at CA1-SC synapses is also NMDA receptor and Ca\(^{2+}\) dependent, but LTD and LTP require different levels of intracellular Ca\(^{2+}\), with LTP requiring a greater influx than LTD (Christie et al., 1996). Since LTD induction requires the action of phosphatases (Mulkey et al., 1993), it has been proposed that in CA1-SC synapses a balance between phosphatase and kinase action (regulated by the level of Ca\(^{2+}\) within the postsynaptic cell) will determine whether a synapse becomes potentiated or inhibited.

8.5.3 Potential roles of mutant huntingtin in influencing LTP

Given the complex nature of LTP, expression of mutant huntingtin could act to disrupt one or a number of components involved in this pathway. Of particular interest is the
Table 8.1: Mouse models of synaptic plasticity

<table>
<thead>
<tr>
<th>Group</th>
<th>mouse/gene</th>
<th>PPF</th>
<th>PTP/STP</th>
<th>LTP</th>
<th>LTD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>mGluR1</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>N</td>
<td>(Aiba et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>mGluR5</td>
<td>N</td>
<td>ND</td>
<td>D</td>
<td>ND</td>
<td>(Lu et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>NMDA-NR1</td>
<td>N</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>(Tsien et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>NMDA-NR2A</td>
<td>N</td>
<td>ND</td>
<td>D</td>
<td>ND</td>
<td>(Sakimura et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>NMDA-NR2B</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>D</td>
<td>(Ito et al., 1997)</td>
</tr>
<tr>
<td>Secondary</td>
<td>Fyn</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>ND</td>
<td>(Ito et al., 1997)</td>
</tr>
<tr>
<td>Messengers</td>
<td>CaMKII</td>
<td>D</td>
<td>N (PTP)</td>
<td>D</td>
<td>D</td>
<td>(Stevens et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>PKCγ</td>
<td>D</td>
<td>ND</td>
<td>D</td>
<td>N</td>
<td>(Silva et al., 1992b)</td>
</tr>
<tr>
<td></td>
<td>Calbindin D28K</td>
<td>ND</td>
<td>ND</td>
<td>D</td>
<td>ND</td>
<td>(Abeliovich et al., 1993a)</td>
</tr>
<tr>
<td></td>
<td>PKA</td>
<td>N</td>
<td>N</td>
<td>N (E-LTP)</td>
<td>D (L-LTP)</td>
<td>(Molinari et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>CREB</td>
<td>N</td>
<td>N</td>
<td>N (E-LTP)</td>
<td>ND</td>
<td>(Abel et al., 1997)</td>
</tr>
<tr>
<td>Retrograde</td>
<td>eNOS/nNOS</td>
<td>N</td>
<td>ND</td>
<td>D</td>
<td>ND</td>
<td>(Bourtchuladze et al., 1994)</td>
</tr>
<tr>
<td>Messengers</td>
<td>BDNF</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>ND</td>
<td>(Son et al., 1996)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>t-PA</td>
<td>N</td>
<td>N</td>
<td>N (E-LTP)</td>
<td>D (L-LTP)</td>
<td>(Korte et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>APP</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>N</td>
<td>(Huang et al., 1996)</td>
</tr>
</tbody>
</table>

*a All mice are knockouts for the respective gene, except APP (amyloid precursor protein) which are transgenics. N: normal; D: decreased; ND: not determined. PPF: Paired Pulse Facilitation. PTP: Post Tetanic Potentiation. STP: Short-Term Potentiation. LTP: Long Term Potentiation. LTD: Long Term Depression. L-LTP: Late LTP. E-LTP: Early LTP*
Figure 8.6 Components involved in SC-CA1 synaptic plasticity.

The components involved in CA1-SC synaptic plasticity. The excitatory amino acid glutamate (Glu) is released from the presynaptic terminal and binds to and activates a variety of receptors; metabotropic glutamate receptors 1 and 5 (mGluR1/5), NMDA receptor (NMDA), and AMPA receptors (AMPA). \( \text{Ca}^{2+} \) ions (entering via the NMDA receptor) and G-coupled proteins associated with the mGluR1/5 receptors activate a series of secondary messenger cascades within the cell, resulting in activation of kinases (fyn, PKA, CaMKII, PKC\( \gamma \)) and other calcium binding proteins (eNOS, nNOS, CD28K) which in turn induce gene transcription (CREB, t-PA) and production of retrograde messengers that diffuse to the presynaptic terminal to enhance Glu release. Details about each of these components can be found in the text. PKA: cAMP dependent protein kinase A. CaMKII: calcium/calmodulin dependent protein kinase. PKC\( \gamma \): calcium-phospholipid dependent protein kinase. eNOS/nNOS: endothelial/neuronal nitric oxide synthase. CD28K: calbindin D28K. CREB: cAMP responsive element binding protein. t-PA: tissue type plasminogen activator. BDNF: brain derived neurotrophic factor.
NMDA receptor since it has already been implicated in excitotoxic cell death in the striatum of HD patients. Huntingtin interactions with the NMDAR in both hippocampal and striatal neurons may provide a common pathway for cell dysfunction in HD. In this light, I will provide a brief overview of the excitotoxic hypothesis and the role of the NMDA receptor.

8.5.4 Excitotoxic Hypothesis

Initial clues implicating an excitotoxic based mechanism of cell death in HD were provided by intrastrial injections of excitatory amino acids (EAA) in rat models. Acute and chronic intrastrial injections of quinolinic acid (QA), a glutamate analogue that preferentially binds to and activates the NMDAR, mimics the neuropathological and neurochemical changes observed in HD (Beal et al., 1986; Bazzett et al., 1993). Selective loss of the neurotransmitters GABA and substance P (found in medium spiny neurons) was observed, with sparing of somatistatin and neuropeptide Y (found in aspiny striatal interneurons) and dopamine. Additionally, histological analyses showed a selective loss of medium spiny neurons, while surrounding aspiny neurons were spared.

The NMDA receptor is of particular interest in HD since studies on post-mortem HD brain tissue have shown a dramatic decrease in levels of this receptor in the striatum (Young et al., 1988). These data, in combination with the QA injection studies, suggest that in HD, cells preferentially expressing the NMDA receptor in the striatum (medium spiny neurons) are particularly susceptible to excitotoxic effects mediated via EAAs. The medium spiny neurons receive massive excitatory glutamatergic input from all regions of the cortex, mediated via Glu receptors, implicating the excitatory amino acid glutamate (Glu) in excitotoxicity.

However, given the fact that the NMDAR is widely expressed in a number of brain regions and the pathology in HD is relatively selective (Hayden and Kremer, 1995), other factors must be taken into account. One suggestion is that striatal neurons are metabolically
compromised in HD and more susceptible to excitotoxic insult. Indeed, studies on cultured neurons have shown that they are more susceptible to death even in the presence of normal EAA release if the cell exists in a metabolically compromised state (Albin and Greenamyre, 1992). Additionally, decreasing neuronal energy metabolism by inhibition of mitochondrial oxidative phosphorylation with the toxin 3-nitropropionic acid (3-NP) causes selective striatal lesions in mice and rats, whether administered by local injection or systemically (Brouillet et al., 1993). Low dose, systemic administration of 3-NP over a 1-month period produced small areas of neuronal loss and astrogliosis in the dorsolateral striatum, highly reminiscent of HD. No lesions were observed outside the striatum. Interestingly, older animals (4 and 12 months) were significantly more susceptible to 3-NP than 1 month old animals, demonstrating an age-related phenomenon, similar to that seen in HD. Since impairment of mitochondrial function accompanies normal aging, it is possible that the delayed onset observed in HD is due to a combination of compromised cell viability due to the polyglutamine expansion and naturally occurring age related decreases in cellular energy production (Beal et al., 1993).

A number of lines of evidence support the observation of defective energy metabolism in HD patients. Positron emission tomography (PET) scans on affected and at risk individuals have demonstrated decreased glucose metabolism in the caudate nucleus that appears normal on MRI scans (Hayden and Kremer, 1995). Furthermore, nuclear magnetic resonance (NMR) spectroscopy studies on at risk and HD patients revealed elevated levels of lactate in occipital cortex and basal ganglia, consistent with impaired energy metabolism (Jenkins et al., 1993). Taking these data together, one potential model (the excitotoxic hypothesis) for neurotoxicity in HD has been proposed (figure 8.7), with the NMDAR and Ca\(^{2+}\) playing a key role as is the case for synaptic plasticity (figure 8.6). Essentially, chronic but subtle increases in intracellular Ca\(^{2+}\) levels (due perhaps to faulty NMDAR function and/or energy impairment) would lead to a series of detrimental molecular events within that cell, ultimately targeting it for death (figure 8.7).
Some proposed mechanisms for excitotoxicity in neurons. Subtle, chronic activation of the NMDA receptor by glutamate (Glu) could occur by over production Glu (1a), impaired clearance from the synaptic cleft by Glu transporters (1b), or a hypersensitive NMDA receptor (1c). This in turn would lead to abnormal elevated intracellular levels of $\text{Ca}^{2+}$ that can exert its toxic effects a number of ways. Oxidative stress is caused by activating enzymes that produce highly reactive metabolites that destroy DNA, lipids, and proteins (2). Other $\text{Ca}^{2+}$ dependent proteins can have the same molecular destroying effects (3). Elevated $\text{Ca}^{2+}$ levels can also impact mitochondrial function thus diminishing ATP production, putting a cell in an even more compromised state (4). Decreased ATP levels would result in the cell’s ability to rapidly clear cytoplasmic intracellular $\text{Ca}^{2+}$ by ATP dependent pumps thus compounding the effect (5). Cells that are already in a metabolically/functionally-compromised state are more susceptible to excitotoxic effects. Therefore, a mutant HD protein may act subtly to disrupt normal cell function enough to allow for a build up of excitotoxic effects even during normal synaptic transmission.
Impaired energy metabolism

1b

Glial Cell

Presynaptic terminal

1a

NMDA R

Glu

5

Ca^{2+}

2 Oxidative stress
- OH^-
- O_2^-

3 Protein Activation
- Phospholipases
- Proteases
- Nucleases

4 Impaired energy metabolism

↓ ATP
Further studies into the cause of the LTP deficits in the HD YAC transgenic mice will help address whether a common link exists between NMDAR mediated excitotoxicity and LTP impairment. It is tempting to speculate that a hyperactive NMDA receptor may be involved in both cases, leading to excess $\text{Ca}^{2+}$ influx. This could cause excitotoxicity in compromised neurons (such as those in the striatum) and saturation of the LTP based mechanisms in hippocampal neurons such that enhancement of synaptic transmission is no longer possible.

8.5.5 Synaptic plasticity and learning and memory

The LTP observations raise interesting questions relating to the learning and memory capabilities of the YAC transgenic mice. Recent animal model work in this area has shed light on the correlation between impairments in synaptic plasticity and certain types of learning and memory which are described below and summarized in table 8.2.

There appear to be 2 classes of associative learning and memory that are either hippocampal dependent or independent (Abeliovich et al., 1993b; Aiba et al., 1994). The first class of learning involves simple associations (referred to as procedural learning) whereby associations are made between 2 particular environmental cues, resulting in a learned behavioral response. For example, pairing a distinct tone with a footshock would result in the learned link between the 2 cues and elicit a fear conditioned response when the tone is heard, regardless of whether the footshock is applied. This type of learning is independent of hippocampal function. A second class of learning is more complex (referred to as configural/declarative learning) and requires that an association be made using multiple sets of cues within the environment. Two subclasses of configural learning have recently been identified: contextual learning and spatial learning. Both subclasses of configural learning require hippocampal function, but it seems that each by be separated into distinct subroutines (Bach et al., 1995).
Table 8.2: Mouse models of synaptic plasticity and learning and memory.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse*</th>
<th>LTP impairment</th>
<th>Learning and memory</th>
<th>Procedural</th>
<th>Reference</th>
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<td></td>
<td></td>
<td>Configural</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Spatial</td>
<td>contextual</td>
<td></td>
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<tr>
<td>Receptors</td>
<td>mGluR1</td>
<td>CA1-SC</td>
<td>ND</td>
<td>D</td>
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</tr>
<tr>
<td></td>
<td>mGluR5</td>
<td>CA1-SC</td>
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<td>D</td>
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<td>ND</td>
<td>N</td>
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<td>PKCy</td>
<td>CA1-SC</td>
<td>D</td>
<td>D</td>
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<tr>
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<td>Calbindin D28K</td>
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<td>calretinin</td>
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<td>CA1-SC</td>
<td>D</td>
<td>N (1hr)</td>
<td>N (1hr)</td>
</tr>
<tr>
<td></td>
<td>CREB</td>
<td>CA1-SC</td>
<td>D</td>
<td>N (0.5hr)</td>
<td>N (.5, 1hr)</td>
</tr>
<tr>
<td></td>
<td>t-PA</td>
<td>CA1-SC</td>
<td>N</td>
<td>N (1,24hr)</td>
<td>D (2,24hr)</td>
</tr>
<tr>
<td>Misc</td>
<td>Thy-1</td>
<td>DG-PP</td>
<td>N</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>APP</td>
<td>CA1-SC</td>
<td>D</td>
<td>ND</td>
<td>N</td>
</tr>
</tbody>
</table>

*All mice are knockouts with the exception of the APP (amyloid precursor protein) transgenic. N: normal; D: decreased; ND: not determined
One of the key findings to come out of these studies is that deficiencies in CA1-SC LTP are usually associated with impairment in spatial learning. CA1-SC synaptic plasticity may also be involved in contextual learning since most mice showing CA1-SC LTP impairment also show deficits in contextual learning.

Given these data, 2 key experiments should help address questions relating to learning and memory in the YAC transgenic mice, contextual fear conditioning (CFC) and the Morris water maze (MWM), since both have been linked to hippocampal based learning and memory. However, a severe limitation here is that FVB/N mice are visually impaired. The MWM requires strong visual capabilities since it tests a mouse’s ability to use distal visual cues to form a representation of its environment and use this information to navigate through the maze. It may be possible to test the mice by CFC since it is not well defined whether blind mice can form a contextual representation of their environment. However, a detailed study of behavioral phenotypes of inbred mouse strains has demonstrated that FVB/N mice are generally poor performers in CFC tests (Crawley et al., 1997). It may be possible to circumvent this problem by crossing the FVB/N YAC mice with a visually sound line (e.g. C57BL6) and studying the F1 hybrids as has been done for a YAC model of Down syndrome (Smith et al., 1997). Given that both parental lines are genetically pure, all F1 mice would be genetically identical except for the presence or absence of the transgene, eliminating effects from differences in mouse background.

8.6 Conclusion

Expression of mutant, but not normal, human huntingtin in YAC transgenic mice does not disrupt normal synaptic transmission or presynaptic release mechanisms but does disrupt maintenance of LTP at SC-CA1 synapses in the hippocampus as early as 6 months of age.
8.7 Reference List


CHAPTER 9 - CAG TRINUCLEOTIDE INSTABILITY
9.1 Hypothesis

The CAG trinucleotide repeat in the HD YAC transgenic mice will be unstable upon transmission through the maternal and paternal germline and will be influenced by CAG size, parental age, and deficiencies in DNA mismatch repair.

9.2 Objective

To assess CAG trinucleotide instability in the YAC transgenic mice with respect to CAG size, parental age, and deficiencies in DNA mismatch repair.

9.3 Introduction

Huntington’s disease (HD) is a dynamic mutation disorder characterized by an unstable CAG trinucleotide repeat that is expanded beyond 35 consecutive repeats in the disease state (HDCRG, 1993). Studies on affected parent-child and normal parent-child pairs show that intergenerational CAG repeat size changes are much more frequent on HD chromosomes (~70%) than on normal chromosomes (<1%) (Kremer et al., 1995). Clearly CAG repeats on HD chromosomes are unstable but factors contributing to this instability remain unclear. CAG size itself is a factor as paternally transmitted large intergenerational expansions (>7 repeats) and CAG size are significantly correlated (Kremer et al., 1995). The potential for larger repeats to form highly stable hairpin structures \textit{in vitro} may account for some aspects of large repeat expansions (Gacy et al., 1995). However, other factors must be involved since intermediate alleles (IA, higher than observed in the general population but premutation, i.e., 30-35 CAG) of similar size on chromosomes derived from the general population versus those from new mutation families display significantly different levels of instability (Goldberg et al., 1995; Chong et al., 1997). Additionally no correlation exists between CAG size and a propensity for smaller expansions or contractions (Kremer et al., 1995).
A growing body of evidence suggests that cis-acting factors play a role in trinucleotide instability in HD and other related disorders. Firstly, haplotype analysis of HD chromosomes using markers in strong linkage disequilibrium with HD reveals that 2 major haplotypes account for ~78% of HD chromosomes (Squitieri et al., 1994). The CAG repeat length on normal chromosomes with these haplotypes is significantly larger than on other normal chromosomes (Squitieri et al., 1994). Additionally, haplotype analysis of new mutation families shows that expansion of the CAG from the intermediate allele range into the HD range occurs on these two haplotypes (Squitieri et al., 1994), and of 5 unstable intermediate alleles observed in the general population, 4 occurred on these same HD haplotypes (Goldberg et al., 1995). Additionally, PCR analysis of single sperm has demonstrated that intermediate alleles of identical sizes have different rates of expansion into the disease range (Chong et al., 1997). Taken together, these data support a model in which a small group of chromosomes with specific haplotypes are prone to expansion. The CAG repeat over multiple generations will gradually approach the intermediate allele range where large intergenerational expansions will cause a jump into the HD range resulting in HD. Similar mechanisms have been proposed for DM (Imbert et al., 1993), fragile X syndrome (Macpherson et al., 1994) and MJD (Takiyama et al., 1995).

A second point supporting the role of cis-acting factors is that a significant association between intergenerational change in CAG size and the level of sperm repeat mosaicism in the offspring has been shown (Telenius et al., 1995). Intergenerational CAG expansion is associated with marked repeat instability in sperm of the offspring suggesting that the factor promoting instability in the previous generation is inherited with the CAG mutation and reflected in the level of mosaicism in the sperm.

Finally, sequence variations that confer instability to repeat sequences have been identified and lie directly within or adjacent to the repeats. Sequence analysis of expanded trinucleotide repeats in fragile X syndrome (Eichler et al., 1994) and SCA1 (Chung et al., 1993) indicate that loss of a trinucleotide interruption within the repeat sequence to produce a contiguous repeat configuration predisposes to expansion. This is also true of HD since sequence changes between the CAG and the adjacent CCG repeat significantly contribute to instability, with a predisposition toward expansion.
(Chong et al., 1997). Additionally, a single G to C transversion 48bp upstream of the MS32 minisatellite repeat unit is associated with a significant reduction in mutation rate at this locus (Monckton et al., 1994).

Trans-acting factors may also play a key role in modulating CAG trinucleotide instability. Of considerable interest are the DNA mismatch repair enzymes since mutations in 2 of these enzymes, PMS1 and MSH2, result in destabilization of CAG trinucleotide repeat tracts in yeast (Schweitzer and Livingston, 1997). Additionally, increased dinucleotide instability was observed in colonic tumors from patients with mutations in the human homologue of the MSH2 gene (Fishel et al., 1993). One mechanism that has been proposed to account for the increased frequency of DNA mismatches in CAG (and other) repeat sequences is DNA polymerase slippage (Kunkel, 1993). During replication of the repeat tract, the primer and template strands temporarily dissociate then reanneal in a misaligned configuration. Deficient mismatch repair would then lead to more frequent expansions or contractions, depending on whether the mismatched bases are found on the primer or template strand.

Thus cis and trans-acting factors may be important components in a pathway conferring CAG trinucleotide instability at the HD locus. In an effort to learn more about these contributing factors, I have assessed CAG repeat length size changes in the YAC transgenic as it is transmitted from parent to offspring. This was done in both the normal FVB/N mouse background and an MSH2 deficient background (Reitmair et al., 1995).

9.4 Results

I assessed CAG size on DNA extracted from parent and offspring tails in a number of YAC transgenic lines (summarized in table 9.1). Differences in CAG size between parent and offspring were scored as an unstable transmission (Figure 9.1). All breedings were done on the FVB/N mouse background, except those that were done on MSH2 heterozygous and null mice (MSH2 mice are
Figure 9.1 Assessment of CAG trinucleotide repeat length.

CAG repeat PCR on maternal parent (P) DNA and 4 offspring (1-4) from YGA2-46 line 668. Offspring 1-3 have no change in CAG size, whereas offspring 4 shows a contraction of a single CAG trinucleotide (arrow).
derived from a C57BL6/129 hybrid background). Unstable transmissions were repeated in duplicate to confirm results.

9.4.1 Rates of Instability and CAG size changes

The results of transmission of the CAG from all YACs are summarized in table 9.2. The overall rates of instability in the FVB/N mouse background were 0% (0/143), 2.3% (5/217), and 8.8% (4/45) for 18, 46/48, and 72 repeats respectively. A comparison of instability between paternal transmissions from 353G6-48 normal (0/33) versus MSH2 null (13/37, 35%) backgrounds revealed a highly significant difference (P<0.0001) suggesting that DNA mismatch repair plays a key role in moderating instability at the HD locus. Contractions were observed more often than expansions (15 contractions vs. 7 expansions) and no expansions greater than 1 repeat unit were seen (table 9.2). In fact, all size changes were by 1 repeat unit with the exception of 1 contraction of 2 units.

9.4.2 CAG size

I compared the effect of CAG size on rate of trinucleotide instability (table 9.3). Comparison of 18 vs. 48 repeats was not significant. However significant differences were seen when comparing 18 vs. 72 (P=0.003) and 48 vs. 72 (P=0.0498) showing that higher CAG lengths (72) are more unstable than normal (18) or moderate (46/48) CAG lengths.

9.4.3 Age of transmitting parent

No significant effect of age was noted in the HD YAC transgenic mice. For 46/48 CAG repeat sizes, results were similar for the number of unstable transmissions less than 24 weeks (3/122)
Table 9.1: Summary of the lines studied for CAG instability

<table>
<thead>
<tr>
<th>YAC</th>
<th>line</th>
<th>Paternal n</th>
<th>Maternal n</th>
</tr>
</thead>
<tbody>
<tr>
<td>353G6-18</td>
<td>B50</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>B51</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B52</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B57</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B60</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>D206</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D209</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>D211</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>D212</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>YGA2-18</td>
<td>15</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>353G6-48</td>
<td>A10</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>A12</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A14</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C772</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>YGA2-46</td>
<td>668</td>
<td>42</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>701</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1747</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1749</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>353G6-72</td>
<td>2494</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2511</td>
<td>16</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 9.2 Summary of CAG trinucleotide instability in HD YAC transgenic mice

<table>
<thead>
<tr>
<th>YAC</th>
<th>Unstable/total transmissions</th>
<th>CAG size change (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paternal</td>
<td>Maternal</td>
</tr>
<tr>
<td>353G6-18</td>
<td>0/44</td>
<td>0/31</td>
</tr>
<tr>
<td>YGA2-18</td>
<td>0/36</td>
<td>0/32</td>
</tr>
<tr>
<td>353G6-48</td>
<td>0/33a</td>
<td>1/29</td>
</tr>
<tr>
<td>YGA2-46</td>
<td>2/92</td>
<td>2/63</td>
</tr>
<tr>
<td>353G6-72</td>
<td>2/36</td>
<td>2/9</td>
</tr>
<tr>
<td>353G6-18 (MSH2 -/-)</td>
<td>0/24</td>
<td>0/22</td>
</tr>
<tr>
<td>353G6-48 (MSH2 -/-)</td>
<td>13/37a</td>
<td>ndb</td>
</tr>
</tbody>
</table>

a Statistically significant by Fisher’s exact test, P<0.0001
b Breedings are presently underway to assess meioses from maternal MSH2 null mice.
Table 9.3: Effect of CAG size on CAG repeat instability

<table>
<thead>
<tr>
<th>CAG size</th>
<th>Paternal</th>
<th>Maternal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>0/80</td>
<td>0/63</td>
<td>0/143(^a,b)</td>
</tr>
<tr>
<td>46/48</td>
<td>2/125</td>
<td>3/92</td>
<td>5/217(^a,c)</td>
</tr>
<tr>
<td>72</td>
<td>2/36</td>
<td>2/9</td>
<td>4/45(^b,c)</td>
</tr>
</tbody>
</table>

\(^a\) Not significant
\(^b\) Statistically significant by Fisher’s exact test, P=0.003
\(^c\) Statistically significant by Fisher’s exact test, P=0.0498

Table 9.4: Effect of age on CAG repeat instability

<table>
<thead>
<tr>
<th>CAG size</th>
<th>&lt;24 weeks</th>
<th>&gt;24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paternal</td>
<td>Maternal</td>
</tr>
<tr>
<td>18</td>
<td>0/42</td>
<td>0/37</td>
</tr>
<tr>
<td>46/48</td>
<td>1/69</td>
<td>2/53</td>
</tr>
<tr>
<td>72</td>
<td>1/24</td>
<td>nd</td>
</tr>
</tbody>
</table>

versus greater than 24 weeks of age (1/95) (Table 9.4, Figure 9.2). This was true for both maternal and paternal transmissions. Additionally, a comparison of the total number of meioses with 72 repeats less than 24 weeks of age (1/24, 4.2%) versus greater than 24 weeks of age (2/9, 22%) was not significant (P=0.17).

9.5 Discussion

9.5.1 Trinucleotide Instability in Transgenic Mice

Initial studies on CAG trinucleotide instability in mice revealed absolute stability of the repeat size upon transmission of parent to offspring. cDNA transgenic mice containing expanded alleles for HD (Goldberg et al., 1996), SCA1 (Burright et al., 1995) and SBMA (Bingham et al., 1995) showed absolute stability of the expanded transgenes in all but 1 of 258 meioses from all
Figure 9.2 CAG size changes in 353G6-72 mice as a function of age.

CAG size observed in offspring from 353G6-72 paternal or maternal transmissions with 72 CAG repeats. The age of the parent at the time of birth of the offspring is represented in weeks. Unstable paternal transmissions (+1, +1) were seen at 23 and 35 weeks. Unstable maternal transmissions (+1, -1) were seen at 36 weeks.
transgenics. The single intergenerational change observed was an expansion by one triplet in an HD cDNA transgenic line (Goldberg et al., 1996). This observation lead to speculation that cis-acting genomic sequences or chromatin structure surrounding the CAG repeat may be important factors in determining repeat instability and may account for the lack of instability observed in the cDNA transgenic mice. Alternatively, species specific differences in DNA replication and/or repair may exist between mice and humans such that mice are not susceptible to instability.

Subsequent studies on transgenic mice containing trinucleotide repeats shed light into factors contributing to instability in mice. Transgenic mice containing a 1.9kb genomic fragment surrounding the CAG repeat at the HD locus showed marked levels of instability (Mangiarini et al., 1997). The fragment, which was derived from an HD patient, contained very high CAG sizes, around 130 repeats, and included 1kb of 5' flanking DNA and 262bp into intron 1. The CAG repeat in these mice tended to increase when transmitted through the paternal germline and contract when transmitted through the maternal germline. Additionally, 1 of the 3 lines studied showed a positive correlation between size of the CAG repeat seen in F1 mice and age of the transmitting founder. Finally, it was noted that no instability was detected in one line that did not express the transgene, suggesting that transcription may be playing a role in mediating the instability in these mice. Most size changes were small (1-4 repeats), the largest being a 10 unit change.

A second study using high repeat sizes (~160 CTG repeats) was conducted in mice containing an ~1kb genomic fragment from the myotonic dystrophy (DM) locus (Monckton et al., 1997). Across multiple lines, unstable meioses were consistently observed 60% of the time from maternal transmissions, consisting primarily of small deletions, up to a maximum of 7 repeat units. The frequency of mutation from paternal meioses was more complex in that it differed amongst lines ranging from 10-70% unstable transmissions, with a bias towards expansion. It was suggested that difference between the lines might be attributable to site of integration and shows that repeat instability is not purely a function of CAG size.
An additional study at the DM locus showed more moderate levels of instability (Gourdon et al., 1997b). A 45kb genomic DNA fragment derived from patient DNA with 55 CTG repeats was used to generate transgenic mice. Instability was observed in 6/7 lines studied, with a change in repeat length observed in 6.8% of all progeny. Most changes were expansions of 1 repeat unit, with 1 change of 6 units observed. No parental bias was observed.

An additional study was conducted on one cDNA transgenic line containing the SCA1 gene with 82 CAG repeats (Kaytor et al., 1997). Little instability was observed from paternal transmissions (3/86). In contrast, 67% of maternal transmissions in this line were observed. All changes in CAG size were contractions, and an increase in magnitude and frequency of unstable transmissions was observed with advanced maternal age. Additionally, it appeared that the instability occurred prior to fertilization since analysis of unfertilized oocytes produced data comparable to that observed in parent to offspring studies.

Finally, CAG trinucleotide instability was assessed in YAC transgenic mice containing the androgen receptor (AR) gene (associated with SBMA) with 45 repeats (La Spada et al., 1998). Moderate levels of instability were noticed in these mice, with an overall rate of instability around 9%. As was the case with the SCA1 cDNA transgenics, the frequency of unstable transmissions increased with advanced maternal age. This was not seen with paternal transmissions. The fact that instability was observed in the SBMA YAC transgenic mice and not in SBMA cDNA transgenics lead the authors to speculate that the surrounding genomic DNA contained the necessary cis-acting elements to confer instability at this locus.

Taking all the above observations into account, some common features of repeat instability in mice begin to emerge. Firstly, contractions rather than expansions appear to occur more frequently in mice and large repeat expansions are rarely seen. Secondly, maternal transmissions, especially as the mouse ages, appear to be more unstable than paternal transmissions. Thirdly, large repeat sizes (>70) appear to be necessary to induce high levels of intergenerational instability, whereas smaller repeat sizes (<70) result in more moderate levels of instability.
9.5.2 Instability in HD YAC transgenic mice

The observations of CAG trinucleotide instability in the HD YAC transgenic mice are consistent with those seen in other mouse models of instability. The similarities and differences are discussed below.

9.5.2.1 Rate of instability and size changes

The overall rates of instability I observed in the HD YAC mice are similar to those seen in DM mice with 55 repeats (DM55) (6.8%) (Gourdon et al., 1997b). The levels of instability lie on either side the DM55 figure, with 2.3% and 8.8% observed for 46/48 and 72 repeats respectively. They do however appear moderate when compared to similar sized repeats (45 CAG) in AR YAC transgenic mice (AR45) (HD 2.3% vs. AR 9.5%) (La Spada et al., 1998). Possible reasons include age of transmitting parent or surrounding genomic sequences, which are discussed in further detail below. The size of CAG changes is also consistent with DM55 and AR45 transgenic mice (La Spada et al., 1998; Gourdon et al., 1997b). The majority of changes in the HD YAC mice were +1/-1 with no evidence of large expansions or contractions. No large CAG/CTG repeat expansions (>7) have been reported in DM55 or AR45 mice. The largest was a single change of +6 units with all others being changes of +1or +2 (Gourdon et al., 1997b). Of note, 2 large paternal contractions (-20 and -18) have been observed in AR YAC transgenic mice which were not seen in the HD YAC transgenics (La Spada et al., 1998).

9.5.2.2 CAG size

The observation that CAG size influences the rate of instability in the HD YAC transgenics is consistent with that observed for trinucleotide repeats in humans (La Spada, 1997) and mice (La Spada
et al., 1998). A CAG size of 72 repeats was more unstable when compared to 18 or 46/48 repeats. Since most of these changes were small (+1 or -1), this may simply reflect an increased propensity of slippage of the DNA polymerase complex during replication. Intuitively, the probability of polymerase slippage during replication would likely increase as the size of CAG repeat increases. This polymerase slippage model has been proposed to account for small expansions and contractions across trinucleotide repeat sequences (Kunkel, 1993).

9.5.2.3 Age of transmitting parent

No significant effect of age was noted in the HD YAC transgenic mice. For 46/48 CAG repeat sizes, results were similar for the number of unstable transmissions less than 24 weeks (3/122) versus greater than 24 weeks of age (1/95) (table 9.3). This was true for both maternal and paternal transmissions. This result is in contrast to AR YAC transgenic mice that showed a significant increase in instability with advanced maternal (but not paternal) age (La Spada et al., 1998). Maternal instability rate in those mice was 3.4% (1/29) prior to 32 weeks of age and 25% (11/44) after 32 weeks of age. Although the comparison between the AR and HD YAC mice is not identical in terms of maternal age cutoff, I have assessed a total of 15 maternal meioses >32 weeks of age (YGA2-46 line 668), none of which were unstable. Clearly, there is a difference between these 2 groups of mice and may be accounted for by differences in mouse background. HD YAC mice are in the FVB/N background whereas AR YAC mice studies were done in a C57BL/6J X SJL/J hybrid background. Alternatively, there may be differences in the cis-acting factors between these 2 genomic loci (see below). Interestingly, when this maternal age related effect of instability in the AR mice is removed, the differences in overall rates of instability for this repeat size range between HD (2.3%) and AR (4.5%) YAC mice are comparable and not significantly different.

A further demonstration of a maternal age related increase in CAG instability was the SCA1 cDNA transgenic study with 82 CAGs (Kaytor et al., 1997). Analysis was carried out on 1 single-copy
transgenic line showing that by 24 weeks of age every observed meiosis was unstable, all of which
were contractions. This is in sharp contrast to the HD YAC-72 mouse line (F2511) that demonstrated
only 2/9 (22%) unstable meioses, all greater than 24 weeks of age. Meaningful comparisons here are a
little difficult for 3 reasons. Firstly, they are different constructs, YAC versus cDNA. Secondly, each
study has assessed maternal transmissions with mid-high sized CAGs (72-82) in only 1 line, leaving
the possibility open that site of integration into the mouse genome is influencing the results in 1 or
both studies. Thirdly, I cannot completely exclude the possibility of a maternal age related issue in the
HD YAC-72 mice since the number of meioses is small (n=9) and no maternal meioses were observed
at less than 6 months of age (table 9.3). Although a comparison of the total number of meioses with 72
repeats less than 6 months of age (1/24, 4.2%) versus greater than 6 months of age (2/9, 22%) is not
significant (P=0.17), a trend toward increased maternal instability with advanced age is apparent.
Assessing further meioses should help clarify this issue.

9.5.2.4 Mismatch repair

I found a significant increase in the level of CAG trinucleotide instability when I compared
paternal meioses on the normal FVB/N mouse background (0/33) versus those on an MSH2 null (-/-)
mouse background (13/37, 35%) (P<0.0001). It is unlikely that this difference is attributable to mouse
background (MSH2 null mice were derived from a C57BL/6J X 129 hybrid background) since no
unstable meioses (0/13) were observed from the heterozygous (+/- at the MSH2 locus) F1 hybrids
(FVB/N X C57BL/6/129). Since MSH2 is involved in DNA mismatch repair (Schweitzer and
Livingston, 1997; Reitmair et al., 1995), it is likely that this difference is due to inefficient repair of
mismatched DNA at the CAG repeat. No instability was noted in 46 meioses from mice on an MSH2
null background with 18 repeats. It is likely that polymerase/strand slippage and mismatch formation
is relatively rare with this number of repeats, hence efficient repair is unnecessary. However, as CAG
size increases (e.g. up to 48 repeats), slippage of the DNA polymerase complex during replication
becomes more common and effective repair machinery is more necessary. These results demonstrate that defective DNA repair mechanisms are significantly associated CAG instability at the HD locus and may provide insight into the mechanisms involved in humans.

9.5.3 Comparison to humans

Consistent with what is observed in humans, CAG size influences the rate of instability in the HD YAC transgenic mice. However, for the most part the results obtained in the YAC transgenic mice are somewhat different to the human data. Changes in CAG size in humans on HD chromosomes are observed in 78% of paternal and 64% of maternal transmissions, with a significantly greater chance of expansions occurring if transmitted through the male germline (Kremer et al., 1995). In the YAC transgenic mice, these numbers are much lower with 2.5% (4/161) of paternal and 5% (5/101) of maternal transmissions showing evidence of instability. Additionally, no large expansions (>7) were seen in the mice whereas this type of change accounts for ~20% of paternal meioses in humans (Kremer et al., 1995). Finally, the tendency in mice is to contract whereas in humans that tendency is toward expansion (Kremer et al., 1995). These data suggest that fundamental differences exist between mouse and humans, perhaps in their DNA replication and/or repair machinery, such that humans are more susceptible to repeat instability. The MSH2 mismatch repair data presented here support this notion since CAG repeat instability was significantly increased in a DNA mismatch repair deficient background. Further experimentation will undoubtedly shed light on other factors that contribute to an increase in instability in mice.

9.5.4 Surrounding genomic sequences

Inclusion of surrounding genomic sequences in the constructs used (YACs, cosmids) does seem to enhance instability in mice when compared to cDNA transgenic mice. However, the changes
observed in the HD YAC transgenic mice (this study), the AR YAC transgenic mice (La Spada et al., 1998), and the DM cosmid transgenic mice (Gourdon et al., 1997a) are minimal compared to humans. In this light, it is interesting to note that the haplotypes of the HD YACs 353G6 and YGA2 are those associated with lower CAG repeat sizes in the general population (Almqvist et al., 1995) and lower intergenerational instability in the IA range (30-35 CAGs) (Chong et al., 1997). This suggests that presence of stability (or lack of instability) conferring factors on these YACs may account for the relative stability in these mice. These results also suggest that the cis acting instability factors lie outside the region used to clone the 72 CAG repeat sequences into the YAC. This region (160bp 5’ and 410bp 3’ of the CAG repeat) was derived from patient DNA. If cis acting instability conferring sequences were present within this region, one might expect higher rates of instability in mice. Perhaps a combination of high CAG sizes in addition to cis-acting instability conferring factors (not present on the YAC transgenes) are required to elevate instability at the HD locus in mice.

9.6 Conclusion

HD YAC transgenic mice exhibit CAG trinucleotide instability, the rate of which is influenced by CAG size and DNA mismatch repair deficiencies but is not influenced by age of the transmitting parent.
9.7 Reference List


CHAPTER 10 - GENERAL DISCUSSION
10.1 Summary

I have generated and characterized a number of YAC transgenic mice expressing normal (18Q) or mutant (46Q and 72Q) human huntingtin (htt) in the FVB/N mouse strain. Human htt is expressed in a spatial and temporal manner identical to that of the endogenous mouse protein. Mice expressing 46Q htt (tg46) show no obvious neurological impairment up to 20 months of age. Behavior in home cages, coordination, and activity were no different to controls, and no obvious pathological abnormalities in the brain were noted at 20 months. Electrophysiological analyses in the hippocampal CA1 region at 10 months revealed a significant reduction in LTP, a form of synaptic plasticity involved in learning and memory. Subsequently, we generated 2 expressing tg72 founder mice, 1 of which has integrated a higher number of YAC copies (4-5X), suggestive of elevated protein expression. This founder (2498) exhibited a neurological phenotype at 6 weeks, characterized by hyperactivity, laterialized circling, and impaired motor control, which were not seen in the lower expressing founder. Analyses of brain tissue showed a dramatic increase in htt nuclear staining in the lateral striatum of mouse 2498 with accumulation of nuclear aggregates and evidence for neurodegeneration. Neurodegeneration was also seen in the low copy number tg72 founder F2511. These results demonstrate that a neurological and morphological phenotype can be achieved in YAC transgenic mice expressing full-length mutant htt consistent with that observed in HD patients. These mice represent an important resource for gaining insight into the causative nature of polyglutamine expansion and disease progression in HD.

10.2 CAG Repeat Mouse Models

The observations made during the course of this study are of critical importance for the field of HD and other trinucleotide repeat disorders. To help generate a feel for how these YAC transgenic mice fit into the CAG mouse model field, I will briefly describe the other models that have been
generated and their underlying phenotypes. I will follow this with a discussion of the general principles that are beginning to emerge in this important field and the implications these have for the future direction of this project.

10.2.1 Mice Lacking a Phenotype

Initial attempts at generating a mouse model for a polyglutamine related disorder met with limited success. Several lines of transgenic mice were generated using the human AR cDNA (the androgen receptor cDNA implicated in SBMA) with 45 CAG repeats under control of the murine neuron specific enolase promoter (NSE) or the inducible murine Mx promoter (Bingham et al., 1995). Mice generated using the NSE promoter produced detectable levels of transcript (determined by RT-PCR) but failed to produce detectable protein on Western blots in all tissues tested. Expression studies in the Mx-AR lines demonstrated inducible levels of protein expression (the mouse Mx promoter is upregulated by administration of interferon) but levels were weak relative to endogenous levels. Mutant NSE-AR and Mx-AR mice were indistinguishable from controls. A detailed morphometric analysis of the phrenic and lumbar ventral root axons at 6 months of age on Mx-AR mice showed no evidence of neurodegeneration. The authors speculated that the absence of phenotype in these animals was a reflection of the low levels of protein expression and/or a reduced susceptibility of the neurons in mice compared to those that are affected in humans.

A second cDNA model was generated using the HD cDNA with 44 CAG repeats under control of the cytomegalovirus (CMV) promoter (Goldberg et al., 1995). A total of 2/6 of the lines showed high levels of the human HD transgene mRNA expression but failed to express protein due to a deletion of a single base pair early in the cDNA sequence. Behaviorally, these mice appeared normal up to 1 year of age and detailed morphometric analysis of the caudate/putamen, globus pallidus, and subthalamic nucleus at 9 months of age showed no changes in neuronal density and no evidence of necrosis or gliosis.
An important message that emerged from these studies was that production of the mutant protein in sufficient quantities appeared essential for the development of an obvious neurological phenotype in mice. Additionally, expansion of the CAG repeat further into the disease-causing range (>70) would perhaps accentuate a phenotype earlier in mice.

10.2.2 Mice That Develop a Neurological Phenotype

One of the first breakthroughs in CAG repeat animal model research came with the development of a mouse model for SCA1 (Burright et al., 1995). Under control of the cerebellar Purkinje cell specific promoter Pcp2, mice expressed the normal (30 CAG) and mutant (82 CAG) protein at levels estimated to range between 10-100 times that of endogenous levels. No mice expressing the normal protein developed a neurological phenotype up to 1 year of age. However, mutant-expressing mice developed adult-onset ataxia and Purkinje cell degeneration reminiscent of human SCA1. The onset of ataxia occurred as early as 12 weeks of age with an apparent dosage effect; lines expressing higher levels of protein developed an earlier onset phenotype. Further studies demonstrated that changes in Purkinje cell structure preceded the onset of ataxia (Clark et al., 1997). At the time of onset of ataxia, there was minimal Purkinje cell loss but obvious changes in cell morphology. Cytoplasmic vacuoles appeared as early as 4 weeks of age and proximal dendrites became more simplified, showing loss of branches and dendritic spines. The changes became more severe and widespread as the animals aged, with cell loss and abnormally-localized Purkinje cells beginning to appear more frequently after the onset of ataxia. These findings led the authors to surmise that cell dysfunction (which precedes cell death) rather than cell loss plays a large factor in the disease observed in SCA1 cDNA transgenic mice.

A second important study in mice came shortly thereafter, which described the production of an MJD/SCA3 model (Ikeda et al., 1996). A series of mice were created using a variety of constructs, again under control of the Pcp2 promoter. The constructs included: (1) a full length MJD cDNA
containing 79 CAG repeats (MJD79), (2) the CAG repeat with only the C-terminal portion of the cDNA with both 79 and 35 repeats (Q79C and Q35C) and (3) a pure 79 CAG repeat tract (Q79). Onset of ataxia and gait disturbance was observed in all Q79C (6/6) and 2/6 Q79 mice as early as 4 weeks of age. In contrast, no such phenotype was observed in MJD79 (n=4) or Q35C (n=10) mice at 23 and 32 weeks of age, respectively. Visual inspection and histological analysis of an 8 week old ataxic Q79C mouse showed a dramatic reduction in cerebellar volume (1/8 that of controls) as well as alterations in Purkinje cell morphology, to the point where they were hardly detectable. An important finding from these studies was that truncated protein products containing polyglutamine tracts (Q79C and Q79) appeared to be more neurotoxic than longer, full-length proteins (MJD79). However, no formal protein expression analysis was done in these lines leaving the possibility that differences in protein expression levels could simply account for the phenotype. However the authors did report that strong ataxic phenotypes were more prominent with higher copy numbers of the Q79C transgene.

Further support for the toxic fragment hypothesis came from studies on transgenic mice expressing the first exon of the HD gene with 115-156 or 18 CAG repeats under control of the first 1kb of HD regulatory gene sequences (Mangiarini et al., 1996). This construct would express the first 69 amino acids of the HD protein in addition to the CAG repeat, which represents ~3% of the total protein. A single founder was produced having integrated the transgene at 5 independent sites. Breeding of this founder produced 5 different lines, 1 of which had lost the CAG repeat, another of which failed to express any detectable protein (R6/0), and 3 of which expressed protein in all tissues tested at levels comparable to endogenous (R6/1, R6/2, R6/5). All expressing lines develop a neurological phenotype with line R6/2 developing symptoms at 2 months of age. The phenotype is complex and includes irregular gait, resting tremor, stereotypic movements, abrupt shuddering movements, and epileptic seizures. Body weight begins to decrease along with onset of other symptoms, and affected mice weigh only 30-40% of their normal weight at the time of death. Breeding of lines R6/1 and R6/5 to homozygosity led to an earlier onset phenotype, again demonstrating that increased levels of protein expression enhance phenotypic progression. Overall brain size was reduced
to 80% of normal across all structures with apparent normal neuronal density. Histological analyses revealed no obvious evidence of cell loss or gliosis in the cerebral cortex, hippocampus, basal ganglia, cerebellum, and spinal chord. Further studies using an N-terminal anti-HD antibody revealed the presence of immunoreactive neuronal intranuclear inclusions (NIIs) with a granular and occasionally fibrillar morphology (Davies et al., 1997). The NIIs first appear prior to onset of a neurological phenotype in the cortex (3.5 weeks) and in the striatum (4.5 weeks). Interestingly, the NIIs are only seen in the projection neurons (the neurons particularly susceptible in HD) and never in glial cells or relatively resistant interneurons. Importantly, no neurological or histological changes were observed in mice expressing the same truncated protein with 18 repeats, showing that the phenotype was not simply due to expression of small protein fragment.

10.3 Emerging Trends in CAG Repeat Mouse Models

Taken together, the data from the CAG repeat mouse models have provided a number of important insights into disease progression in the CAG trinucleotide repeat disorders in mice. Firstly, translation of the mutant protein is necessary to induce a phenotype (Bingham et al., 1995; Goldberg et al., 1995). Additionally, increasing levels of protein expression induces an earlier-onset phenotype in mice. This was accomplished by either increasing copy number in different lines (Burright et al., 1995; Ikeda et al., 1996) or by breeding lines to homozygosity (Mangiarini et al., 1996; Burright et al., 1995). An additional factor in ensuring early onset in mice is to express proteins with long polyglutamine tracts (>70Q). The data here are a little less clear since no mice have been generated that express high levels of mutant protein with moderately expanded repeats (40-50Q). It is likely that a combination of these 2 criteria will stress the system in mice to ensure phenotypic onset. The data on these HD YAC transgenic mice are consistent with this trend. The only mouse that developed an early-onset neurological phenotype and had obvious striatal pathology (F2498) had a high CAG repeat size (72) and elevated protein expression levels. The low 72Q expressing founder (F2511) did not
develop an obvious neurological phenotype up to 1 year of age. Additionally, all low copy number 46Q expressing YAC transgenics failed to develop any phenotypic changes up to 20 months of age. Thus, expressing high levels of mutant protein with high repeat sizes appears to be a critical component of generating a polyglutamine-mediated disease in mice. Breedings of these lines to homozygosity will help boost expression levels (see below).

Secondly, expressing truncated polyglutamine-containing peptides appears to enhance neurotoxicity in vivo (Ikeda et al., 1996; Mangiarini et al., 1996). It is still unclear exactly how these fragments disrupt cell function despite the observation that they tend to aggregate and form intranuclear inclusions (Davies et al., 1997). Additionally, it is not obvious at this point how this information may relate to the human condition. If the HD protein (htt) is cleaved in humans within a subset of neurons liberating a toxic polyQ fragment, what are the mechanisms by which this occurs? Is cleavage of htt and liberation of this fragment causative of neurotoxicity or is it simply a by-product of a process that is causing cell dysfunction? The presence of N-terminal immunoreactive intranuclear microaggregates in 353G6-72 founder F2498 is of interest since it is the only mouse that has developed an early onset disease. However, neurophysiological dysfunction was detected in YGA2-46 YAC mice despite the lack of development of neuronal nuclear aggregates, suggesting that cleavage and subsequent aggregate formation are not necessary for cellular toxicity. Thus the obvious neurological phenotype in founder 2498 is likely independent of aggregate formation and likely due to elevated protein expression levels and an aggressive cellular progression of the disease.

Thirdly, cell dysfunction and obvious neurological onset precede cell death in mice (Clark et al., 1997; Mangiarini et al., 1996). These findings are interesting in that the phenotype observed in HD patients has traditionally been thought to be the result of loss of certain populations of neurons. These data suggest that this may not be the case, and the HD phenotype may be, in part at least, due to cell dysfunction. The data on the YGA2-46 YAC transgenics are consistent with this view. Clear changes in neuron function (impaired synaptic plasticity) occur long before any changes in behavior or cell death. In fact, LTP deficits were observed in the YAC transgenic mice as early as 6 months of age,
whereas no behavioral phenotype or evidence for cell loss was observed up to 20 months of age. Thus these findings have set the stage for thinking of new ways to treat HD. Research can be geared towards understanding and blocking cell dysfunction in addition to attempting to find ways of blocking what is likely to be the final stage of disease progression, cell death.

Finally, an important and often overlooked consideration is that of mouse strain. The C57BL/6 mouse strain is relatively resistant to excitotoxic mediated cell death compared to the FVB/N strain (Schauwecker and Steward, 1997). This observation likely accounts for the lack of neurodegeneration in some CAG repeat models (Mangiarini et al., 1996; Ordway et al., 1997) but not in others (Burright et al., 1995) despite clear neurological and behavioral abnormalities. The HD YAC transgenic mice used in this study are consistent with this observation in that marked neurodegeneration was observed in 353G6-72 mutant mice at 1 year of age, both in the FVB/N mouse background. This observation has great potential for studying modifying factors involved in polyglutamine-mediated neuronal cell death in mice. Breeding 353G6-72 mice onto the C57BL/6 background would help find factors (other genes) involved in reducing neurotoxicity and provide important insight into the mechanisms of cell death.

10.4 Breedings to regulate the phenotype

The development of the YAC transgenic model for Huntington disease creates unique opportunities to investigate several aspects of HD pathology in an in vivo setting by breeding the YAC transgenic animals to mice with defined genetic changes. This will establish whether the phenotype of the YAC transgenic mice can be specifically enhanced or reduced in crosses that test different hypotheses for HD pathogenesis.
10.4.1 Breeding to homozygosity

The studies on CAG mouse models have demonstrated that increased levels of protein expression enhance phenotypic onset. Thus protein levels would be boosted 2-fold by breeding mouse lines to homozygosity. To this end, two YGA2-46 lines (668 and 1747) have been bred to homozygosity and known homozygotes (based on breeding results from line 668) have still not manifested with obvious signs of imbalance, chorea, or other neurological signs up to 19 months of age. Breeding 353G6-72 line 2511 to homozygosity has begun and potential homozygous offspring are now being screened.

10.4.2 Breeding onto the Hdh null background

It is possible that the presence of the normal endogenous mouse protein has a neuroprotective effect and may suppress expression of the mutant human protein. To address this question, I bred the mutant YGA2-46 mutant YAC onto the Hdh null background and rescued the embryonic lethal phenotype. Rescued mice have not shown any phenotypic differences compared to YGA2-46 transgenic mice on a normal Hdh background up to 16 months of age, suggesting that the normal endogenous mouse protein does not play a role in disease progression. However, during breeding to generate the rescued knockout mice, genetic background was altered. HD YAC transgenics are in the FVB/N strain whereas Hdh knockout mice are in the C57BL/6 background, thus direct comparison of YAC transgenics on a normal and null Hdh background is not possible.

10.4.3 Breeding to other transgenic/knock-out lines

Apoptosis, excitotoxicity and production of reactive oxygen species, are each implicated in the pathogenesis of HD. The importance of each of these factors can be evaluated in specific crosses with
the YAC transgenic mice by determining whether the observed phenotype is enhanced or delayed. A good example of such a cross would be the Cu/Zn superoxide dismutase-deficient mice since they are phenotypically normal under physiological conditions and survive well into adulthood (up to 6 months reported) (Reaume et al., 1996). However, these mice display enhanced cell death in response to nervous system injuries such as ischemia (Kondo et al., 1997) and axonal injury (Reaume et al., 1996). If free radical production plays a role in mediating neuronal cell death in HD, exacerbation of neurotoxicity in HD YAC transgenic mice may be expected when crossed onto the Cu/Zn SOD null background. Another example is the Bclw knockout mouse. Bclw is an apoptotic cell death protecting member of the Bcl2 family of proteins which when knocked out in mice produces testicular degeneration and results in male infertility (Ross et al., 1998). However, apart from this phenotype, the mice appear normal and develop as well as controls (up to 15 months of age reported). Since Bcl2 is expressed in brain, these mice would make excellent candidates to assess the role of apoptosis in HD YAC transgenic mice. These examples and other feasible breeding lines would help tease out the nature of cell death in HD.

10.5 Future direction

The HD YAC transgenic mice produced and characterized during the course of this study have laid an important foundation for future studies in HD animal models. The protein expression pattern and phenotypes observed in these mice have many similarities to HD that make them a particularly attractive model. Clearly, many questions need to be addressed and some key experiments should be done in the near future to help draw further knowledge of disease progression in these mice. Here are some suggestions:

1) Generate additional high copy number 353G6-72 YAC transgenic lines to produce lines that develop an early-onset neurological phenotype.
2) Produce YAC clones with larger CAG repeat sizes (e.g. 120-130 CAG repeats) to stress the system in mice. 353G6 is likely the best candidate YAC since it is smaller than YGA2 and easier to manipulate and handle during preparation of the YAC for microinjection. Additionally, the results of this thesis suggest that generating high copy number mice is more feasible if smaller YACs are used. No high copy number mice were generated with YGA2.

3) Assess the molecular basis of the LTP deficiencies in the YGA2-46 mice. One quick way to assess NMDA receptor involvement is to perform whole-cell voltage-clamp experiments on hippocampal slices obtained from mutant mice. Under the whole-cell voltage clamp technique the AMPA and NMDA component of the synaptic response can be isolated. A direct comparison of NMDA receptor function in mutants and controls is then possible.

4) Assess the procedural and configural learning and memory in the mutant YAC transgenic mice. Careful consideration must be taken here since FVB/N mice are visually impaired and these tests have been designed for visually acute rodents.

5) Perform receptor binding autoradiography, immunoblotting for receptor proteins, and *in situ* hybridization assays on the HD YAC mice as has been reported for the HD exon 1 transgenics (Cha et al., 1998). Such assays will detect changes in neuroreceptor levels between mutant and control mice and provide important clues into the neuronal pathways involved in HD.

6) Breed the YAC transgenics with other knock-out/transgenic lines to exacerbate or delay onset of phenotype.

Undoubtedly, these mice will provide an important tool in the quest to find the cause and ultimate cure for HD.
10.6 Reference List


