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Amyloid in Huntington Disease: Identification, Purification and Partial Characterisation of Protein Constituents.

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This thesis is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

February 2002.
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Abstract

Huntington disease (HD) is a progressive, autosomal dominantly inherited, neurodegenerative disease that is characterised by involuntary movements (chorea), cognitive decline and psychiatric manifestations. HD is one of a number of late-onset neurodegenerative diseases caused by expanded glutamine repeats, with a likely similar biochemical basis. It has been suggested that the disease-causing mechanism in Huntington disease (and the other polyglutamine disorders) is the ability of polyglutamine to undergo a conformational change that can lead to the formation of very stable anti-parallel β-sheets; more specifically, amyloid structures. This hypothesis is supported by evidence that polyglutamine forms amyloid-like protein aggregates in vitro, which stain with Congo red (a histological stain for amyloid) and exhibit green birefringence under polarized light. Further support for this hypothesis is provided by evidence of Congo red staining with green birefringence, of purified aggregates from HD brain captured on cellulose acetate filters.

This study describes the first finding of amyloid-like inclusion bodies in situ, in HD brain. Inclusions possessing an amyloid-like structure were identified by Congo red staining and polarized-light microscopy in the cortex and striatum of HD brain, but these were absent in both normal control brains and Alzheimer’s disease brains. Amyloid-like inclusions were of a similar size to the immunohistochemically-detected neuronal inclusions reported by other investigators, however the frequency of the former was far lower than the latter and the relationship between the two types of inclusion is uncertain.

Efforts to purify and characterise the amyloid-forming protein(s) in HD utilised the methodologies used to purify amylin from amyloid plaques in the diabetic pancreas, with a number of refinements. Internal fragments of three different proteins, glial fibrillary acidic protein (GFAP), histone H3 and hypothetical protein FLJ20623 were co-purified with the amyloid component of HD brain based on their increased abundance relative to control brains, and subsequently characterised. The role of these proteins in HD is investigated and discussed. In addition, an ~4285 dalton protein was co-purified with the amyloid component of HD brain; this protein was noticeably
absent in control brain and displayed unusual biochemical properties, however an amino-acid sequence could not be determined. Interestingly, a protein of the same approximate size, and displaying similar properties, was purified from poorly soluble protein aggregates formed in polyglutamine-expressing cultured cells.

SDS-PAGE and Western analysis of sub-cellular fractions of HD and control brain revealed a novel pattern of proteolysis at the N-terminus of huntingtin in HD relative to control, that is supported by recent literature. Myelin basic protein was demonstrated to co-migrate with an N-terminal huntingtin-positive peptide, with apparent molecular weight of ~20 kDa, using tryptic in-gel digestion and protein sequencing. This N-terminal huntingtin-positive peptide and the ~4285 dalton protein described above were both purified from amyloid-containing fractions and both resisted N-terminal protein sequencing. Due to the aberrant migration of polyglutamine-containing proteins in polyacrylamide gels, a possible identity between these peptides is proposed. Laser capture microscopy and tandem mass spectrometry were investigated as potential methods for the purification and characterisation of amyloid-forming proteins in HD and the further development of these techniques may enable the realisation of these aims in the future.

The major contribution of this study has been the finding of amyloid-like inclusions in Huntington disease brain. This finding places HD and by association the other polyglutamine disorders into the category of amyloid diseases, and suggests that strategies to prevent amyloid accumulation, a focus of Alzheimer’s Disease research, may be of much wider application to glutamine repeat disorders such as HD or vice versa.
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First and foremost I would like to thank all of the people who have so kindly donated their brains to the New Zealand Neurological Foundation Brain Bank, and their families. Without this resource the work described in this thesis would simply not have been possible.

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1D-PAGE</td>
<td>1-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>2D-PAGE</td>
<td>2-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>α-CHC</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance units</td>
</tr>
<tr>
<td>APAF</td>
<td>Australian Proteomics Analysis Facility</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CnBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic-AMP response element-binding protein</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DRPLA</td>
<td>dentatorubral pallidoluysian atrophy</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetra-acetic acid</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino-butyric acid</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GHCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>GPl</td>
<td>globus pallidus, internal segment</td>
</tr>
<tr>
<td>GPe</td>
<td>globus pallidus, external segment</td>
</tr>
<tr>
<td>HAP</td>
<td>Huntingtin associated protein</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HD</td>
<td>Huntington disease</td>
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<tr>
<td>HD</td>
<td>Huntington disease gene</td>
</tr>
<tr>
<td>Hdh</td>
<td>mouse homologue of HD gene</td>
</tr>
<tr>
<td>HIP</td>
<td>Huntingtin interacting protein</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>iodoacetic acid</td>
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<tr>
<td>IEF</td>
<td>isoelectric focussing</td>
</tr>
<tr>
<td>iGFAP</td>
<td>internal fragment of GFAP</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>M</td>
<td>moles per litre</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionisation-time-of-flight</td>
</tr>
<tr>
<td>MJD</td>
<td>Machado-Joseph disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>N-CoR</td>
<td>nuclear receptor co-repressor</td>
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<td>NI</td>
<td>neuronal inclusion</td>
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<tr>
<td>NII</td>
<td>neuronal intranuclear inclusion</td>
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<td>N-terminal</td>
<td>amino-terminal</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PB</td>
<td>phosphate buffer</td>
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<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
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<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
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<td>PrP</td>
<td>prion protein</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidenfluoride</td>
</tr>
<tr>
<td>Q</td>
<td>one letter code for glutamine</td>
</tr>
<tr>
<td>Q-TOF MS/MS</td>
<td>quadrupole-time-of-flight tandem mass spectrometry</td>
</tr>
<tr>
<td>SBMA</td>
<td>spinal and bulbar muscular atrophy</td>
</tr>
<tr>
<td>SCA</td>
<td>spinocerebellar ataxia</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>SELDI-TOF</td>
<td>surface-enhanced laser-desorption/ionisation-time-of-flight</td>
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<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS plus Tween-20</td>
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<tr>
<td>TEMED</td>
<td>$N,N,N',N'$-Tetramethylene diamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>tTG</td>
<td>tissue transglutaminase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</table>
Chapter 1

General Introduction

1.1 Overview

1.1.1 Research Overview
The research described in this thesis was designed and performed to investigate the molecular basis of the lesion in Huntington disease (HD), a devastating, progressive neurodegenerative disease. A wide range of biochemical techniques was applied to purify and characterise the protein or proteins responsible for forming amyloid structures in HD. These include immunohistochemical and histological methods, high performance liquid chromatography, SDS-polyacrylamide gel electrophoresis, MALDI-TOF mass spectrometry and laser-capture microdissection. The work described in this thesis was performed on human brain tissue obtained from the New Zealand Neurological Foundation Human Brain Bank, cultured cells and transgenic mouse tissue. There are six separate research projects described in this thesis that comprise chapters 2-7. The present chapter (Chapter 1) gives a review of the HD field and puts the aims of the research into context. This chapter provides a description of the current knowledge of HD biology, and will both explain and justify the research approaches. Finally, a discussion chapter (Chapter 8) amalgamates the results and conclusions from the different facets of the research and discusses the direction of HD research.

1.1.2 General Disease Overview
HD is a progressive, autosomal dominantly inherited neurodegenerative disease affecting about 4-9 in 100,000 in European populations (Harper, 1996). HD is characterized by involuntary movements (chorea), cognitive decline and psychiatric manifestations, progressing inexorably to death by 15-20 years following onset (Harper, 1991). Clinical aspects of the disease are discussed in section 1.2. There are
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widespread morphological changes in the brain, but the area primarily affected by the pathology in HD is the striatum, consisting of the caudate nucleus and putamen (see figure 1.1); there is a marked loss of neurons, particularly the GABAergic medium spiny neurons, and atrophy in this area of the brain (Martin & Gusella, 1986). In addition there are marked neurotransmitter and receptor changes in the globus pallidus and substantia nigra (Nicholson et al., 1995). These morphological changes are the subject of section 1.3. The genetic mutation that causes HD is a CAG repeat expansion in exon one of the gene IT15 located on chromosome 4 (4p16.3) (Huntington's Disease Collaborative Research Group, 1993). Molecular genetic aspects of HD including possible mechanisms of repeat expansion and correlations between repeat length and phenotype, are discussed in section 1.4. The CAG repeat expansion in the HD gene is translated into an expanded polyglutamine stretch in the gene product called ‘huntingtin’. The normal function of the huntingtin protein is yet to be established, however aspects of huntingtin biology including intracellular localisation and protein interactions are the subjects of section 1.5.

HD is one of a number of late-onset neurodegenerative disorders caused by expanded glutamine repeats, with a likely similar biochemical basis. Section 1.6 summarises important similarities and differences between these diseases and HD. A number of other disorders, mostly neurodegenerative, resemble HD in that they are characterised by aggregated protein as plaques or inclusion bodies; these disorders are discussed in section 1.7. Recent research describing protein aggregates in HD is discussed in section 1.8, and various models of HD are examined in section 1.9. Hypotheses relating to the toxicity of polyglutamine are examined in section 1.10. Lastly, some recent findings relating to the function of huntingtin and the aims of the research project are outlined in sections 1.11 and 1.12 respectively.

1.2. Clinical aspects of HD

Huntington disease takes its name from George Huntington, who provided a succinct and accurate although not the first description, of this hereditary chorea (Huntington, 1872). Although the term Huntington’s chorea is still sometimes used to describe the disorder, the term Huntington disease has become more favoured due to clinical features of the disease other than movement disorder. A triad of clinical features have been described associated with HD: dyskinesia (including chorea and other motor
abnormalities), a nonaphasic dementia, and disorders of mood and perception (particularly depression) (Folstein, 1989). The movement disorder or dyskinesia consists of involuntary movements (chorea) and abnormalities of voluntary movement (including clumsiness, bradykinesia, slowing of response time and the inability to sustain a voluntary movement) (Folstein, 1989). The second clinical feature of HD consists of cognitive defects that begin early in the course of the disease and increase in severity with disease progression. These defects include trouble with memory, calculation, fluency, visuospatial abilities, judgement and speed of performance (Folstein, 1989). This cognitive decline represents dementia, however there is no corresponding aphasia or agnosia: features of the dementia observed in Alzheimer’s disease. The third clinical feature of HD, mood and perception disorder, describes “the tendency to insanity, and sometimes... to suicide” that was noted by Huntington (Huntington, 1872). For this reason, patients with HD were until recently cared for in psychiatric hospitals and facilities. High rates of depression and an increased likelihood of schizophrenia relative to the normal population have been described for patients with HD (Dewhurst, 1970; Garron, 1973). In addition to depression, personality changes, withdrawal and disinhibition are also noted (Mendez, 1994). It has been suggested that HD results in organic mental disorders from dysfunction of prefrontal-subcortical circuits coursing through the caudate nuclei (Mendez, 1994). Despite the single cause of this disease, symptoms can vary considerably in severity and even occurrence between patients even from the same family.

Death generally occurs between 12-15 years from the time of symptomatic onset of the disease (Vonsattel & DiFiglia, 1998). HD is usually apparent in the third to fourth decade of life, with a progression in the severity of motor symptoms until the patient becomes rigid (Snell, 1993). A juvenile form of HD (onset before age 20 years) has been recognised for a long time, and clinical and morphological differences have been noted in this type of HD (Goebel et al., 1978). Patients with the juvenile-onset disorder usually have CAG repeat lengths exceeding 70, inherit the disease paternally, and display rigidity as the most common movement abnormality (Vonsattel & DiFiglia, 1998).
1.3 HD Pathology

An overview of normal chemical neuroanatomy will be presented (section 1.3.1) to provide a framework in which the changes occurring in the HD brain (section 1.3.2) can be appreciated. Only the areas of the brain that are primarily affected by HD, the connections between them and the known neurotransmitters they use to communicate will be covered here.

1.3.1 Neuroanatomy of the Basal Ganglia

The basal ganglia consist of five large subcortical nuclei that participate in the control of movement (Cote & Crutcher, 1991). These nuclei – caudate nucleus, putamen, globus pallidus, substantia nigra and subthalamic nucleus – are extensively interconnected. The caudate nucleus and putamen are collectively known as the neostriatum (commonly termed the ‘striatum’), and act as input nuclei for the basal ganglia. The globus pallidus is divided into internal and external segments (denoted GPi and GPe respectively). The subthalamic nucleus lies below the thalamus, and the substantia nigra (also divided into two segments, the pars reticulata and the pars compacta) is located in the midbrain. Figure 1.1 illustrates the positions in the brain of the basal ganglia nuclei.

![Figure 1.1. Coronal section of the brain illustrating the positions of the basal ganglia nuclei. (From: Cote & Crutcher, 1991).](image-url)

The neostriatum is the major input compartment of the basal ganglia receiving input from both the cerebral cortex and the thalamus (McGeorge & Faull, 1989; Nauta &
Domesick, 1979). The major output compartments of the basal ganglia include the internal segment of the globus pallidus (GPi) and the pars reticulata of the substantia nigra, and these project to the thalamus.

![Schematic illustration of the direct and indirect pathways through the basal ganglia.](after Cote & Crutcher, 1991).

There are two pathways (a direct and an indirect) connecting the input and output compartments of the basal ganglia nuclei. The direct pathway arises from inhibitory neostriatal efferent neurons projecting to the GPi. The indirect pathway also arises in the neostriatum projecting first to the external segment of the globus pallidus (GPe), from there to the subthalamic nucleus, and finally to the output nuclei of GPi and substantia nigra pars reticulata (Vonsattel, Ge & Kelley, 1997). Figure 1.2 shows these two pathways schematically, including the neurotransmitters that mediate excitation or inhibition of the various nuclei. The two pathways have opposite effects on the output nuclei: the direct pathway leads to activation of thalamocortical neurons.
(neurons that project from the thalamus to areas of motor cortex), whilst the indirect pathway leads to inhibition of these neurons. A dopaminergic projection from the substantia nigra pars compacta has the effect of exciting the striatal neurons of the direct pathway, and inhibiting the striatal neurons of the indirect pathway, thereby facilitating movement (Albin, Young & Penney, 1989). There is evidence suggesting that there is a selective loss of the striatal neurons that give rise to the indirect pathway, early in the course of HD (Albin et al., 1989; Vonsattel et al., 1997; Glass et al., 2000). This neuronal loss may ultimately lead to a reduction in the inhibitory action of the GPi upon the thalamus, and this disinhibition in turn leads to the choreiform movements typical of HD. Disturbances to this finely balanced circuitry, are the cause of a number of other diseases of the basal ganglia, including HD, Parkinson’s disease (PD), ballism and tardive dyskinesia (Cote & Crutcher, 1991).

### 1.3.2 Neuropathology of HD

Although the mechanism(s) of pathology occurring in HD are not well understood, there are certain anatomical hallmarks of the disease. These hallmarks include patterns of neuronal loss in particular regions of the brain, changes in the abundance of neurotransmitters and their receptors, and cognitive defects. Vonsattel and DiFiglia (1998) provide a thorough description of the neuropathology observed in HD. The pathological hallmark in HD, seen in 95% of HD brains, is atrophy in the striatum. In addition, upon gross examination 80% of HD brains show atrophy in the frontal lobes. In HD striatum, the neuronal loss is also associated with reactive astrogliosis, a proliferation of glial cells called astrocytes. Additionally, early and progressive accumulation of reactive microglia was recently observed in HD brain (Sapp et al., 2001). The neuronal loss is progressive, proceeding along caudo-rostral and dorso-ventral gradients, with the degenerative process beginning in the tail of the caudate nucleus (see figure 1.3; Vonsattel & DiFiglia, 1998). A grading system, with 5 grades (0-4) of severity of striatal degeneration, has been developed by Vonsattel et al. (1985). Figure 1.3 shows the ordered, topographic distribution of striatal degeneration in HD brains and their associated grades.
Within the striatum, there are three known groups of GABAergic medium-sized spiny projection neurons (possessing spiny dendrites) that project to distant connections, and together make up the predominant cell type in the neostriatum (Glass, Dragunow & Faull, 2000). These neurons are particularly vulnerable in HD.

The enkephalin-containing, GABAergic spiny neurons projecting to the GPe are the first affected population of striatal neurons (Albin et al., 1992; Storey & Beal, 1993; Sapp et al., 1995). Also affected but to a lesser degree, are the substance P-containing, GABAergic medium spiny neurons projecting to the substantia nigra (Reiner et al., 1988). Substance P-containing, GABAergic medium spiny neurons projecting to the GPi are relatively spared being affected only in late stages of the disease (Storey & Beal, 1993). In addition, there are numerous types of aspiny (with smooth dendrites) interneurons with local connections. Those containing somatostatin and neuropeptide Y are spared even in late stages of the disease (Augood, McGowan & Emson, 1994). Calretinin-containing, GABAergic interneurons are generally also relatively spared (Cicchetti & Parent, 1996). Lastly, cholinergic and parvalbumin-containing GABAergic interneurons are variably affected, generally being lost by the end-stages of the disease (reviewed by Cicchetti et al., 2000).

It has been proposed that the selective vulnerability of medium spiny neurons can be attributed to impaired energy metabolism and cell death by glutamate excitotoxicity.
(Beal, Hyman & Koroshetz, 1993; Hannan, 1996). This hypothesis is further described in section 1.10.1. A more recent finding that may explain the selective vulnerability of medium spiny neurons comes from a knock-in mouse model of HD that will be described further in section 1.9.1 (Wheeler et al., 2000). In brief, the authors present a scenario in which medium spiny neurons, in contrast to most other types of cells, possess an alternate form of huntingtin with an 'exposed' N-terminus that appears to be redistributed to the nucleus.

Studies of HD biopsy tissue have identified a number of ultrastructural changes in HD brain, but these have been largely ignored until quite recently. These changes include the presence of a nuclear inclusion (Roizin, Stellar & Liu, 1979), nuclear membrane indentations (Roos & Bots, 1983), abnormal mitochondria (Goebel et al., 1978), and an irregular distribution of nuclear pores (Tellez-Nagel, Johnson & Terry, 1974). The recent finding of neuronal intranuclear inclusions in post-mortem HD brain (Becherer et al., 1998; DiFiglia et al., 1997), and a mouse model of the disease (Davies et al., 1997), has renewed interest in these features. These are discussed further in section 1.8.

Loss of neurotransmitter receptors is another pathological hallmark of HD brains that mirrors the observed pattern of cell loss. Glass et al. (2000) investigated the pattern of neurodegeneration in HD, in a comparative study of receptor changes. Low grades of the disease are characterized by a major loss of cannabinoid CB₁, dopamine D₂ and adenosineA₂a receptor binding in the striatum and GPe, and an increase in GABAₐ receptor binding in the GPe. Intermediate grades showed further loss of CB₁ receptors and loss of dopamine D₁ receptors in the striatum, and loss of both in the substantia nigra. Advanced grades show further loss of both CB₁ and D₁ receptors in the striatum, and loss of both associated with increased GABAₐ receptor binding in the GPi. Similar changes, typical of early grade HD are observed in R6/2 mice, an HD transgenic mouse line that is discussed in section 1.9.1.

1.4. Molecular Genetics

1.4.1 The HD Mutation

The genetic defect responsible for HD is an intragenic expansion of a CAG repeat in the gene IT15 on chromosome 4. This gene was mapped to chromosome 4p16.3 and
subsequently discovered by a collaborative effort in 1993 using a reverse genetic approach (Huntington’s Disease Collaborative Research Group, 1993). IT15 is now more commonly referred to as the huntingtin gene or ‘HD’. The huntingtin locus is very large, spanning 180 kilobases (kb) of DNA and consisting of 67 exons. It is expressed as two alternatively spliced forms due to different polyadenylation sites, with different relative abundance in various adult and fetal tissues. The longer 13.7 kb transcript is expressed predominantly in adult and fetal brain, whereas the smaller 10.3 kb transcript is more widely distributed. The transcript length variation does not affect the coding part of the gene as it occurs in the 3’ untranslated region of mRNA (Lin et al., 1993). HD mRNA has been detected in striatum, cortex, hippocampus, cerebellum, olfactory bulb, lung, testis, ovaries, liver and pancreas. The gene encodes a protein (huntingtin) of 3144 amino acids with a molecular mass of 348 kDa (Huntington’s Disease Collaborative Research Group, 1993). Figure 1.4 schematically illustrates the chromosomal location and structure of HD and huntingtin.

The CAG repeat region that is expanded in the disease form, is close to the 5’ end of the gene (beginning at nucleotide 367), and is translated into an expanded polyglutamine stretch starting at residue 18 of huntingtin. The CAG repeat length in normal individuals varies between 10-35 units, and in HD patients ranges from about 36 to 400 (Human Gene Mutation Database, Cardiff). However, alleles with between 36-39 repeats (a very small proportion of alleles in the population) can sometimes variably result in HD. This phenomenon is described as incomplete penetrance, a feature of HD that is further described in section 1.4.3. The dominant pattern of inheritance of the disease, combined with the observation that there is no difference in phenotype between homozygotes or heterozygotes for the mutation (Wexler et al., 1987), has led to the proposal of a true dominant or ‘gain of function’ hypothesis. According to this hypothesis, the altered gene or gene product acquires a toxic function in HD. This toxicity might cause neuronal dysfunction leading to death, however the mechanism involved is not clear. To distinguish between ‘loss of function’ and ‘gain of function’ hypotheses for HD, the murine HD homologue (Hdh) was inactivated by gene targeting. Whilst mice that were heterozygous for this inactivation were phenotypically normal, homozygotes displayed abnormal gastrulation at (embryonic day) E7.5 and rapid degeneration between E7 and E8.5 (Duyao et al., 1995). This result tends to favour a gain of function model for HD, and
suggests that huntingtin is critical early in embryonic development, before the emergence of the nervous system.

Figure 1.4. Chromosomal location and structure of HD and huntingtin. (adapted from: Haque, Borghesani & Isacson, 1997)

1.4.2 Anticipation

Anticipation, the earlier onset of disease symptoms among affected individuals in succeeding generations, is a characteristic of HD and other trinucleotide repeat expansion disorders (Rubinsztein et al., 1994). Other trinucleotide-repeat disorders where the repeat is not within a coding region, such as myotonic dystrophy and the fragile X syndromes, also display the phenomenon of anticipation (Richards & Sutherland, 1994; Warren, 1996). Following the discovery of the repeat expansion in HD, this feature was explained: the repeat is unstable in transmission with a tendency to further expansion (Snell, MacMillan & Cheadle, 1993; Jones, Wood & Harper, 1997). Section 1.4.3 further describes this instability. Furthermore, there is an inverse correlation between repeat size and age of onset. The largest repeats, which are mostly paternally transmitted usually result in juvenile onset cases of the disease (age of onset < 20 years of age). The correlation is strongest at higher repeat length (and low age of onset) suggesting that CAG repeat length is a major determinant of age of onset in juvenile cases.

The weaker correlation in older onset cases implies that factors in addition to the CAG repeat length might be important in determining the age of onset of symptoms
(Nance, 1996). Supporting this, polymorphisms adjacent to the CAG repeat have been shown to be associated with variations in clinical features at onset of HD (Vuillaume et al., 1998). Despite the correlation of triplet repeat expansion and age of onset, at least one study suggests that there is no correlation between repeat expansion and the nature of symptoms at onset or mode of progression of HD (Claes et al., 1995).

1.4.3 Meiotic Instability and Incomplete Penetrance

A general model for the evolution of new mutations for triplet diseases is now becoming apparent (Almqvist et al., 1995). In fragile X syndrome, myotonic dystrophy and HD, the evolution of mutations is thought to occur on ancestral chromosomes with large repeat lengths. Linkage disequilibrium studies suggest that these alleles underwent gradual increases in size and eventual conversion to premutations or intermediate alleles (discussed below), which are capable of large expansions to the full mutation (Rubinsztein et al., 1994). There are two other trinucleotide polymorphisms in the HD gene that are associated with this process (Rubinsztein et al., 1995a; Rubinsztein et al., 1995b).

New mutations can be explained by meiotic instability. Whereas less than 1% of normal alleles (10-35 repeats) show CAG repeat expansion or contraction, most parent-child transmissions of an HD allele (≥36 repeats) manifest meiotic instability in the form of repeat expansion or contraction (De Rooij et al., 1995). In all studies there is also a sex-of-parent effect; repeat expansion is more than twice as common in cases of paternal transmission than maternal transmission, and very large CAG repeat expansions occur almost exclusively among paternal transmissions (Goldberg et al., 1993; McNeil et al., 1997). This explains the observation that juvenile onset HD patients most often acquire the HD gene from an affected father (Nance, 1996). A single-sperm analysis of CAG repeat tract length within the HD gene revealed a very high mutation frequency for disease alleles (92-99%) relative to that for normal alleles (<1%) (Leeflang et al., 1995). There was observed a marked elevation in both the size and frequency of repeat expansions with increased repeat number.

In addition to such gonadal mosaicism of the HD gene CAG repeat length, somatic mosaicism has also been reported (Telenius et al., 1994). CAG repeat expansion was analysed in different tissues from affected individuals. All tissues displayed some degree of repeat mosaicism with the greatest levels being detected in the brain and
sperm. Furthermore, somatic mosaicism of expanded CAG repeats has also been reported in the brains of patients with dentatorubral pallidoluysian atrophy (DRPLA, another trinucleotide repeat expansion disorder that will be described further in section 1.5) (Takano et al., 1996). Thus, both meiotic and mitotic instability of expanded repeats have been demonstrated. However, the pattern of somatic mosaicism does not account for the specific pattern of neurodegeneration observed in either HD or DRPLA.

1.2% to 3% of HD cases represent a new mutation, arising as a result of expansion of the huntingtin CAG repeat tract in a normal allele into the disease range (Goldberg et al., 1993). Often these expansions occur from 'intermediate alleles'. The term intermediate allele refers to an allele that does not cause HD in an individual carrying it, but is meiotically unstable enough to cause HD in the next generation (McNeil et al., 1997). The range of intermediate alleles is thought to be between 30-35 repeats (Goldberg et al., 1995). Parents, especially fathers, with a repeat length in this range have a relatively higher probability of passing on an allele in the disease range to offspring.

A number of individuals with HD have been shown to possess 36 repeats, confirming that this repeat size is associated with HD. However, several individuals with repeat lengths of 36 to 39 with no apparent symptoms of HD at advanced ages have been identified (Rubinsztein et al., 1996). Most remarkable was the identification of a 95-year old man with 39 repeats who showed none of the features of HD. The observation that individuals with HD alleles of 36-39 repeats may survive unaffected past common life expectancy suggests that the HD mutation is not always fully penetrant. Therefore, it has been suggested that alleles with repeats in this size range (36-39) should be referred to as belonging to a range of reduced or incomplete penetrance (McNeil et al., 1997; Nance, 1996).

Replication slippage, where slippage of the elongated strand during polymerization can result in the addition (or deletion) of a few repeats, is the most likely model explaining repeat expansion (Richards & Sutherland, 1994). Trinucleotide repeat expansions are just one type of repeat expansion with repeats of varying lengths being involved in a range of different disorders, including certain types of cancer (mono-, di-, and tri-nucleotide repeat expansions) (Richards & Sutherland, 1994). The cis-acting elements discussed above (i.e. the two other polymorphisms in the HD gene) might
also be involved in the mutation mechanism on the basis of their linkage to a disease haplotype.

1.5 Huntingtin

In this section the localisation, putative function and binding partners for huntingtin are discussed. Throughout this thesis ‘mutant’ huntingtin refers to huntingtin protein containing a polyglutamine tract that is expanded into the disease range (ie. 36 or greater). Normal huntingtin refers to huntingtin with 35 or fewer glutamines in this tract. Despite a large research effort since the discovery of the HD gene, the native function(s) of normal huntingtin, and the acquired function of mutant huntingtin, remain unknown. Understanding both of these could be critical for the effective targeting of therapeutic strategies in the future.

1.5.1 Localisation and putative function

*HD* messenger RNA (mRNA) is widely expressed throughout the brain with no apparent difference in expression level in the vulnerable neurons in HD (Strong *et al.*, 1993). In the brain, the level of expression of mRNA is greater in neurons than glia for all areas, and mRNA is also expressed in lung, liver, pancreas, ovaries and testis as already discussed. The expression pattern of *HD* does not therefore account for the regional specificity of neuropathology in HD.

The native function of huntingtin remains unknown, although the near-ubiquitous expression of this protein suggests a possible housekeeping role. Normal huntingtin is located in the cytoplasm and appears to be expressed in neuronal perikarya, nerve fibres, and nerve endings (DiFiglia *et al.*, 1995; Gutekunst *et al.*, 1995; Sharp *et al.*, 1995). DiFiglia *et al.* (1995) found huntingtin associated with both microtubules and vesicles, suggesting a possible role in vesicle trafficking. This hypothesis will be further investigated below. Like the distribution of mRNA, mutant protein was not limited to vulnerable neurons. This suggests that the presence of mutated huntingtin alone is not sufficient to cause cell death. Gourfinkel-An *et al.* (1997) report a different intraneuronal distribution of normal and mutated forms of huntingtin in the brains of HD patients. Normal and mutant huntingtin were both observed in some neuronal perikarya and proximal nerve processes. Mutant huntingtin, however, was absent from nerve endings where the normal form of huntingtin was observed even in
HD patients. This observation has led to the suggestion of a specific alteration in the transport of mutant huntingtin (Gourfinkel-An et al., 1997).

A large number of studies describe the presence of non-mutated huntingtin in neuronal cytoplasm only (DiFiglia et al., 1995; Gutekunst et al., 1995; Persichetti et al., 1996; Sharp et al., 1995; Trottier et al., 1995a). In contrast, two studies describe the presence of normal huntingtin in the nuclei of cells from a range of tissue types (Hoogeveen et al., 1993) and mammalian cell lines (De Rooij et al., 1996). This aberration in apparent distribution in immunohistochemical studies is likely to be due to differences in the antibodies used and/or preparation of brain tissue material. Support for a nuclear role for normal huntingtin is provided by the presence of a polyglutamine stretch and a leucine-zipper motif in huntingtin, features that are commonly found in DNA-binding proteins and transcriptional activators. However, the polyglutamine repeat length in huntingtin from other species is much shorter, suggesting that such a role for huntingtin is not evolutionarily conserved.

Both N-terminal fragments of mutant huntingtin and full-length mutant huntingtin have been detected within neuronal nuclei as aggregates (De Rooij et al., 1996; Gutekunst et al., 1999; Hoogeveen et al., 1993). It is not known whether huntingtin is cleaved within the nucleus or in the cytoplasm and then transported to the nucleus. Regardless, the presence of aggregates of huntingtin and other proteins within or surrounding the nucleus is now regarded as a pathological hallmark of HD. However, the role of these inclusion bodies in the neuropathology of HD remains unknown. These aggregates, termed neuronal inclusions are described further in section 1.8.

Vesicle trafficking has been proposed as one putative function for huntingtin (Block-Galarza et al., 1997). In this study, forceps were applied to the sciatic nerve 1 mm distance apart. The crush sites were marked with a suture and the wounds were closed with staples. At intervals between 6-48 hours following the crush, animals were sacrificed, sciatic nerves were removed and huntingtin accumulation at the crush sites was investigated by SDS-PAGE and Western analysis. The results showed a rapid enrichment of full-length huntingtin and an N-terminal fragment, on both proximal and distal sides of the crush site relative to unligated nerve. Anterograde accumulation (shown by enrichment on the proximal side of the crush) is consistent with axonal transport on vesicular membranes. Retrograde movement (seen by accumulation on the distal side of the crush) may be necessary for degradation in the cell body or may suggest a function in retrograde membrane trafficking. More evidence for such a role
for huntingtin is provided by the finding of an actin-binding vesicular protein related to huntingtin-interacting protein (Hip1) (Engqvist-Goldstein et al., 1999), and the co-fractionation of huntingtin with synaptophysin (Wood et al., 1996).

The role of huntingtin in hematopoietic development was recently investigated on the basis of the embryonic lethality of HD−/− mutants (Duyao et al., 1995; Nasir et al., 1995), and expression of huntingtin in both spleen and thymus. Metzler et al. (2000) demonstrated that huntingtin is required for normal hematopoietic progenitor cell development using an embryonic stem (ES) cell in vitro differentiation model. There was a reduction in the number of hematopoietic progenitors at various stages of differentiation in their model, in both HD+/− and HD−/− cell lines relative to a wild type cell line. Cell expansion in the presence of hematopoietic cytokines was also impaired in HD+/− and HD−/− cell lines relative to the wild type. The defect was found to be gene dosage dependent with homozygous gene-targetted embryos more severely affected than heterozygous embryos. It is possible that the defect in hematopoiesis in the absence of huntingtin may be a consequence of impaired signal transduction, transcriptional regulation, or iron homeostasis (Metzler et al., 2000). This last effect of huntingtin absence is also suggested by very recent research that describes changes in numerous cellular organelles associated with a lack of huntingtin (Hilditch-Maguire et al., 2000; Trettel et al., 2000). This research, is discussed in section 1.11.

### 1.5.2 Protein Interactions

Table 1.1 summarises the huntingtin interactors discussed in this section, and their characteristics. The application of the yeast two-hybrid system has led to the identification of a number of proteins (mostly of unknown function) that interact with huntingtin. Using this system, Li et al. (1995) isolated rat huntingtin-associated protein (rHAPl) from a rat brain cDNA library on the basis of its interaction with a cDNA encoding the first 230 amino acids of huntingtin containing a polyglutamine tract of 44 residues. The transcript was shown to be brain-specific.

The human homologue (hHAPl) was subsequently isolated from caudate nucleus by RT-PCR. hHAPl shares 96% of its amino acids with rHAPl, and both proteins interacted more strongly with huntingtin as the polyglutamine length was increased in length, consistent with a gain-of-function hypothesis. Co-immunoprecipitation...
experiments confirmed the interaction of full length HAP1 with huntingtin in vivo, however a function for HAP1 has not been determined.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Method</th>
<th>Expression</th>
<th>Interaction is.</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP1</td>
<td>Yeast two-hybrid</td>
<td>Brain</td>
<td>Repeat length dependent</td>
<td>Possible protein trafficking – associated with cytoskeleton and synaptic vesicles. Interacts with Duo (see text).</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Affinity chromatography</td>
<td>Ubiquitous</td>
<td>Not modulated by repeat length</td>
<td>Essential enzyme in glycolysis</td>
</tr>
<tr>
<td>HIP1</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Stronger at shorter repeat lengths</td>
<td>Possible cytoskeletal function – by homology to Sla2p (actin-binding vesicular protein)</td>
</tr>
<tr>
<td>N-CoR</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Repeat length dependent</td>
<td>Transcriptional repression</td>
</tr>
<tr>
<td>E2-25K</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Not modulated by repeat length</td>
<td>Ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>Family of WW domain proteins</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Repeat length dependent, mediated by prolines</td>
<td>Non-receptor signalling, pre-mRNA splicing, proteosome</td>
</tr>
<tr>
<td>Cystathionine β-synthase</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Outside of polyQ region</td>
<td>Key enzyme in generation of cysteine</td>
</tr>
<tr>
<td>SH3GL3</td>
<td>Yeast two-hybrid</td>
<td>Ubiquitous</td>
<td>Repeat length dependent, mediated by prolines</td>
<td>Unknown, expressed in brain and testis</td>
</tr>
</tbody>
</table>

Also using the yeast two-hybrid system, a novel protein called Duo (because of its homology with the protein Trio) was found to interact specifically with HAP1. Duo is expressed predominantly in the brain, has characteristics of membrane cytoskeletal proteins, spectrin-like repeat units, a GEF (guanine exchange factor) domain and adjacent PH (pleckstrin homology) domain, suggesting that it is likely to activate the GTP-binding protein rac. These results support the hypothesis that huntingtin is involved in vesicle trafficking and cytoskeletal functions, and suggest that it may act by regulating a ras-related signalling pathway.

Shortly after the discovery of the HAP1-huntingtin interaction, it was hypothesised that the polyglutamine domain in huntingtin (and other proteins containing expanded
polyglutamine stretches) may mediate protein-protein interactions. This would support a possible common mechanism for the pathophysiology of expanded polyglutamine repeat neurodegenerative disorders. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was purified from normal brain homogenate and microsequenced after being retained by an immobilised 60-glutamine synthetic peptide (Burke et al., 1996). Affinity chromatography was then used to show an interaction between immobilised GAPDH and both huntingtin and atrophin (the protein affected in dentatorubral-pallidoluysian atrophy (DRPLA) – see section 1.6). GAPDH has an essential enzymatic role in glycolysis; impairment of its function due to affinity with expanded polyglutamine domains would be consistent with the excitotoxicity hypothesis of neuronal death (section 1.10.2). However, the ubiquitous nature of GAPDH and the near-ubiquitous expression pattern of huntingtin, leave unresolved the tissue-specific neurodegeneration in HD brain.

Two different groups independently identified a further huntingtin-interacting protein (HIP1 – huntingtin-interacting protein 1) by yeast two-hybrid screening using an amino-terminal fragment of huntingtin as bait (Kalchman et al., 1997; Wanker et al., 1997). HIP1 is the human homologue of a yeast protein (Sla2p) with known roles relating to membrane and cytoskeletal functions. Northern analysis reveals the presence of the approximately 9 kb transcript in all tissues examined, and it appears that this transcript is enriched in the brain. The interaction between HIP1 and huntingtin is stronger at shorter repeat lengths and if critical, it may be that loss of this interaction in HD patients could lead to neuronal dysfunction.

The C-terminal region of the nuclear receptor co-repressor (N-CoR) was shown by yeast two-hybrid experiments to interact with the N-terminus of huntingtin (Boutell et al., 1999). This interaction was shown to be repeat length-dependent and specific to huntingtin. The N-CoR has an important role in transcriptional repression of ligand-activated receptors and other nuclear receptors. The expansion of the polyglutamine tract in HD causes a redistribution of N-CoR and other repressor proteins, limiting them to the cytoplasm in cortex and caudate nucleus. The alteration of transcription and transcriptional repression that is likely to result from this redistribution may therefore be ultimately involved in the pathology of HD (discussed in section 1.10.1). Other proteins that have been shown to interact with huntingtin include a ubiquitin-conjugating enzyme termed E2-25K (Kalchman et al., 1996), a family of WW domain proteins (Faber et al., 1998), cystathionine β-synthase (Boutell et al., 1998), and
SH3GL3 (Sittler et al., 1998). E2-25K was found by yeast two-hybrid screening to interact with huntingtin in a manner that was not modulated by repeat length. However, the finding that inclusions in HD brain and transgenic mice show ubiquitin immunoreactivity indicates a possible defect in the degradation of huntingtin and this may result from the interaction between huntingtin and E2-25K.

The WW domain proteins that were found to interact with huntingtin by the yeast two-hybrid system included a novel protein, a transcription factor and human FBP-11, a protein implicated in spliceosome function. The WW domain that was common to all three represents an ancient protein motif involved in non-receptor signalling, channel functioning, protein processing and pre-mRNA splicing. WW domains are small protein modules found in various proteins that participate in cell signalling or regulation (Lu et al., 1999). The interaction of these modules with signalling proteins is usually via short, proline-rich motifs (Sudol & Hunter, 2000). Similarly, the interaction between huntingtin and WW domain proteins is mediated by huntingtin’s proline-rich region, and this interaction is enhanced by longer polyglutamine tracts (Passani et al., 2000).

Cystathionine β-synthase, which was also identified by yeast two-hybrid screening to interact with huntingtin, is a key enzyme in the generation of cysteine from methionine. Absence of this enzyme is associated with homocystinuria which is known to affect various physiological systems including the central nervous system. Metabolites of homocysteine (which accumulates in this disorder), include excitotoxic amino acids which may be involved in HD. Therefore, the interaction of huntingtin with this enzyme could provide a mechanism for excitotoxic damage (discussed in section 1.10.2), however the site of interaction is outside the polyglutamine tract.

Finally, SH3GL3 represents a further huntingtin interactor. The C-terminal SH3 domain in this protein binds to the proline-rich domain immediately downstream of the polyglutamine tract in huntingtin. The interaction selectively occurs with huntingtin moieties containing a glutamine repeat length in the pathological range. Furthermore, the interaction appears to promote the formation of polyglutamine-containing protein aggregates and both proteins co-immunoprecipitated from transfected cells, indicating that SH3GL3 could be involved in the formation of neuronal inclusions in HD.
Table 1.1 illustrates that most of the known huntingtin interactors are expressed more or less ubiquitously throughout the body. Even HAP1, which is limited to the brain, is expressed widely within the brain. Therefore, no interactor of huntingtin yet discovered can explain the tissue specific pattern of neurodegeneration observed in HD. Lastly, it has been demonstrated that huntingtin is a substrate for caspase-3, a proapoptotic enzyme, and the extent of protein cleavage is modulated by polyglutamine repeat length (Goldberg et al., 1996; Wellington et al., 1998). Caspases are activated in cultured cells expressing expanded polyglutamine-containing proteins (Wang et al., 1999). Cleavage products of these enzymes, containing expanded polyglutamine peptides, may be involved in neuronal dysfunction and formation of inclusions. Apoptosis has been demonstrated in HD and HD models (Portera-Cailliau et al., 1995; Rosen, 1996; Saudou et al., 1998), however it is not yet clear whether this process is a cause or effect of the neurodegeneration in HD. It has been suggested that inhibition of caspases may slow disease progression (Ona et al., 1999; Chen et al., 2000).

1.6 Trinucleotide Repeat Expansion Disorders

Huntington disease is just one of a number of neurological disorders caused by a CAG repeat expansion in the coding region of the associated gene (Masino & Pastore, 2001). Other disorders in the same family include dentatorubral-pallidoluysian atrophy (DRPLA; Takano et al., 1996), spinocerebellar ataxia 1 (SCA1; Orr et al., 1993), SCA2 (Trottier et al., 1995b), SCA3 (also known as Machado-Joseph disease (MJD); Kawaguchi et al., 1994), SCA7 (Gouw et al., 1995) and SCA17 (Nakamura et al., 2001). In each of these disorders, autosomal dominant inheritance and anticipation are observed, a gain of toxic property is implicated for the relevant protein, and nuclear inclusions are observed in affected cell types (Chai et al., 1999; Holmberg et al., 1998; Klement et al., 1998). In addition to these disorders, Kennedy’s disease (also known as spinal and bulbar muscular atrophy (SBMA)) and SCA6 are also caused by CAG repeat expansions in coding regions. Kennedy’s disease is an X-linked recessive disorder that is thought (like HD) to be due to a gain of toxic function of the androgen receptor (La Spada et al., 1991). SCA6 is an autosomal dominant disorder with a stable CAG repeat expansion within a calcium channel protein that most likely causes channel misfunction (Zhuchenko et al., 1997).
Table 1.2 summarises the characteristics of each of these diseases, which are reviewed in Orr (2001) and Robitaille et al. (1997).

### Table 1.2. CAG/polyglutamine repeat diseases and their characteristics. (Updated from Lunkes et al., 1998). HD, Huntington disease; DRPLA, dentatorubral-pallidoluysian atrophy; SCA, spinocerebellar ataxia; SBMA, spinal and bulbar muscular atrophy. TBP, TATA-binding protein.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Sites of Neuropathology</th>
<th>Normal Repeat Length</th>
<th>Disease Repeat Length</th>
<th>Causative Protein</th>
<th>Normal Protein Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>Striatum (medium-spiny neurons), cortex in late-stage.</td>
<td>6-35</td>
<td>36-121</td>
<td>Huntingtin</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>DRPLA</td>
<td>Globus pallidus, dentatorubral and subthalamic nuclei</td>
<td>7-35</td>
<td>49-88</td>
<td>Atrophin</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>SCA1</td>
<td>Cerebellar cortex (Purkinje cells), dentate nucleus and brainstem</td>
<td>6-39</td>
<td>40-81</td>
<td>Ataxin-1</td>
<td>Nuclear, Cytoplasmic</td>
</tr>
<tr>
<td>SCA2</td>
<td>Cerebellum, pontine nuclei, substantia nigra</td>
<td>15-29</td>
<td>35-64</td>
<td>Ataxin-2</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>SCA3/MJD</td>
<td>Substantia nigra, globus pallidus, pontine nucleus, cerebellum</td>
<td>13-42</td>
<td>61-84</td>
<td>Ataxin-3</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>SCA6</td>
<td>Cerebellar and mild brainstem atrophy</td>
<td>4-18</td>
<td>21-30</td>
<td>Ca^{2+} channel subunit</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>SCA7</td>
<td>Photoreceptors, bipolar cells, cerebellar cortex, brainstem</td>
<td>7-17</td>
<td>37-120</td>
<td>Ataxin-7</td>
<td>Nuclear</td>
</tr>
<tr>
<td>SCA17</td>
<td>Striatum, thalamus, frontal and temporal cortex, Purkinje cells</td>
<td>29-42</td>
<td>47-55</td>
<td>TBP</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Kennedy’s Disease/ SBMA</td>
<td>Motor neurons and dorsal root ganglia</td>
<td>11-34</td>
<td>40-62</td>
<td>Androgen receptor</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>

Remarkably, all nine of these disorders share numerous similarities despite no homology (outside of the CAG tract) between their causative proteins. For example, disease occurs only when the CAG length expands over a certain threshold, which is similar in all. Furthermore, each disorder has a unique pattern of neuronal degeneration despite the wide expression of the causative proteins. It is possible that
polyglutamine expansion results in a similar pathogenic process in each disorder, with the different diseases' unique characteristics being due to the protein context in which the polyglutamine is expressed.

As in HD, many proteins have been found that interact with the causative proteins of these disorders. However, the cellular distribution of these interactors also does not correlate with the pathological profiles of these diseases. RNA-binding activity was recently observed for ataxin-1, which decreased with increasing polyglutamine length (Yue et al., 2001). This result suggests that ataxin-1 might play a role in RNA metabolism that is altered by the expansion of its polyglutamine tract.

In addition to the well-characterised expanded polyglutamine diseases listed in table 1.2, it is thought that expansion of polyglutamine tracts could play a role in other disorders. For example, a polyglutamine expansion in a novel acidic protein has been associated with childhood onset schizophrenia (Morinieres et al., 1999). Polyglutamine-containing proteins have also been detected in protein extracts from lymphoblastoid cell lines of schizophrenic patients (Joober et al., 1999). These proteins were absent in cell lines from normal controls. CAG repeat expansion has also been linked to autosomal dominant pure spastic paraplegia (ADPSP) (Nielsen et al., 1997). It is likely that the list of polyglutamine-associated disorders will get longer in the future, and knowledge of these disorders may lead to further insight into the mechanism(s) of pathology of long tracts of polyglutamine.

1.7 Other Related Disorders

In addition to diseases that are genetically related to HD by virtue of a CAG repeat expansion and polyglutamine-containing proteins, there are a number of other diseases that could fall into a larger related family by virtue of the aggregative nature of a causative protein (Tran & Miller, 1999). These diseases include Alzheimer's disease (AD), the prion diseases (including Creutzfeld-Jacob disease), Parkinson's disease (PD) and type II diabetes mellitus (Esiri & Morris, 1997; Goldsbury et al., 1997; Walker & LeVine, 2000). Most of these are neurological disorders with the exception of diabetes, which affects the islet \( \beta \)-cells of the endocrine pancreas, thought to be of neuronal lineage. In addition, most of these diseases (with the exception of PD) are associated with a polymeric proteinaceous structure known as
Amyloid. A wide range of proteins has now been shown to have the capacity to form amyloid structures under certain conditions (Chiti et al., 1999; Lansbury, 1999). These disorders are of particular relevance to the research described in this thesis which is aimed at the purification and characterisation of aggregated protein in HD. It should be noted that amyloid is extremely resistant to proteolysis and is also difficult to detect with antibodies due to its insolubility. Hence, methods that have been developed for investigating the role of amyloid in these other diseases may be useful for similar studies on HD.

1.7.1 Alzheimer’s Disease
Alzheimer’s disease (AD) is a very common (affecting 5% of the population over 65 years of age and as much as 20% of the population of 80 years of age), progressive neurodegenerative disease that severely affects memory and personality in those afflicted (Mortimer, Schuman & French, 1981; Roher et al., 1986). It is the most frequent cause of dementia in the United States and the fourth most common cause of death (Glenner & Wong, 1984). Apart from a number of hereditary forms representing a minority of sufferers, the ultimate cause of AD remains unknown. Modifying genes have been identified that affect the age of onset of AD such as the E4 allele of apolipoprotein E. Despite this however, many aspects of AD biology have been thoroughly researched and this research can provide clues into the pathological mechanisms of other neurodegenerative diseases such as HD. Selkoe (2001) provides a recent review of the various genes and proteins involved in AD.

The potential relevance of AD research to HD, besides the fact that both are late-onset, progressive neurodegenerative diseases, lies in the amyloid deposits that are found in AD post-mortem brain (Glenner & Wong, 1984; Morris, 1997). The two main neuropathological features of AD are intracellular neurofibrillary tangles consisting mostly of the protein tau, and extracellular amyloid plaques consisting mostly of the protein β-amloid. Although β-amloid protein is present in both of these features, within plaques this protein stains with Congo red and displays amyloid characteristics such as birefringence under polarized light and fibrillar morphology (Glenner, 1980). β-amloid protein is 39-43 amino acids in length and is derived from the much larger amyloid precursor protein (APP). APP is a trans-membrane protein of unknown function, and the β-amloid protein is partly located in the trans-membrane
region of this protein (Morris, 1997). Some of the hereditary forms of AD may be due to an aberration in the proteolytic cleavage of APP. The methods that were used to purify and characterise β-amyloid from AD brain are similar to those used in the research for this thesis to purify and characterise aggregated protein from HD brain (discussed in Chapter 3) (Glenner & Wong, 1984). In addition, tissue sections from AD brains were used in the research described in Chapter 2 as a positive control for amyloid staining.

1.7.2 Prion Diseases

Prion diseases such as Creutzfeld-Jacob disease in humans and numerous spongiform encephalopathies in animals are also associated with the aggregation and deposition of an amyloidogenic protein (reviewed by Hsiao, 1997; Prusiner et al., 1998). Collectively known as transmissible spongiform encephalopathies (TSE), these diseases were novel in that the infectious agent was demonstrated to be small proteinaceous particles (Prusiner, 1982). In these disorders, the prion protein PrP undergoes a change in conformation to a pathological state that induces a similar change in other PrP moieties and deposition into plaques. Mutations in the PrP protein are associated with familial cases of CJD, however there are forms of CJD (iatrogenic, new variant) that are not familial and appear to be contracted through a currently unknown form of transmission. The gene encoding PrP is located on chromosome 20, however other loci may also be involved in familial cases (Hsiao, 1997). The normal function for PrP is unknown, and the mechanism whereby deposition into amyloid plaques leads to massive neurodegeneration remains a mystery. There is however a very large research effort dedicated to dissecting these mechanisms (DeB Burman et al., 1997; DePace et al., 1998; Glover & Lindquist, 1998; Supattapone et al., 1999; Jackson et al., 1999). Much of this work is focussed on the mechanism(s) of prion protein folding. It is conceivable that developments in our understanding of protein aggregation in HD could offer insights into the aggregative mechanism in prion disease. Conversely, new discoveries in prion research offer HD researchers a paradigm whereby the conformational change of a normally functioning protein to an extremely 'sticky', aggregating protein, possibly via a toxic intermediate, can be investigated.
1.7.3 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease characterised by tremor, bradykinesia, rigidity and postural instability (Engelender et al., 1999). PD is also associated with protein aggregation in the form of Lewy bodies, a cardinal feature of the disease (reviewed by Esiri & McShane, 1997). Although in this case the aggregates are not of an amyloid nature (as defined by Congo red staining and birefringence under polarized light), the presence of Lewy bodies (highly insoluble, dense protein aggregates) within the cytoplasm of affected neurons is reminiscent of the inclusions observed in HD (discussed in section 1.8). Lewy bodies are usually spherical, intracytoplasmic structures 15-30 μm across (reviewed by Esiri & McShane, 1997). Single or multiple Lewy bodies may be found in substantia nigra neurons in PD. A number of familial cases of PD have been linked to mutations in the protein α-synuclein (Polymeropoulos et al., 1997). Immunoelectron microscopy of extracted Lewy body filaments later demonstrated α-synuclein to be the major filamentous component of Lewy bodies and Lewy neurites (Spillantini et al., 1998).

The physiological role of α-synuclein is unknown, however it has been proposed that the aggregation of this protein into Lewy bodies might be a result of misfolding into an alternative, aggregative conformation (Mezey et al., 1998). In addition to α-synuclein, immunostaining has revealed a host of other antigens in Lewy bodies including phosphorylated neurofilament, ubiquitin, tubulin, APP and others (Galloway, Mulvihill & Perry, 1992; Yamada, McGreer & McGreer, 1992). In addition to the potential role of α-synuclein in PD, this protein has been shown to aggregate in the senile plaques of AD, the glial cytoplasmic inclusions of multiple system atrophy, and within the glial cells of patients of amyotrophic lateral sclerosis (Mezey et al., 1998). More recently, α-synuclein immunoreactivity was observed in huntingtin- and polyglutamine-positive aggregates in striatum and cortex of HD patients and transgenic mouse models (Charles et al., 2000). Interestingly, despite the absence of amyloid staining in situ in PD brain, α-synuclein was shown to form amyloid fibrils in vitro (Conway, Harper & Lansbury, 2000; Serpell et al., 2000). The discovery of this protein has established PD as a disease with abnormal protein aggregation at or close to the root of its pathogenesis (Golbe, 1999), putting PD in the same category as AD, prion disorders and HD.
1.7.4 Type II Diabetes Mellitus

Type II diabetes mellitus is a disease affecting the \( \beta \)-islet cells of the pancreas, which secrete the peptide hormones insulin and amylin (Cooper, 1994). These hormones are involved in glucose metabolism, and the hormone deficiency in diabetes can lead to hyperglycaemia if not managed. Pathologically, amylin may contribute to insulin-resistance, and it is likely to play a significant role in the initiation and/or progression of type II diabetes mellitus (Cooper, 1994; Verchere et al., 1996). The deposition of amyloid deposits containing amylin in pancreatic islets is somewhat analogous to the deposition of \( \beta \)-amyloid in AD, and as such represents another paradigm for HD researchers to investigate the mechanisms of protein aggregation. Advances in the characterisation of the fibrillar assembly of human amylin, and how these fibrils grow, may be of some relevance to the other fibrillar amyloid disorders (Goldsbury et al., 1997; Goldsbury et al., 1999).

The original purification method used in the research described herein was taken from Cooper et al. (1987), developed to enable the purification of islet amyloid from diabetic pancreas. This method was similar to the original method used for the purification of \( \beta \)-amyloid protein from amyloid plaques in AD brain (Glenner & Wong, 1984). Finally, a further parallel between HD and diabetes is drawn from the observation that R6/2 transgenic mice (an HD model discussed in section 1.9.1) develop diabetes at 9 weeks of age, preceeding death at approximately 13 weeks of age (Hurlbert et al., 1999). Diabetes has been reported as being more common in HD (with up to a 4-fold increase in incidence) and other trinucleotide repeat disorders (Farrer, 1985). The exact mechanism of diabetes in HD is unknown, however huntingtin is expressed in the pancreas.

1.8 Neuronal Intranuclear Inclusions

In 1997, neuronal intranuclear inclusions (NIIs) were first described in a mouse model (R6/2) for HD (Davies et al., 1997). Since this time, a number of reports have described the presence of NIIs in post-mortem HD brain (DiFiglia et al., 1997; Becher et al., 1998), as well as cell culture (Li et al., 1999) and other animal models of these diseases (Warrick et al., 1998; Wheeler et al., 2000). These reports describe perinuclear inclusions and inclusions located within dystrophic neurites, in addition to the intranuclear inclusions described above. In addition, research on several other
polyglutamine repeat disorders including SCA1, SCA3, SCA7 and DRPLA describe intranuclear inclusions in diseased brains, cell culture and transgenic animal models of those diseases (Holmberg et al., 1998; Igarashi et al., 1998; Skinner, Koshby & Cummings, 1997). Neuronal intranuclear inclusions have now become the neuropathological hallmark of the polyglutamine disorders, however their role in the disease progression is still debated (Yamada, Tsuji & Takahashi 2000). Immunohistochemical studies using antibodies raised to the N-terminal region of huntingtin (adjacent to the repeat) and ubiquitin have located the highest density of inclusions in the cerebral cortex, caudate, putamen and thalamus of HD patient’s brains (DiFiglia et al., 1997; Becher et al., 1998; Gutekunst et al., 1999). The inclusions were seen within both dystrophic neurites (processes) and densely stained neurons. Furthermore, Becher et al. (1998) describe a correlation between the frequency of NIIs and the associated polyglutamine repeat length in the neocortex but not in the striatum. These inclusion bodies contain proteolytic fragments of huntingtin incorporating the polyglutamine tract. Neuropil aggregates have recently been observed associated with synaptic vesicles (Gutekunst et al., 1999). There has been a developing debate over the functional significance of inclusions, with some evidence suggesting that they may be secondary to the disease process and may arise due to a proteolytic protective mechanism (Klement et al., 1998; Saudou et al., 1998). This issue has yet to be resolved and further research is required to ascertain the role that inclusions play in HD pathogenesis. However, it is apparent that the distribution of inclusions, or huntingtin aggregates, does not correlate with the selective pattern of striatal neuron loss (Kuemmerle et al., 1999). A recent study demonstrated the presence of a host of proteins within intracellular aggregates purified from cells expressing exon 1 of huntingtin containing an expanded polyglutamine tract (Suhr et al., 2001). These included: ubiquitin, the cell cycle-regulating proteins p53 and mdm-2, HSP70 (a proteasomal component), TBP (a universal transcriptional regulator that also contains a polyglutamine tract), the cytoskeletal proteins actin and 68-kDa neurofilament, and proteins of the nuclear pore complex. The results of this study indicate that polyglutamine aggregates are highly complex structures containing many sequestered proteins.
1.9 Huntington Disease Models

1.9.1 Mouse Models

There are now a large number of well-characterised transgenic and knock-in mouse models for HD. The nature of the transgene or knock-in, and the effects on the mice expressing them, are summarised in Table 1.3.

This section will discuss some recent findings in mouse models potentially relevant to the disease process in HD. The models of Hayden, Ross and Tagle are not described in any more detail than is provided by the table, as these are essentially similar in phenotype to the R6/2 model which has been extensively characterised. The other models listed in Table 1.3 are discussed in the text. The R6/2 model in which neuronal intranuclear inclusions were first identified has been referred to previously. These mice express exon-1 of the human HD gene with a polyglutamine stretch of 144-190 glutamine residues, under the control of the HD promoter (Mangiaroni et al., 1996). It is thought likely that this line originated as a three-copy integrant (Mangiaroni et al., 1996). Onset of neurological symptoms including resting tremor, involuntary movements, stereotypic grooming and handling-induced seizures, is at approximately 2 months (Mangiaroni et al., 1996; Bates et al., 1997), however subtle deficits are apparent at 5-6 weeks (Carter et al., 1999) as are changes in gene expression (Luthi-Carter et al., 2000).

A recent report describes a late-onset pattern of neurodegeneration in these mice, within anterior cingulate cortex, dorsal striatum and Purkinje cells of the cerebellum (Turmaine et al., 2000). The neurodegeneration was neither apoptotic nor necrotic and mimicked the appearance of degenerated cells in the same regions of brains of patients who died with HD. Gene expression analysis using DNA arrays was used to profile approximately 6000 striatal mRNAs in the R6/2 mouse (Luthi-Carter et al., 2000). This study demonstrates that mutant huntingtin directly or indirectly reduces the expression of a distinct set of genes involved in signalling pathways known to be critical to striatal neuron function. In particular, this study suggests a possible role for impaired retinoid signalling in HD, as at least 22 of the 104 genes that showed decreased expression in the R6/2 mouse are regulated by retinoic acid signalling.
Table 1.3. Mouse models of HD. Prom. = promoter, 72Q= 72 glutamine residues or CAG repeats, Ptype = phenotype, Cx. = cortex, N171 = N-terminal 171 amino acids, htt = huntingtin, CMV = cytomegalovirus, kb = kilobase, TetO = operator element (controls expression by CMV), Hdh = mouse huntingtin homologue, hom = homozygote, het = heterozygote. This is a conditional model of HD, i.e. transgene expression can be switched on and off. The inactivation model of Dragatsis et al. (2000) discussed in the text is not shown here as it is neither a transgenic or knock-in model.

<table>
<thead>
<tr>
<th>Lab</th>
<th>Promoter /Size/ CAG#</th>
<th>Protein level</th>
<th>Ptype Onset (mo.)</th>
<th>Symptoms</th>
<th>NII pathology</th>
<th>Cell loss</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transgenic HD models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bates (R6 lines)</td>
<td>HD prom., exon 1, 144-190Q</td>
<td>Higher than endogenous</td>
<td>2 (R6/2) 5 (R6/1) &gt;12 (R6/5)</td>
<td>Tremors, abnormal gait, learning deficit, diabetic at 12 weeks</td>
<td>NII and neuropil aggregates in most areas of the brain</td>
<td>Late stage; Frontal Cx., dorsal striatum, Purkinje cells</td>
</tr>
<tr>
<td>Hayden</td>
<td>HD prom., Full-length, 18Q, 46Q, 72Q, (YAC)</td>
<td>Double endogenous</td>
<td>3</td>
<td>Hyperactive, circling</td>
<td>Inclusions in striatum</td>
<td>Limited to the striatum</td>
</tr>
<tr>
<td>Ross</td>
<td>PrP prom., N171, 18Q, 44Q, 82Q</td>
<td>1/5-1/10-fold endogenous</td>
<td>5</td>
<td>Tremors, abnormal gait, weight loss</td>
<td>Inclusions in most areas of the brain</td>
<td>Lateral striatum</td>
</tr>
<tr>
<td>Tagle</td>
<td>CMV prom., Full-length, 16Q, 48Q, 89Q</td>
<td>5-fold endogenous</td>
<td>4</td>
<td>Hyperactive, circling, end-stage urinary incontinence</td>
<td>Rare inclusions throughout the brain</td>
<td>20% in striatum of some</td>
</tr>
<tr>
<td>CMV prom., exons 1-3, 89Q</td>
<td>1 to 2-fold endogenous</td>
<td></td>
<td>4</td>
<td>Prolonged hyperactivity</td>
<td>As above</td>
<td>Not reported</td>
</tr>
<tr>
<td>Hen/Yamamoto*</td>
<td>CMV prom. controlled by TetO, exon 1, 94Q</td>
<td>Slightly higher than endogenous</td>
<td>2.5</td>
<td>Late-onset tremor and abnormal gait</td>
<td>Inclusions in striatum, Cx., hippocampus + astrocytes</td>
<td>Not reported</td>
</tr>
<tr>
<td><strong>Targeted Hdh knock-in models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lin/Detloff</td>
<td>Hdh prom., 80Q, 150Q</td>
<td>endogenous</td>
<td>6 (hom) 14 (het)</td>
<td>Motor and gait abnormalities plus reactive gliosis</td>
<td>Predominantly striatal, also in the nucleus accumbens</td>
<td>None reported</td>
</tr>
<tr>
<td>Shelbourne</td>
<td>Hdh prom., 72-80Q; describe 'mutation instability'</td>
<td>endogenous</td>
<td>3</td>
<td>Aggressive behaviour (especially in males)</td>
<td>None; No neuro-pathology after 18 months</td>
<td>None; but decreased brain weight</td>
</tr>
<tr>
<td>MacDonald/Wheeler</td>
<td>Hdh prom., 50Q, 92Q, 111Q</td>
<td>endogenous</td>
<td>None</td>
<td>None, subtle reduction in nuclear volume</td>
<td>Nuclear re-localisation of htt in striatum at 1.5 mo., NII after 10 mo.</td>
<td>None observed</td>
</tr>
<tr>
<td>neo and Hdh prom., 20Q, 111Q</td>
<td>Less than half endogenous</td>
<td>2</td>
<td>Similar to Bates model</td>
<td>Nuclear re-localisation of htt and inclusions</td>
<td>As above</td>
<td></td>
</tr>
</tbody>
</table>

28
Additional studies have shown reduced neurotoxicity and delayed mortality in R6/2 mice where caspases are inhibited (Andreasson et al., 2000; Chen et al., 2000). Minocycline, was found to inhibit caspase-1 and caspase-3 mRNA upregulation leading to a delay in disease progression and a decrease in inducible nitric oxide synthetase activity. Delayed onset has also been demonstrated for R6/1 mice kept in an enriched environment (cages containing cardboard, plastic and paper objects that were changed every 2 days from the age of 4 weeks; van Dellen et al., 2000). These mice are related to the R6/2 mouse line, but express a single copy of exon-1 of HD, containing 116 repeat units (Mangiarini et al., 1996).

The embryonic lethality of huntingtin knock-out mice has already be mentioned (Nasir et al., 1995). More recently, the mouse Hdh (HD homologue) gene was selectively inactivated in the forebrain and testis, resulting in a progressive degenerative phenotype and sterility (Dragatsis, Levine & Zeitlin, 2000). On the basis of this result, it was proposed that huntingtin is required for neuronal function and survival and that a loss-of-function may contribute to the mechanism of HD pathology.

A number of knock-in models for HD have been described. The insertion of extra CAG repeats into the murine homologue of the HD gene resulted in mice that express full-length huntingtin appropriately, and display behavioural and pathological features reminiscent of early HD (Shelbourne et al., 1999; Wheeler et al., 2000; Lin et al., 2001). The model of Shelbourne et al. (1999) shows dramatic repeat length instability in mouse striatum, suggesting the possibility that polyglutamine load may contribute to the tissue-specificity of neuronal cell death in HD (Kennedy & Shelbourne, 2000). The model of Lin et al. (2001) shows increased glial fibrillary acidic protein immunoreactivity in the striatum, suggesting that these mice have a similar neuronal injury to that found in the course of HD. In another model, reported by Wheeler et al. (2000), a novel form of full-length huntingtin with an accessible N-terminus is described that is localised to the nuclei of medium-spiny striatal neurons. It was suggested that long glutamine tracts may change huntingtin's physical properties, producing HD-like correlates in the striatum such as the nuclear localisation of this novel form of huntingtin and the subsequent formation of N-terminal inclusions and insoluble aggregate. The result of this might be the prevention of the interaction of huntingtin with other critical cellular constituents or alternatively the loss of an essential functional form of huntingtin in medium-spiny neurons. This research
suggests that a loss-of-function hypothesis for HD should not be ruled out (Cattaneo et al., 2001).
Lastly, a conditional HD-gene targeted mouse model demonstrated that turning off expression of the mutant HD gene at an age when neuropathological and behavioural phenotypes are displayed, results in either a halt or reversion of the different phenotypes (Yamamoto, Lucas & Hen, 2000). This model suggests that a continuous influx of the mutant protein is required to maintain inclusions and symptoms, raising the possibility that HD may be reversible.

1.9.2 Cell culture and other models
In addition to the mouse models described above, several cell lines expressing mutant huntingtin have been established.
A recent study of a clonal mouse striatal cell line, described huntingtin-enriched cytoplasmic vacuoles that internalised the lysosomal enzyme cathepsin D in proportion to the polyglutamine length in huntingtin transgenes (Kegel et al., 2000). These vacuoles had little or no ubiquitin, proteasome or HSP70 immunoreactivity, but instead displayed the ultrastructural features of early and late autolysosomes. Golgi and nuclear membranes, and mitochondria were also rearranged. It was speculated that the accumulation of mutant huntingtin in HD activates the endosomal-lysosomal system, which contributes to huntingtin proteolysis and to an autophagic process of cell death.
Investigations into the function(s) and sub-cellular localisation of huntingtin have been performed using cultured cells, as well as human HD and transgenic mouse tissue as discussed in previous sections (Gutkeunst et al., 1995; Hackam et al., 1999).
The results of these studies support a cytoskeletal function for huntingtin, with the nucleus and cytoplasm as sub-cellular sites of pathogenesis. PC12 cells expressing exon-1 of huntingtin containing either 20 or 150 glutamines in the repeat region, have been used to study cellular defects and altered gene expression in HD (Li et al., 1999). This study showed that cells expressing N-terminal huntingtin with a polyglutamine tract of 150Q display abnormal morphology including clumping, irregular shapes, and fewer and shorter processes. In addition, a lack of normal neurite development, a tendency to apoptosis, and an alteration of gene transcription were demonstrated.
Neuronal intranuclear inclusions have been reported in human HD brain (DiFiglia et al., 1997; Becher et al., 1998), transgenic mouse brain (Davies et al., 1997), and cell culture models of HD (Scherzinger et al., 1997; Cooper et al., 1998; Hazeki et al., 1999), however the role of these is still debated. One study (Saudou et al., 1998) suggests that mutant huntingtin induces neurodegeneration by an apoptotic mechanism. Blocking nuclear localisation of mutant huntingtin suppressed the formation of intranuclear inclusions and cell death, however these two effects did not correlate with each other; blocking inclusion formation only led to an increase in neurodegeneration, whilst blocking of apoptosis led to an increase in the number of inclusions. These results led to the suggestion that inclusions may reflect a cellular protective mechanism (Saudou et al., 1998). Other cell culture studies also demonstrate that nuclear targeting of mutant huntingtin increases cellular toxicity, by the addition of either a nuclear export signal (NES), or nuclear localisation signal (NLS) to N-terminal huntingtin peptides (Peters et al., 1999).

Lastly, with respect to cell culture models of HD, caspase activation by the expression of expanded polyglutamine has been demonstrated in cell culture (Miyashita et al., 1999; Wang et al., 1999). Similar to the studies of caspase inhibition in mice discussed in the previous section, caspase inhibition in a cell culture model led to apparent dissociation of inclusions and increased neuronal survival (Kim et al., 1999). In addition to the mouse and cell culture models for polyglutamine diseases that have been described, a small number of invertebrate models demonstrate that the cellular mechanisms of human polyglutamine disease are conserved in invertebrates. Expression in Drosophila of a fragment of the human MJD1 gene (defective in SCA3/MJD), containing an expanded polyglutamine tract led to intranuclear inclusion formation and late-onset cell degeneration (Warrick et al., 1998). Neurons were demonstrated to be particularly vulnerable. A more recent Drosophila model from a different group demonstrated that long polyglutamine peptides alone (ie. removed from a disease gene context), are intrinsically cytotoxic and cause neurodegeneration (Marsh et al., 2000). Nuclear inclusions were not reported in this model. Lastly, C. elegans were engineered to express within sensory neurons a fragment of huntingtin (amino acids 1-171) containing 2, 23, 95 and approximately 150 glutamines in the polyglutamine tract (Faber et al., 1999). These worms demonstrated progressive degeneration of sensory neurons and formation of cytoplasmic protein aggregates.
within affected neurons. Other cell culture or animal models of HD will be or have been described in other sections where they are relevant.

1.10 Polyglutamine Toxicity Hypotheses

There are several different hypotheses to describe the mechanism by which huntingtin containing an expanded polyglutamine tract (and other proteins with expanded polyglutamine tracts) might lead to specific neuronal cell death. These hypotheses are by no means mutually exclusive and a combination of different mechanisms is likely. Some of these hypotheses are briefly outlined below.

1.10.1 Transcriptional Interference

Recent research describes the inhibition of CREB-dependent (cAMP-responsive element-binding protein) transcriptional activation by expanded polyglutamine tracts (Shimohata, Onodera & Tsuji, 2000). Additionally, both huntingtin and ataxin-1 have now been demonstrated to interfere with CREB-binding protein (CBP)-mediated transcription, leading to cellular toxicity (Steffan et al., 2000; Nucifora et al., 2001). Nucifora et al. (2001) showed that CBP was depleted from its normal nuclear location and present in polyglutamine aggregates in HD cell culture models, HD transgenic mice and human HD post-mortem brain. Furthermore, over-expression of CBP rescued polyglutamine-induced neuronal toxicity. CBP is an important transcriptional coactivator that coordinates the nuclear responses to a host of cell signalling cascades, particularly to neurotrophic factors. Sequestration of CBP in polyglutamine diseases could therefore lead to a diminished response to neurotrophic factors which are essential for neuronal survival (Beal & Hantraye, 2001).

Steffan et al. (2000) demonstrated an interaction between an N-terminal region of huntingtin with p53 (an apoptotic inducer and transcriptional regulator), CBP, and mSin3a (a transcriptional corepressor). These authors suggest that huntingtin containing an expanded repeat might cause aberrant transcriptional regulation, which may result in neuronal dysfunction and cell death in HD.

The interaction of huntingtin with the transcription factor N-CoR (Boutell et al., 1999), and the finding within polyglutamine aggregates of TATA-binding protein (TBP) (Huang et al., 1998), also support a role for the inhibition of transcription as
one of the underlying pathogenic mechanisms in HD. Finally, the very recent discovery of an interaction between huntingtin and the Gln-Ala repeat-containing transcriptional activator CA150 may provide further evidence for this hypothesis (Holbert et al., 2001). CA150 is a WW domain protein that interacts with full-length huntingtin, has increased expression in HD and displays aggregate formation.

1.10.2 Excitotoxicity

A potential mechanism for the neuronal dysfunction and cell death in HD has been proposed whereby subtle alterations in glutamine, and subsequently glutamate levels, could induce chronic excitotoxicity and slow cell death in neurons possessing specific glutamate receptors (Hannan, 1996). It was proposed that translation and/or degradation of the mutant protein (ie. containing an expanded polyglutamine tract) could cause subtle alterations in the uptake and/or release of glutamine. This amino acid is converted to glutamate in a reaction catalysed by the enzyme glutaminase, and glutamate can be neurotoxic when released into the extracellular space under hypoxic-ischaemic conditions, even at low concentrations (Choi, Malucci-Gedde & Kriegstein, 1987). Furthermore, inhibition of glutamate uptake and a subsequent increase in extracellular glutamate concentration has been shown to induce slow neurotoxicity in cultured neurons (Rothstein et al., 1993); quinolinic acid (a glutamate analogue) has been shown to induce selective striatal neuron cell death in a pattern similar to HD (Beal et al., 1993); glutamate NMDA receptors are decreased in HD striatum (Young, Greenamyre & Hollingsworth, 1988); and glutamate levels are increased relative to controls in HD cerebral spinal fluid (Perry & Hansen, 1990). It is possible that regional differences in susceptibility to glutamate-induced excitotoxicity might account for the specific pattern of neurodegeneration in HD.

This hypothesis supports the gain-of-function mechanism for neuropathology, which is suggested by the dominant pattern of inheritance. Additionally, this hypothesis is consistent with the inverse correlation between repeat length and age of onset described in section 1.4. How glutamate causes cell death is unclear, however it is thought that the generation of free radicals such as superoxides, and a transmembrane influx of calcium may be involved leading to dysfunction of mitochondria and energy metabolism (Hannan, 1996; Wellington et al., 1997).
1.10.3 Amyloid Hypothesis

A theory has been proposed that the disease mechanism in HD (and the other polyglutamine disorders) is the conformational change of polyglutamine (Perutz, 1996). Such a change could lead to self-association between hydrogen-bonded hairpins which form very stable anti-parallel beta-sheet structures. The 'amyloid hypothesis' suggests that these structures could be of an amyloid nature, similar to the plaques and inclusions observed in Alzheimer's and prion diseases and diabetes mellitus. A thorough description of the physical and chemical properties of amyloid is given in Chapter 2. Amyloid formation by polyglutamine is supported by data from peptide association (Perutz et al., 1994), cell culture expression studies (Scherzinger et al., 1997), and post-mortem HD brain (Huang et al., 1998). These studies reveal that polyglutamine and polyglutamine-containing molecules can indeed form the predicted structures. These studies will also be discussed in Chapter 2.

1.10.4 Tissue Transglutaminase Hypothesis

A number of studies have investigated the role of the calcium-dependent enzyme tissue transglutaminase (tTG), in HD and other polyglutamine disorders. Polyglutamine was found to confer excellent tTG substrate properties on any soluble peptide, resulting in the formation of insoluble aggregates containing glutamyl-lysine cross-links (Kahlem et al., 1996). The formation of covalent cross-links between polyglutamine peptides and polyamines was shown to yield high molecular weight aggregates more favourably with longer polyglutamine stretches (Gentile et al., 1998). Additionally in this study, endogenous GAPDH (a known huntingtin interactor – see section 1.5) was shown to form cross-links with a 60-glutamine polypeptide, in a tTG-catalysed reaction in cultured fibroblast cells.

Further evidence for a possible role for tTG in HD, is provided by the finding of increased levels of this enzyme in HD brain (Lesort et al., 1999). The levels of tTG expression and activity were both shown to be increased in HD brains in a grade-dependent manner, with higher levels of expression and activity found in higher grades. The increase in activity was further shown to be brain-specific, with decreased activity in lymphoblastoid cells from HD patients (Karpuj et al., 1999). These authors demonstrate the aggregation of huntingtin into non-amyloidogenic polymers, catalysed by tTG.
Lastly, and more recently, evidence suggests that tTG is not involved in the formation of mutant huntingtin aggregates (Chun et al., 2001). This was demonstrated by stable transfection of human neuroblastoma cells with truncated N-terminal huntingtin constructs containing either 18 or 82 glutamines in the repeat region. Within cells expressing expanded (82 glutamines) polyglutamine, SDS-resistant aggregates formed within the cytoplasm and nucleus as previously described. tTG did not co-precipitate with the truncated huntingtin molecules, nor could tTG be detected immunohistochemically within the aggregates – tTG immunoreactivity was completely excluded from these. Significantly increasing tTG expression had no effect on the number or location of aggregates that formed, and aggregate formation was observed in cells that were stably transfected with a tTG antisense construct, expressing no detectable tTG. This report suggests that tTG is unlikely to play a role in the formation of mutant huntingtin aggregates in HD.

1.11 Towards an understanding of huntingtin function: normal and abnormal

Recent evidence indicates an essential role for huntingtin in RNA biogenesis, vesicle trafficking and iron homeostasis. By surveying 19 classes of organelle in embryonic stem cells (wild-type and knock-out), Hilditch-Maguire et al. (2000) showed dramatic changes in 6 classes including both nuclear organelles (nucleoli, transcription speckles) and perinuclear membrane organelles (mitochondria, endoplasmic reticulum (ER), Golgi and recycling endosomes) where huntingtin was absent. Additionally, an essential function for huntingtin in the proper regulation of the iron pathway was shown by its requirement for the regulation of the transferrin receptor (an iron transport protein) and its upmodulation in response to iron need. Moreover, an excess of huntingtin (as demonstrated by a transient over-expression cell culture model) produced similar abnormalities to the huntingtin-deficiency phenotype (as demonstrated by a knock-out cell culture model), indicating the potential importance of huntingtin level to its normal function.

The same group have shown alternate versions (distinguished by epitope availability) of full-length normal huntingtin in distinct subsets of nuclear and cytoplasmic organelles, distinguished on the basis of epitope availability (Trettel et al., 2000) in striatal cells. This finding supports the previous report of distinct immunoreactive
versions of full-length huntingtin in medium-spiny neurons of the knock-in mouse model striatum (Wheeler et al., 2000). More specifically, an amino-terminal accessible form of the protein (but with inaccessible internal epitopes) was prominent in the nucleus and in cytoplasmic dots. This form of huntingtin co-localized with markers for pre-mRNA splicing speckles and nuclear matrix, supporting a proposed role for huntingtin in mRNA and rRNA biogenesis (Faber et al., 1998; Passani et al., 2000). In contrast, an internal accessible form (with inaccessible amino-terminal epitopes) was found in the nucleolus, in the cytoplasm surrounding the nucleus and in dispersed cytoplasmic dots. This form of huntingtin colocalized with markers for nucleolus, rough ER and Golgi membrane, implicating this version of the protein in normal perinuclear membrane function, consistent with the protein's vesicle interactors (Kalchman et al., 1997; Wanker et al., 1997).

Although mutant protein also exhibited these forms, abnormal forms of full-length mutant huntingtin with aberrant polyglutamine accessibility were mislocalized in both nucleus and cytoplasm. This observation implies compartment-specific structural properties, modifications or interactors. The abnormal phenotypes specific to mutant striatal cells, which are dominant over the wild-type, include elevated levels of p53, enlarged ER and heightened activity of the iron pathway, all pointing towards potentially harmful metabolic stress.

In summary, the latest research into the normal function(s) of huntingtin suggest that it is an iron-response protein implicated in cellular homeostasis and upmodulated in response to hypoxia (Hilditch-Maguire et al., 2000). Mutant protein is also upmodulated in response to hypoxia (Trettel et al., 2000). There are at least two structural isoforms, distinguished by epitope availability, and these are localized to different sets of nuclear and perinuclear organelles involved in RNA biogenesis, and membrane trafficking respectively. These isoforms are exhibited by mutant as well as normal huntingtin. In addition, abnormal forms of mutant huntingtin are associated with dominant phenotypes that are different to those phenotypes caused by either excess or deficiency of huntingtin. These results suggest an abnormal toxic activity unique to mutant huntingtin, which may be exacerbated by its upregulation in response to metabolic stress caused by its own toxicity. As striatal neurons are particularly sensitive to oxidative stress, the combination of these two aspects of mutant huntingtin could explain the selective vulnerability of striatal neurons in HD.
1.12 Aims and Presentation of Thesis Research

Much of the research described in the preceding sections was published during the course of research for this thesis. At the beginning of the research, neuronal intranuclear inclusions were a recently observed phenomenon and their significance was uncertain. The aggregation of proteins to form these inclusions, and the biochemical properties of polyglutamine-containing proteins (in particular their potential for forming amyloid) pointed towards a possible role for protein aggregation in the neuropathology of HD.

Based on this research, we hypothesised that HD and probably the other expanded polyglutamine disorders, are amyloid-associated disorders like Alzheimer's disease, prion diseases such as Creutzfeld-Jacob disease, and type II diabetes mellitus. We further hypothesised that knowledge of the disease-causing, amyloid-forming monomer in HD could provide a potential avenue for therapy and/or prevention of the disease. The principal aim at the beginning of the project was to purify and characterise the aggregated protein species in HD brain.

We decided to take a biochemical approach to achieve these aims, with the ultimate objective of applying this knowledge to the prevention of aggregation. Because neuronal inclusions were known to be relatively small and rare, it was decided to first investigate different regions of the HD brain to find areas of abundance. At the same time we planned to attempt to determine whether or not these inclusions were of an amyloid nature. The results of this research are presented in Chapter 2. Once it was established that amyloid-like inclusions are present in HD brain, we proceeded to purify and characterise poorly soluble proteins from amyloid-enriched samples, based on the extreme insolubility and protease-resistance of amyloid in other diseases. The results of these biochemical investigations are presented in Chapter 3. Chapter 4 describes the investigation of the role in HD of a protein fragment (of glial fibrillary acidic protein) that was purified and characterised by methods described in Chapter 3. Chapter 5 describes experiments with cultured cells designed to investigate and verify the usefulness of the various biochemical methods used for the purification of aggregated proteins. The results of biochemical investigations using polyacrylamide gel electrophoresis are presented in Chapter 6. Finally, after the application of conventional biochemical techniques would not allow the characterisation of purified
peptides, some new and highly sensitive techniques were applied towards achieving the same goal. The results of this work are presented in Chapter 7.

The thesis is presented in project format. Rather than having a chapter dedicated to materials and methods and other chapters dedicated to only results, the six related research projects performed for this thesis are presented as separate chapters (Chapters 2-7). These six chapters each have an overview, introduction, materials and methods section, results section and a discussion based on those results. Methods common to more than one chapter are cross-referenced.
Chapter 2
Amyloid-like Inclusions in Huntington Disease

2.1 Overview

Before attempting to isolate, purify and characterise aggregated protein species from neuronal inclusions in Huntington disease (HD) brains, it was decided to identify areas of abundance of such protein aggregates in the HD brain. Furthermore, because amyloid is a substance associated with a number of other neurodegenerative disorders including Alzheimer's disease and prion disorders, and because it had been suggested that either full-length or fragments of mutant huntingtin could form amyloid-like aggregates (Perutz et al., 1994; Perutz, 1996), it was decided to look for signs of amyloid in HD brain. This chapter describes the discovery of amyloid-like inclusions in HD brains, and their characteristics.

The hypothesis that amyloid-like protein aggregation could be involved in the pathogenesis of HD was first suggested by Dr Max Perutz (MRC Laboratories, Cambridge, U.K.). Perutz (1996) proposed that the expanded polyglutamine tract in mutant huntingtin could act as a polar zipper, leading to the formation of protein oligomers. After demonstrating that poly(L-glutamine) can form paired antiparallel β-strands linked together by hydrogen bonds between main-chain and side-chain amides (Perutz et al, 1994), Perutz inferred that a polyglutamine sequence might undergo a conformational change from random-coil to β-sheet causing self-association into large protein aggregates. This hypothesis is supported by the identification of an antibody that specifically binds to the pathogenic polyglutamine expansions (Trottier, 1995b). Proteolytic cleavage of GST-huntingtin fusion proteins containing exon-1 of huntingtin with an expanded polyglutamine stretch (into the pathogenic range) resulted in the formation of highly insoluble amyloid-like protein aggregates in vitro (Scherzinger et al., 1997), also lending support to the hypothesis of Perutz.
The term ‘amyloid’ refers to a disease-associated mammalian polymer exhibiting fibrillar structures and green birefringence after Congo red staining (Glenner, 1980). On a finer scale, amyloid deposits are composed of paired twisted β-pleated sheet fibrils formed from various proteins by several different pathogenic mechanisms. Importantly, the polypeptide chains of the β-pleated fibrils are oriented perpendicularly to the axis of the fibrils, creating axial grooves on the filament surfaces that are the binding sites for dye molecules such as Congo red (Modis, 1991). The two most commonly used defining features of amyloid are hence: (a) fibrillar structures, and (b) green birefringence after Congo red staining. Congo red is the most commonly used histological stain for amyloid, resulting in pink-red coloured deposits that demonstrate green birefringence under polarized light. The two factors that are important in Congo red staining and birefringence are the linearity of the dye molecule (with a recurring active group at 10.3Å intervals) and the β-pleated sheet configuration of the substrate (Francis, 1977). The same features should also generate a positive reflectance signal by confocal microscopy. Figure 2.1 illustrates the physical structure of amyloid fibrils with Congo red dye binding sites also indicated.

![Figure 2.1. Diagramatic representation of twisted β-pleated sheet-like, paired amyloid filaments. (From: Glenner, 1980).](image)

Birefringence is a modification of polarized light by a structurally and optically anisotropic material - i.e. a material whose properties are different in different directions (Modis, 1991). In polarized-light microscopy, a polarizer mounted below
the condenser produces linearly polarized light by which the specimen is illuminated. The oscillating electric field of this linearly polarized light interacts with the valence electrons of anisotropic, birefringent material, characterised by two distinct refractive indexes perpendicular to each other. The linearly polarized light splits into two components (the ordinary and extraordinary rays) which pass through the sample at different velocities. Both beams will be slowed down inside the structure, but one (extraordinary) will be slowed down more than the other. The phase difference thus created between these two beams is observed in the eyepiece when these beams have passed through an analyzer, a second polaroid oriented perpendicular to the polarizer. When analyzer and polarizer are crossed in this way, the light coming from the polarizer is extinguished, the microscopic field appears completely dark, and birefringent specimens (such as highly ordered, Congo red-stained, twisted β-pleated sheet fibrils) appear bright. Congo red binding acts to augment the phase difference, and therefore the level of birefringence, that is detected. In the case of Congo red staining of amyloid, the birefringence is a light green colour.

At the beginning of this project there was no report describing in situ amyloid-like protein aggregates in HD brain, despite the observation of amyloid-like polyglutamine aggregates in vitro (Scherzinger et al., 1997). Neuronal inclusions that are immuno-positive for ubiquitin and N-terminal fragments of huntingtin had been identified in HD brain (Becher et al., 1998; DiFiglia et al., 1997; Gutekunst et al., 1999). More recently, studies have shown that neuronal inclusions in polyglutamine disorders are also immuno-positive for a number of transcription factors including TATA-binding protein (TBP), cAMP-responsive element-binding protein (CREB), and CREB-binding protein (CBP) (Shimohata, Onodera & Tsuji, 2000; Yamada, Tsuji & Takahashi, 2000). Other proteins that have been detected within neuronal inclusions are the cytoskeletal proteins actin and neurofilament (68 kDa), the cell cycle-regulating proteins p53 and mdm-2, HSP70 (a proteasomal component), and proteins of the nuclear pore complex (Suhr et al., 2001). We wished to purify and characterise the aggregated protein species within these inclusions, with a view towards finding ways of preventing aggregation and possibly disease progression. To determine regions of abundance of these inclusions, and to determine if these inclusions displayed amyloid-like staining characteristics, we examined HD and control tissue from a number of different brain regions, by Congo red staining with polarized light.
and confocal microscopy. The methods used and the results of this investigation are described in the following sections.

In addition to the investigation of HD brain tissue for amyloid-like protein aggregation, brains from R6/2 transgenic mice (Mangiarini et al., 1996) expressing exon 1 of the human huntingtin gene with expanded polyglutamine stretches of 144-190 residues, and unaffected littermate controls, were also examined by the same techniques. The R6/2 mice exhibit a progressive neurological phenotype with many of the features of HD and onset at approximately 2 months (Mangiarini et al., 1996; Bates et al., 1997). As described in section 1.9.1 (Chapter 1), these mice possess neuronal intranuclear inclusions and neuropil aggregates in most areas of the brain (Davies et al., 1997). The appearance of these inclusions and aggregates, which are immunoreactive for huntingtin and ubiquitin, precedes the onset of neurological symptoms (Davies et al., 1998). Because of the presence of these inclusions, it was decided to look for signs of amyloid in transgenic mouse brains, to determine if the amyloid hypothesis proposed for polyglutamine diseases was relevant to the R6/2 model of HD.

2.2 Materials and Methods

Normal control brain tissue and Alzheimer's disease brain tissue were used in all cases for comparison. Preliminary staining experiments used a Congo red staining protocol after Benhold (cited in Prophet et al., 1992; section 2.2.2.1) and a cresyl violet counterstain. The results of these attempts (summarised in section 2.3.1) led to the hypothesis that any Congo red-stained birefringent intranuclear inclusions that may have been present were being masked by the relatively strong counterstain. Subsequent attempts included staining sections with Congo red only (ie. minus the counterstain), co-staining with Congo red and neuron-specific antibodies, experimenting with section thickness and length of counterstain, and finally the adoption of a different Congo red staining protocol (M. McAnulty-Smith, personal communication) (section 2.2.2.2). Once Congo red-stained birefringent 'inclusions' were identified, attempts were made to determine if these were the same inclusions that had previously been identified using immunohistochemistry (section 1.8, Chapter 1). Immunohistochemistry methods are described in section 2.2.3. All chemicals (throughout this thesis) were supplied by BDH (NZ) Ltd unless otherwise specified.
2.2.1. Preparation of tissue for staining and microscopy

The human brain tissue was obtained from the New Zealand Neurological Foundation Human Brain Bank (School of Medicine, University of Auckland), and in the case of HD tissue the diagnosis of HD was confirmed unequivocally by genetic analysis of the CAG repeat length. Ethical approval for use of this tissue was obtained from the University of Auckland’s Human Subjects Ethics Committee. The tissue was perfused with phosphate-buffered saline and 1% sodium nitrite followed by 15% formalin in 0.1 M phosphate buffer (PB), pH 7.4, cut into blocks and postfixed in the same formalin fixative. It was then transferred to 25% sucrose in 0.1 M PB, 0.1% sodium azide for two to three days, then to 30% sucrose, 0.1% sodium azide for two to three days. The tissue blocks were then dehydrated through a series of alcohols from 70% to absolute, followed by chloroform, then impregnated with wax and blocked into paraffin wax. The blocks were cut on a rotary microtome (Leitz) at a thickness of 10 μm in preliminary experiments, or 8 μm in subsequent experiments, and mounted on Apes-coated glass slides (2% aminopropyltrimethoxysilane in acetone). Mounted sections were dewaxed in xylene and then hydrated through a series of alcohols (100%, 95%, 70%, 50%) to water.

Immunohistochemistry was performed on both paraffin-embedded tissue (processed as described above) and on frozen tissue. Perfused frozen tissue for immunohistochemistry was dissected into blocks and cryoprotected. Sections of 50 μm were cut on a freezing sledge microtome and collected in PBS with 0.1% sodium-azide. These sections were immuno-labelled and rinsed floating in wells. For immunohistochemistry performed on paraffin sections, a wax pen was used to create a boundary for antibody and rinse solutions.

Congo red staining was also performed on R6/2 transgenic mouse brains and littermate controls with no neurological symptoms. Frozen, perfused brains from R6/2 transgenic mice were obtained from the laboratory of Professor Gillian Bates (Guy’s Hospital, London). Mice were perfused with 4% paraformaldehyde. The brains were removed, fixed overnight and transferred to PBS with 0.02% azide for transport. Once received, mouse brains were dehydrated through a series of alcohols from 70% to absolute, followed by chloroform, then impregnated with wax and blocked into paraffin wax. The blocks were cut on a rotary microtome (Leitz) at a thickness of 8 μm and mounted on Apes-coated glass slides (2% aminopropyltrimethoxysilane in
acetone). Mounted sections were dewaxed in xylene and then hydrated through a series of alcohols (100%, 95%, 70%, 50%) to water. Congo red staining of mouse brain sections was performed using the McAnulty-Smith method described in section 2.2.2.2.

2.2.2 Congo red staining protocols
Two different staining protocols were used for Congo red staining. Preliminary staining experiments on 10 μm-thick sections, were performed as follows:

2.2.2.1 Benhold Method (cited in Prophet et al., 1992)
1. Stain sections in filtered Congo red solution (1% (w/v) in distilled water; Gurr) for 1 hour.
2. Rinse in distilled water.
3. Rapidly differentiate in alkaline alcohol solution (1% NaOH, 50% Ethanol in distilled water).
4. Wash in running tap water for 5 minutes.
5. Counterstain in 0.4% (w/v) Mayer’s haematoxylin solution (BDH) for 5 minutes.
6. Wash in running tap water for 15 minutes.
7. Dehydrate (in alcohols), clear (in xylenes) and coverslip (in Histomount mounting media (Hughes and Hughes Ltd., England)).

The results of staining experiments using this method are described in sections 2.3.1-2. An alternative method, kindly supplied by Mrs Michelle McAnulty-Smith (Department of Anatomy, University of Auckland) was used in all subsequent Congo red staining. This was performed as follows:

2.2.2.2 McAnulty-Smith Method.
1. Incubate slides in alkaline salt solution (0.01% NaOH in NaCl-saturated 80% alcohol) for 20 minutes.
2. Incubate slides in a 3% Congo red solution (3% Congo red (w/v), (Gurr); 0.01% NaOH; NaCl-saturated 80% alcohol) for a further 20 minutes.
3. Rinse slides in tap water.
4. Counter-stain sections in 0.1% (w/v) cresyl violet solution (length of this stain was varied – see section 2.3.3).
5. Rinse slides in water.
6. Dehydrate, clear and coverslip as above.

2.2.3 Immunohistochemistry

Antibodies raised against a neuron-specific nuclear protein (NeuN), ubiquitin and huntingtin were used in this study. Neurons were localised with a mouse monoclonal antibody termed NeuN (Chemicon). Ubiquitin was localised with a rabbit polyclonal ubiquitin antibody (DAKO Corporation). For detection of the huntingtin protein, a rabbit polyclonal antibody (HN1) was used. This antibody was raised against amino acids 1-17 of huntingtin protein (kind gift of Dr A.L. Jones).

**Double labelling.** 50 µm sections were incubated in 0.2% (v/v) triton in PBS (PBS-triton) at 4°C overnight, transferred to 1% (v/v) H₂O₂ in 50% (v/v) methanol for 20 minutes, washed in PBS-triton (3x10 minutes), and then incubated for 3 days at 4°C in primary antibodies diluted in 1% (v/v) goat serum in PBS with 0.2% (v/v) triton and 0.4% (w/v) merthiolate. Primary antibodies were used at a dilution of 1:100 for NeuN, and 1:1000 for ubiquitin. The sections were washed with PBS-triton (3x10 minutes) and then incubated in secondary antibody overnight at room temperature. Goat anti-mouse secondary antibody directly linked to CY3 (1:500, Chemicon), or goat anti-rabbit FITC secondary antibody directly linked to FITC (1:100, Sigma) were used. The labelled sections were then washed, mounted on slides with Citifluor (Agar Scientific, Stanstead, England) and viewed by confocal microscopy (section 2.2.4).

**Single labelling.** 8 µm paraffin sections were prepared and incubated in primary antibodies as described above, with a wax pen used to create a boundary for antibody and rinse solutions. Primary antibodies were used at a dilution of 1:1000 for ubiquitin and 1:500 for HN1. The sections were washed with PBS-triton (3x10 minutes) and then incubated with donkey anti-rabbit Ig biotinylated secondary antibody (1:500, Amersham, Life Science) overnight at room temperature. The sections were washed, incubated in Streptavidin-biotinylated horseradish peroxidase complex (1:500, Amersham, Life Science) for 3 hours, washed again in PBS-triton and the antigen was visualised with 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% (v/v) H₂O₂ to produce a brown reaction product. Floating sections were
mounted on chrome alum coated slides and air-dried. Finally, all sections were then dehydrated, cleared and coverslipped.

2.2.4 Microscopy
Slides were examined on a Leitz Dialux 20 EB polarizing microscope or a Leica TCS 4D laser scanning confocal microscope.

2.2.5 Photography
Photographic slides of Congo red-stained and immuno-labelled sections, were taken using a WildLeitz (Switzerland) MPS 46/52 Photoautomat equipped with E-6 slide film.

2.2.6 Progression of methodology
The flow-chart illustrated in figure 2.2, demonstrates the progression of methodologies that was followed to reach a method that enabled the identification of amyloid-like inclusions in HD. The results of each of these different sets of experiments will be described in the following sections.
**CHAPTER TWO – AMYLOID-LIKE INCLUSIONS IN HUNTINGTON DISEASE**

<table>
<thead>
<tr>
<th>Staining of 10µm, paraffin-embedded sections from different regions of HD, normal and Alzheimer’s disease brains, with Congo red (following Benhold protocol) and cresyl violet (about 20 minutes in accordance with standard histological procedure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining of 10µm, paraffin-embedded HD sections with Congo red only (Benhold protocol)</td>
</tr>
<tr>
<td>Double labelling of 50µm unfixed, floating HD sections, using fluorescent secondary antibodies, with: (a) neuronal-N + ubiquitin antibodies (b) neuronal-N antibody + Congo red (c) ubiquitin antibody + Congo red (Benhold protocol in both)</td>
</tr>
<tr>
<td>Staining of paraffin embedded, HD and normal control sections (cut at 8µm) with Congo red (McAnulty-Smith protocol) and cresyl violet (at 15 seconds, 60 seconds and 120 seconds).</td>
</tr>
<tr>
<td>Co-staining of 8µm, paraffin-embedded HD and Alzheimer’s disease sections with an antibody to ubiquitin (using fluorescent secondary), Congo red (McAnulty-Smith protocol) and cresyl violet (~15 seconds)</td>
</tr>
<tr>
<td>HN1 and ubiquitin labelling (separately) of HD and normal control brain sections, using DAB system, and weakly counter-stained with cresyl violet (~15 seconds)</td>
</tr>
</tbody>
</table>

**Figure 2.2.** Schematic illustration demonstrating the logical progression of experimentation that led to the identification of amyloid-like inclusions in HD.
2.3 Results

2.3.1 Congo red staining (Benhold protocol) with and without counter-staining

In preliminary experiments, a wide range of sections were stained with Congo red and counter-stained in cresyl violet for a period of up to 20 minutes in accordance with standard histological procedures. Cresyl violet is a commonly used counterstain of neuronal tissue detecting 'Nissl' substance or more specifically the aggregates of rough endoplasmic reticulum. When these slides were examined, no Congo red staining was apparent for HD and normal sections, and Nissl substance was strongly stained. The Alzheimer's disease (AD) cases showed typical extracellular plaque morphology (micrographs not shown). Based on the presumption that the strong counter-stain could be masking intracellular and particularly intranuclear Congo red staining and birefringence, further sections were stained with Congo red without the cresyl violet counter-stain. These sections appeared pink to the naked eye, and when examined by light microscopy most of the tissue was seen to be stained pink-red. Both normal control and HD sections exhibited this Congo red staining without birefringence, although there was a hint of birefringent particles in some of the HD sections. However, due to the lack of a counter-stain and the continuity of Congo red staining across the section, it was very difficult to recognise cell boundaries and cell types and we were unable to determine whether the birefringent particles were artefactual or associated with neurons. Even with differential interference contrast (DIC) microscopy and high power objectives it was not possible to determine the nature of the birefringent particles.

2.3.2 Double-labelling results.

Unfixed floating sections (50 µm) of HD brain, double labelled with (a) neuronal-N and ubiquitin antibodies, (b) neuronal-N antibody and Congo red, and (c) ubiquitin antibody and Congo red, were examined by confocal microscopy. Differential interference contrast (DIC) was used to visualise the cell bodies as the counter-stain step was left out due to possible blocking of signal from intranuclear inclusions. There appeared to be some localisation of ubiquitin immunoreactivity to neuronal nuclei,
however the localisation of Congo red staining was again uncertain, and no reflectance signal was observed in sections stained with Congo red (micrographs not shown).

2.3.3 Congo red staining (McAnulty-Smith protocol) with reduced counter-staining.

During the course of subsequent experimentation, a new Congo red staining protocol was applied and the period of counter-staining was gradually reduced. 8 μm sections of HD and normal control tissue were stained with Congo red and counter-stained with cresyl violet for 2 minutes, 1 minute and 15 seconds. The 1-minute and 2-minute counter-stained slides were much fainter in colour than the slides described in section 2.3.1, but no Congo red staining was observed under either staining regime. With a 15-second counter-stain, sections were invisible to the naked eye. However, cresyl violet-stained nuclei were visible by microscopy and in these sections Congo red staining and birefringence became apparent. Congo red-stained birefringent inclusions, were observed in cortical and striatal neurons in five HD cases (table 2.1), but were absent in two normal brains and two cases of AD. Inclusions were observed both within neuronal nuclei (figure 2.3 a-d) and peri-nuclear (figure 2.3 e & f).

Regions that contained these inclusions were middle-frontal gyrus, middle-temporal gyrus, caudate nucleus, amygdaloid nucleus and globus pallidus, with cortical regions (particularly middle-frontal gyrus) having the greatest abundance and being invariably affected in the five cases examined. No Congo red-stained birefringent inclusions were observed in cerebellum or hippocampus. There was no clear correlation between the repeat length and the number of Congo red-stained, birefringent inclusions, although the number of cases was small. As before, the Alzheimer’s disease (AD) cases showed typical extracellular plaque morphology however no intracellular birefringent inclusions were observed (micrographs not shown).

When the HD sections were examined by confocal microscopy, using transmitted light (figure 2.4 a) and reflection modes (figure 2.4 b), the reflectance signal was seen to co-localize with Congo red-stained neuronal inclusions.
CHAPTER TWO - AMYLOID-LIKE INCLUSIONS IN HUNTINGTON DISEASE

Table 2.1. Brain number, age at death, repeat-length, HD grade (Vonsattel & DiFiglia, 1998) and post-mortem delay of HD, AD and normal cases that were examined for amyloid. PM = post-mortem. MFG = middle-frontal gyrus; GP = globus pallidus; MTG = middle-temporal gyrus; CN = caudate nucleus; A = amygdaloid nucleus. N.A. = not applicable.

<table>
<thead>
<tr>
<th>Brain Number</th>
<th>Type</th>
<th>Sex/ Age at Death</th>
<th>Repeat lengths</th>
<th>HD grade</th>
<th>PM Delay (hours)</th>
<th>Areas with amyloid-like inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC74</td>
<td>HD</td>
<td>F/42</td>
<td>17/42</td>
<td>1</td>
<td>12</td>
<td>MFG, GP</td>
</tr>
<tr>
<td>HC75</td>
<td>HD</td>
<td>M/41</td>
<td>18/52</td>
<td>3</td>
<td>5.5</td>
<td>MFG, MTG</td>
</tr>
<tr>
<td>HC76</td>
<td>HD</td>
<td>M/71</td>
<td>17/42</td>
<td>2</td>
<td>16</td>
<td>MFG, GP</td>
</tr>
<tr>
<td>HC86</td>
<td>HD</td>
<td>M/46</td>
<td>17/37</td>
<td>1</td>
<td>18</td>
<td>MFG, CN</td>
</tr>
<tr>
<td>HC87</td>
<td>HD</td>
<td>M/45</td>
<td>21/50</td>
<td>3</td>
<td>18</td>
<td>MFG, CN, A</td>
</tr>
<tr>
<td>AZ27</td>
<td>AD</td>
<td>F/79</td>
<td>N.A.</td>
<td>N.A.</td>
<td>8</td>
<td>None</td>
</tr>
<tr>
<td>AZ31</td>
<td>AD</td>
<td>M/86</td>
<td>N.A.</td>
<td>N.A.</td>
<td>7.5</td>
<td>None</td>
</tr>
<tr>
<td>H78</td>
<td>Normal</td>
<td>F/48</td>
<td>N.A.</td>
<td>N.A.</td>
<td>11.5</td>
<td>None</td>
</tr>
<tr>
<td>H111</td>
<td>Normal</td>
<td>M/46</td>
<td>N.A.</td>
<td>N.A.</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>H112</td>
<td>Normal</td>
<td>M/79</td>
<td>N.A.</td>
<td>N.A.</td>
<td>8</td>
<td>None</td>
</tr>
<tr>
<td>4466</td>
<td>Normal</td>
<td>F/53</td>
<td>N.A.</td>
<td>N.A.</td>
<td>17</td>
<td>None</td>
</tr>
<tr>
<td>4499</td>
<td>Normal</td>
<td>M/53</td>
<td>N.A.</td>
<td>N.A.</td>
<td>20</td>
<td>None</td>
</tr>
</tbody>
</table>

These results provide evidence that the inclusion bodies contain amyloid-like protein aggregates. The intracellular inclusions observed averaged 1.5-2 μm in diameter, a size consistent with the immunohistochemically detected inclusions reported by other investigators (Becher et al., 1998; DiFiglia et al., 1997; Gutekunst et al., 1999). However, they were very infrequent occurring at a frequency that ranged between 5 and 20 per 150 mm² section (n=11 inclusion-containing regions as listed in table 2.1).

The upper end of this frequency range (20 inclusions per 150 mm² section) was most commonly associated with the regions of cortex that were examined; i.e. middle-frontal and middle-temporal gyri. The lower end of the range (5 inclusions per 150 mm² section) was most commonly associated with the striatum. Given the effect of the counter-stain on our ability to detect Congo red-stained, birefringent inclusions, it is entirely possible that many more amyloid-like inclusions remained undetected due to masking by cresyl violet.

Larger Congo red-stained, birefringent bodies (5-6 μm in diameter) were also observed in non-neuronal cells within middle-temporal and middle-frontal gyri of two HD cases (HC74 and HC 76 – case details in table 2.1). These appeared to be groups of the smaller inclusions gathered within astrocyte-like cells (figure 2.4 c & d). Such aggregates are not described in immunohistochemical studies and the relevance of these is unknown.
Figure 2.3. Amyloid-like inclusion bodies in 8 μm paraffin sections from HD brain. A neuronal intranuclear inclusion (arrowhead) in the caudate nucleus of a Grade 3 HD case (HC87; 21/50 repeats) stained with Congo red, when viewed in bright-field (a) and polarized light (b). Scale bars = 5 μm. Congo red-stained neuronal intranuclear inclusion (arrowhead) in the middle-frontal gyrus of a Grade 1 HD case (HC86; 17/37 repeats), when viewed in bright-field (c) and polarized light (d). Scale bars = 15 μm. (e) Bright-field and polarized-light (f) images of a Congo red-stained perinuclear inclusion (arrowhead) in a middle-frontal gyrus neuron of a Grade 2 HD case (HC76; 17/42 repeats). Scale bars = 10 μm.
Figure 2.4. Confocal microscopic images of Congo red-stained amyloid-like inclusions and large birefringent bodies in 8 μm paraffin sections from HD brain. (a) Transmitted light image (using DIC optics) of a Congo red-stained neuronal intranuclear inclusion in the caudate nucleus of a Grade 3 HD case (HC87; 21/50 repeats), corresponding to the reflectance signal generated by confocal microscopy on the same section (b). (c) Transmitted light image (using DIC optics) of two inclusion-filled non-neuronal cells in the middle-temporal gyrus of a different Grade 3 HD case (HC75; 18/52 repeats) corresponding to the reflectance signal generated by confocal microscopy on the same section (d). An unaffected neuron can be seen below centre in (c) (arrowhead). Scale bars = 10 μm.
2.3.4 Are these amyloid-like inclusions the same neuronal inclusions reported in the literature?

Following the observation of amyloid-like inclusions in HD tissue, the question remained whether or not these corresponded in any way to the immunohistochemically-detected neuronal inclusions described in the literature (see section 1.8). This latter class of inclusion has been shown in a number of studies to be immuno-positive for ubiquitin, N-terminal epitopes of huntingtin, and TBP among other antigens (Becher et al., 1998; Suhr et al., 2001). At this time it is not clear whether or not immunohistochemically detected inclusions can be split into subclasses based on their antigenicities, or if all of these antigens are present within the same inclusions. Although there was a good correlation between the two sets of inclusions based on size and localisation, the amyloid-like inclusions we detected were considerably less frequent than any that had been described in immunohistochemical studies. In an effort to determine whether these two sets of inclusions were related or not, we attempted to Congo red-stain sections that had previously been labelled with ubiquitin and huntingtin antibodies, and vice versa. Unfortunately, due to methodological incompatibilities, it was not possible to obtain unequivocal evidence that the Congo red-stained, birefringent inclusions contain any proteolytic fragments of huntingtin. Also, because of the small size of these inclusions we were unable to find a bisected inclusion in serial sections that was immunohistochemically labelled in one section, and Congo red-stained in the adjacent section.

In order to demonstrate the presence of immunohistochemically-detectable neuronal inclusions in the tissue that was stained with Congo red, 8 μm paraffin sections from some of the blocks that were shown to contain amyloid-like inclusions, were labelled with antibodies raised against the N-terminal 17-amino acid region of huntingtin, and ubiquitin. Sections that were examined for immunohistochemistry include middle-frontal gyrus and caudate nucleus from HC87, middle-frontal gyrus from each of HC74, HC76 and HC86, and middle-temporal gyrus from HC75. The repeat length, HD grade and PM delay for each of these cases is listed in table 2.1. In addition to these HD cases, 8 μm paraffin sections from five control brains with no history of neurodegenerative disorders were labelled with the same antibodies. No huntingtin- or
ubiquitin-positive inclusions were observed in any of the control cases examined. Characteristic immunohistochemically-detected inclusions are illustrated in figure 2.5.

Figure 2.5. Neuronal intranuclear inclusions immunoreactive for N-terminal huntingtin and ubiquitin in 8 μm sections from HD brain. (a) Micrograph of an N-terminal huntingtin-positive neuronal intranuclear inclusion (arrowhead), within the middle-frontal gyrus of a Grade 3 HD case (HC87; 21/50 repeats). (b) Micrograph of a different, ubiquitin-positive neuronal intranuclear inclusion (arrowhead), on a different section taken from the same region of the same brain as (a). The immunoreactive material within the oval is likely to represent dystrophic neurites. Antibodies were detected using biotinylated secondary antibodies, streptavidin-biotinylated horseradish peroxidase complex, and DAB to produce a brown reaction product. Scale bars = 15 μm.

2.3.5 Congo red staining of R6/2 transgenic mouse brain

Nine mouse brains were prepared as described in section 2.2.1 and sent from the laboratory of Professor Gillian Bates (Guy’s Hospital, London) in vials containing PBS with 0.02% azide. These included four that had been sacrificed at five weeks of age (two R6/2 transgenics and two littermate controls), and five that were sacrificed at eight weeks of age (three R6/2 transgenics and two littermate controls). This line of transgenic mouse is described in section 1.9.1, Chapter 1. Eight-micron paraffin-embedded, coronal sections were taken at a level of the rostro-caudal axis where cortex and striatum could be easily visualised, and stained with Congo red as described. Eight-micron paraffin sections from human HD middle-frontal gyrus were stained in parallel as a positive control.

No Congo red staining with birefringence was observed for either transgenic mice or littermate controls, despite clear and abundant immunohistochemically-detected inclusions reported by other investigators (Davies et al., 1997; H. Waldvogel, personal communication). Amyloid-like inclusions were observed in the human HD tissue as previously described. A number of possible reasons for the absence of amyloid-like inclusions in the R6/2 mouse brains are discussed below, however it is
likely to be an important observation distinguishing amyloid-like inclusions from previously reported inclusions with the former being restricted to human HD brains.

2.4 Discussion

Prior to the beginning of this research, it had been proposed that polyglutamine could form amyloid structures. We have identified amyloid-like structures in HD brains however the presence of polyglutamine could not be confirmed, and at the limits of our resolution their frequency is very low. This area of work, described in this chapter, has now been published in the journal *Neuroscience* (McGowan et al., 2000). Further support for amyloid-like protein aggregation occurring in HD is now provided by evidence of Congo red staining with birefringence, of purified insoluble material from HD brain, captured on cellulose acetate filters (Huang et al., 1998).

The structures we observed appeared in the form of intranuclear and perinuclear inclusions of similar size to the immunohistochemically-detected inclusions reported in the literature (Becher et al., 1998; DiFiglia et al., 1997; Gutekunst et al., 1999). Their frequency was however estimated to be much lower (being between 5 and 20 inclusions per 150 mm² section). A comprehensive, quantitative survey of amyloid-like inclusions in the HD brain was not performed as part of this study, however the results of such a study would be very interesting. In addition to being rare, Congo red-stained inclusions were only apparent when counter-staining was sufficiently weak. It is likely that in preliminary experiments using the Benhold method, the strong counter-stain masked amyloid-like inclusions. It is also possible that masking of inclusions by the counter-stain is responsible for the low frequency of amyloid-like inclusions we observed. Section thickness was also found to be an important factor in detection of amyloid-like inclusions. The optimum section thickness for polarized-light microscopy and birefringence is proposed to be 8 μm (Modis, 1991). No inclusions were seen in the 10 μm sections, although this is likely to be at least partly a result of counter-staining as discussed above. Nor were they seen in the 50 μm sections that were additionally labelled with a neuronal antibody or an antibody to ubiquitin, despite there being no counter-stain on these sections. This is most likely to be due to absorption of the polarized light by the tissue section.

In a separate study in our laboratory, the accumulation of TBP, huntingtin and ubiquitin was investigated in the HD middle-frontal gyrus. The same ubiquitin and N-
terminal huntingtin antibodies described in this thesis were used. Ubiquitin-labelling of 50 μm sections revealed a much higher frequency of inclusions than was observed following Congo red staining (W. van Roon-Mom, personal communication). These inclusions were observed in intranuclear, perinuclear and cytoplasmic locations. The frequency was estimated to be approximately forty to fifty thousand ubiquitin-positive inclusions per 150 mm² section (n=2). However, staining of adjacent sections with an antibody raised against the N-terminal region of huntingtin, detected a much lower frequency of inclusions (in the same locations) estimated to be in the vicinity of nine hundred N-terminal huntingtin-positive inclusions per 150 mm² section (n=2; van Roon-Mom et al., submitted). Also in this study, seven different morphological forms of TBP-positive inclusions were described and collectively these were shown to be approximately four times more frequent than huntingtin-positive inclusions (approximately four thousand per 150 mm² section; n=2).

It is apparent from this study and others (Becher et al., 1998; Gutekunst et al., 1999) that there is a very large range in the frequency of immunohistochemically-detected inclusions depending on the particular antibodies used. Other factors, such as the use of antigen retrieval methods (ie. treatment of sections prior to immuno-staining), may also play a role in the variability between studies. The reason for and relevance of this observation is not yet understood, however it is unlikely that small n-values would contribute such a massive variation. Regardless, work in our laboratory suggests that at least within HD middle-frontal gyrus, Congo red-stained, birefringent inclusions represent approximately 2% of huntingtin-positive inclusions, which in turn represent approximately 2% of ubiquitin-positive inclusions. Some of the difference in frequency described here may be due to masking of a proportion of Congo red stained inclusions by the counterstain, and some may be due to the difference in section thickness; 8 μm for Congo red staining and 50 μm in van Roon-Mom et al.

Because HD is a late-onset, slowly progressive disorder, it may be that at any one point in time the number of live cells containing a polyglutamine β-sheet structure is low, because at a critical point they die. The observation that membrane-trapped aggregates purified from HD brain stain with Congo red, display birefringence, and react with antibodies to N-terminal epitopes of huntingtin (Huang et al., 1998), supports the theory that there may be some overlap between amyloid-like inclusions and the immunohistochemically-detected inclusions. Alternatively, if these two
different types of inclusion represent separate populations, it is possible that they were co-purified using the filter trap assay. It may be that the presence of TATA-binding protein and ubiquitin in the immunohistochemically-detected inclusions could prevent either an ordered aggregation of huntingtin monomers, or the linear binding of the Congo red dye molecule, thereby impeding staining and birefringence in the majority of inclusions. Such a hypothesis could explain the relatively low abundance of amyloid-like inclusions when compared with the immunohistochemically-detected inclusions described in the literature.

The absence of Congo red staining and birefringence in the R6/2 transgenic mice was a surprising result following the discovery of amyloid-like inclusions in human HD brain, indicating that detectable amyloid did not form in these mice. Ubiquitin- and huntingtin-positive inclusions have previously been demonstrated to be present in abundance in this line of transgenic mice. Electron microscopic studies of these inclusions reveal a mixture of fine granules and occasional filamentous structures (Davies et al., 1997). This description is in contrast with electron microscopic studies of neuronal intranuclear inclusions in the human brain, which showed a mixture of granules, filaments and parallel and randomly oriented fibrils (DiFiglia et al., 1997).

The lack of fibrils observed by electron microscopy in the R6/2 mice provides further evidence for the absence of detectable amyloid.

This absence could be due to the incorporation into aggregates of other proteins, or possibly smaller undetectable β-sheet inclusions in the mice. In vitro studies have shown the aggregation of polyglutamine-containing proteins to be length-dependent; longer repeat lengths lead to faster and more complete protein aggregation (Li & Li, 1998; Martindale et al., 1998). The repeat length in the transgenic mice (141-157 repeats in a truncated protein) that were examined is in the range of the more severe juvenile-onset disease in humans. The rate of aggregation in these mice is therefore much faster than in the normally slowly progressive human disorder, as also indicated by the rate of disease progression. This rapidity of aggregation could potentially lead to the incorporation of other proteins into the aggregates, thereby preventing linear binding of Congo red – an important factor for generating positive birefringence.

Conceivably, the age at which these mice were sacrificed (5-8 weeks) may precede formation of the amyloid-like ordered aggregates observed in human disease tissue. Abundant immunohistochemically-detected inclusions had previously been observed.
throughout the brains of other R6/2 mice of this age. The age at death of R6/2 mice is generally 12-13 weeks, and brains from 12-week old mice were obtained at a later stage in the research project. These brains were homogenised, subjected to an amyloid-enrichment protocol (section 3.2.1.1, Chapter 3) and pellets were stained with Congo red as described in section 3.2.1.2 (Chapter 3). No detectable amyloid was observed in smears of Congo red-stained tissue homogenates from these brains suggesting that amyloid had not formed, however this was not confirmed by Congo red staining of brain sections.

Taken together, the independent observations in HD tissue of fibrils in NIIs (DiFiglia et al., 1997), and Congo red staining with birefringence of inclusions that we have described, support the hypothesis of an amyloid-type aggregation event occurring in HD. Although this is the first description of amyloid-like inclusions in HD, other groups have previously looked for in situ evidence of amyloid in HD brain tissue (Huang et al., 1998; Karpj et al., 1999). Whilst the boundaries between amyloid-like inclusions and immunohistochemically-detected inclusions are yet to be determined, the observation of amyloid structures in HD lends support to the ‘polar zipper’ hypothesis of Perutz discussed in section 2.1. This provides evidence for the hypothesis that HD and the other polyglutamine disorders, fit into the category of amyloid diseases together with AD, prion disease, and type 2 diabetes mellitus. Whilst the role of aggregates remains contested in all of these diseases including HD, our finding that the inclusions in HD have an amyloid-like structure raises the possibility that strategies to prevent amyloid accumulation being developed for one neurodegenerative disease (ie. AD) may be of much wider application to CAG repeat disorders such as HD.

2.5 Future research

Any future work in the area of amyloid-like inclusions should involve a more accurate quantification of this type of inclusion in a variety of brain regions from a variety of different grade HD cases than we describe here. Subsequent statistical analysis could potentially reveal a correlation between repeat-length and inclusion-number as has been hypothesised for immunohistochemically-detected inclusions (Becher et al., 1998). The observation by both Becher et al. (1998) and that described in this thesis, of a greater abundance of amyloid-like inclusions in middle-frontal gyrus over
striatum, suggests that there is no correlation between the frequency of inclusions and the location of neuropathology. However, it must be remembered that the cases examined were all post-mortem brains obtained at autopsy, following progression of HD. Hence, all descriptions of human tissue in this thesis represent the end-stage of a neurodegenerative disease.

The remaining chapters of this thesis describe our efforts to purify and characterise the protein constituents of amyloid-like inclusions.
Chapter 3

Purification and Characterisation of Poorly Soluble Proteins from Huntington Disease Brains

3.1 Overview

The identification of amyloid-like inclusions in Huntington disease (HD) brain and their relative abundance in middle-frontal gyrus raised the possibility that this material could be purified and characterised. Because amyloid is highly insoluble in most known solvents and resists cleavage by proteases (Glenner, 1980), it was thought that these physical properties could be exploited to enrich samples in the amyloid-like material in HD brain (prior to solubilisation and characterisation) according to published amyloid purification procedures (Glenner & Wong, 1984). The first protocol used in this study had been successfully applied for the purification and characterisation of amylin from amyloid plaques in the diabetic pancreas (Cooper et al., 1987). The methodology was refined as the work progressed in an attempt to allow for the nuclear localisation of many inclusions, as well as their small size and scarcity.

The solvents used in the solubilisation of amyloid-associated proteins are too harsh for affinity chromatography, consequently high performance liquid chromatography (HPLC) was used to separate the proteins and peptides within tissue homogenates. The protein elution profiles of samples from control and affected brains were
compared and variant peaks of absorbance (within chromatograms) were selected and characterised.

HPLC involves the injection of a mixture of compounds dissolved in liquid phase onto an HPLC column, transport of these compounds along the column by the pumping of a suitable buffer, and separation and purification of these compounds based on their difference in retention by the stationary phase (column packing). There are numerous types of HPLC, two of which were used in the research performed for this thesis – gel filtration and reverse-phase. Only these two techniques will be discussed here. The type of column and buffer(s) used are specific to each different technique.

Gel filtration is also known as size-exclusion chromatography (SEC). This technique separates molecules by exploiting differences in their apparent molecular sizes in solution. The column packing materials consist of particles containing pores of well-defined size (Bradshaw, 1998). Those proteins with a molecular weight that is larger than the exclusion limit of a particular bead pore size will not enter the pores and will elute in the void volume \( (V_v) \) of the column. Proteins that are small enough to enter these pores will take longer to pass through the column and as such will elute at a later point known as the elution volume \( (V_e) \). The volume within the pores of the gel is called the internal volume \( (V_i) \), and the total permeation volume \( (V_t) \) of a column is defined as the sum of the void volume and the internal volume. There is a linear relationship between the elution volume (as long as this is between the void volume and total permeation volume of the column) of a protein and the logarithm (base10) of its molecular size, making it possible to estimate the molecular weight in daltons of that protein. In the present study a strong denaturant (6 M guanidine hydrochloride (GHCl)) was used as the buffer of choice due to the expected poor solubility of the protein(s) of interest. Because in gel filtration there is no direct interaction between proteins and the column packing, proteins that are smaller than the exclusion limit of a column have two ‘choices’ each time they encounter a pore. Proteins can either enter the pore and be slightly retained (relative to void volume), or they can pass by the pore and therefore not be retained. Because a very large number of pores will be encountered as a protein passes through a gel filtration column, a distribution of elution volumes \( (V_{es}) \), detected by UV spectroscopy of the eluting buffer, will be seen on the chromatogram for that protein. A chromatogram from gel filtration HPLC is a graph of absorbance against elution time or volume. The elution volume for a
particular protein is therefore read as the elution volume at its maximum absorbance ($A_{\text{max}}$). In a complex mixture of proteins however, the distribution of $V_e$s for each protein may overlap. For this reason, gel filtration was followed by reverse-phase HPLC as a further purification step.

Reverse-phase HPLC, unlike gel filtration, relies on a chemical interaction between solute and column packing (also known as the stationary phase). Most column packings in this form of HPLC are silica-based and contain covalently bonded alkyl chains of differing lengths (Bradshaw, 1998). The two types of column used in this project, C$_5$ and C$_{18}$ have a slightly different chromatographic character based on this chain length. These hydrophobic groups retain proteins and peptides on the column as a function of their relative hydrophobicities. Proteins and peptides are adsorbed onto the non-polar surface and remain bound until a sufficiently strong non-polar organic solvent displaces it from the solid support. Therefore, a gradient of increasing organic solvent strength is applied to the column to promote the elution of solutes. An organic modifier such as trifluoroacetic acid (an ion-pairing reagent) is usually included to minimise ionic interactions between solute and stationary phase and to maintain a low pH. A number of different mobile phases and gradient profiles were tested in this research and these will be described in the relevant sections. Similarly to gel filtration, UV spectroscopy is used for the detection of proteins and peptides in the eluting buffer and a chromatogram displaying absorbance, elution time, and %B (buffer B containing the organic solvent) is produced. Reverse-phase HPLC provides far greater resolution than gel filtration. Purified proteins were characterised by mass spectrometry and N-terminal protein sequencing as described below. Numerous modifications were made to the original purification protocol and these are also described below.

### 3.2 Methods and Materials

A wide range of different methods, often variations on a common theme, were attempted based on methods that had been successfully employed to purify the aggregating monomer in amyloid from Alzheimer's disease brain and the diabetic pancreas. However, there are some very important differences between the amyloid-like inclusions observed in HD, and the amyloid of other disorders. These are briefly discussed here to put the development of the methods described in this thesis into
context. Firstly, virtually all other known forms of amyloid including amylin and β-amyloid are extracellular in location. Secondly, their aggregates are all relatively large and abundant when compared with the 1.5-2 μm inclusions in HD. Because of these factors, much of the research performed for this thesis involved methodology development.

Brain tissue samples that were used in the purification experiments described in this chapter were obtained from the Neurological Foundation (NZ) Human Brain Bank, School of Medicine, University of Auckland. Ethical approval for use of this tissue was obtained from the University of Auckland’s Human Subjects Ethics Committee. The tissue had been perfused and blocked as described in section 2.2.1, Chapter 2, for storage by the Brain Bank staff. A summary of the progression of methodology used for the research described in this chapter is provided in figure 3.2, section 3.2.7.

3.2.1 Cooper Method

The protocols described in this section had previously been applied for the successful purification of amylin from amyloid plaques within the diabetic pancreas (Cooper et al., 1987). They were modified for the purification of aggregated protein within amyloid-like inclusions in HD brains, as described in subsequent sections. For the purification experiments according to the methods of Cooper et al. (1987), tissue from 2 control and 2 HD brains (table 3.1) was used.

Table 3.1. Brain number, type, region and HD grade (Vonsattel, Ge & Kelley, 1997) of tissue processed according to Cooper protocol. PM =post-mortem. Control and HD brains were age and sex matched. N.A. = not applicable.

<table>
<thead>
<tr>
<th>Brain</th>
<th>Type</th>
<th>Sex / Age at Death</th>
<th>Region</th>
<th>Repeat # / (HD Grade)</th>
<th>PM Delay (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H111</td>
<td>Normal control</td>
<td>M/46</td>
<td>Middle-frontal gyrus</td>
<td>NA</td>
<td>10</td>
</tr>
<tr>
<td>H112</td>
<td>Normal control</td>
<td>M/79</td>
<td>Middle-frontal gyrus</td>
<td>NA</td>
<td>8</td>
</tr>
<tr>
<td>HC76</td>
<td>HD</td>
<td>M/71</td>
<td>Middle-frontal gyrus</td>
<td>17/42 (2)</td>
<td>16</td>
</tr>
<tr>
<td>HC87</td>
<td>HD</td>
<td>M/45</td>
<td>Middle-frontal gyrus</td>
<td>21/50 (4)</td>
<td>18</td>
</tr>
</tbody>
</table>
3.2.1.1 Enrichment of tissue homogenates in amyloid-like particles

The first step in the purification process was the enrichment of amyloid-like particles within tissue homogenates. This was performed as follows:

1. Tissue was homogenized in ice-cold 150 mM NaCl using a teflon-glass homogenizer.
2. Homogenates were spun at 10 000 x g for 30 minutes (Sorvall RC-5 centrifuge, SS-34 rotor).
3. The supernatant was discarded and the pellet was re-homogenized and re-pelleted twice more, as before. Pellets were stored at -80°C.
4. Crude collagenase solution was prepared as follows: Lyophilised collagenase (Sigma, Cat.# C9891) was dissolved 1:100 (wt/vol) in buffer (50 mM Tris-Cl, 150 mM NaCl, 3 mM CaCl₂, 2% (vol/vol) Nonidet P-40, pH 7.4). Insoluble material was removed by ultracentrifugation (Beckman-L8 centrifuge, SW 40 Ti rotor, 150 000 x g for 2 hours). The solution was kept at 4°C, and considered crude due to the presence of probable contaminating enzymes including clostripain, trypsin and caseinase.
5. 100 mg of the pellet obtained following tissue homogenisation and saline extraction was dissected into a siliconized eppendorf tube using a scalpel blade and forceps.
6. The pellet was heated to 70°C for 10 minutes (to dissociate proteins of the extracellular matrix including collagen) and rapidly cooled on ice. Collagenase solution was then added to the pellet and shaken overnight at 37°C.
7. Pellets were washed twice with ice-cold 150 mM NaCl and once with distilled water, spinning tubes at 13 000 x g for 10 minutes each time in between.
8. A portion of each pellet was removed for Congo red staining (section 3.2.1.2) to determine the purity of amyloid particles.
9. Remaining pellet was resuspended in 70% (w/v) formic acid and sonicated with 4 x 30-second bursts at 8 μm wavelength using a Soniprep 150 probe-sonicator (MSE), with cooling on ice between exposures.
10. Following sonication, formic acid was removed by vacuum evaporation, and the near-dry remaining pellet was resuspended in phosphate-buffered 6 M GHCl (prepared as described in section 3.2.1.3).
11. Sonication was repeated as before and particulate matter pelleted by centrifugation at 13,000 x g for 5 minutes.

12. Solubilized samples were filtered to 0.2 μm using nitrocellulose (Sartorius) and injected onto a gel filtration column (section 3.2.1.3).

### 3.2.1.2 Congo red staining of tissue homogenates

Congo red staining of tissue homogenates was performed as described by Cooper et al. (1987) to determine the purity of amyloid-like particles within them.

1. An aliquot of the homogenate was placed in an Eppendorf tube.

2. 700 μl of NaCl-saturated, 80% ethanol (ethanol:distilled water, 80:20 (v/v), saturated with NaCl; filtered before use) was added and mixed vigorously by vortex until finely suspended.

3. Suspensions were mixed by rotation for 30 minutes, then centrifuged at 13,000 x g for 10 minutes.

4. The supernatant was removed and discarded.

5. 500 μl of saturated alkaline Congo red solution [NaCl-saturated, 80% ethanol solution (described above) was saturated with Congo red (Gurr), filtered and brought to 2.5 mM NaOH (final concentration) before use] was added and mixed vigorously by vortex until finely suspended.

6. Suspensions were centrifuged at 13,000 x g for 10 minutes.

7. The supernatant was removed and discarded.

8. The pellet was washed in 1 ml of distilled water and re-pelleted as above.

9. Final pellet was resuspended by trituration in 20 μl of distilled water and a large drop of this suspension was placed on a clean glass slide.

10. The drop was overlaid with a clean coverslip as if making a blood smear, the edges were sealed with nail varnish, and slides were examined by polarized-light microscopy for presence of amyloid.

### 3.2.1.3 Gel filtration

Once the presence of amyloid in tissue homogenates had been confirmed and the purity estimated (by Congo red staining of aliquots), gel filtration high performance liquid chromatography (HPLC) was applied to separate the proteins and peptides that were solubilized by sonication in 70% formic acid, on the basis of their molecular
size. This technique has been described in section 3.1 and was performed using a Waters 600E solvent delivery system (Millipore) and a microUVIS20 UV absorbance detector (Carlo Erba Instruments). The mobile phase used in all gel filtration separations was phosphate-buffered 6 M GHCl, which was prepared as follows:

573 g of guanidine-HCl (Sigma) was dissolved in a mixture of 406 ml of 0.4 M Na2HPO4 and 38 ml of 1 M NaH2PO4. The pH was adjusted to 7.5, and the total volume was brought to 1 L with milliQ water. The buffer was filtered to 0.2 μm using nitrocellulose (Sartorius).

All gel filtration separations were performed using a Phenomenex SEC-2000 size exclusion column. Chromatograms were recorded on a Yokogawa 3057 portable data recorder. Molecular weight standards were used to create a standard curve based on their elution times. These included: blue dextran (2 000 000 Da – used to calculate the void volume (V0) of the column; Sigma); bovine serum albumin (66 430 Da; Serva); cytochrome C (12 360 Da; Sigma); human insulin (5808 Da; Novo Nordisk Pharmaceuticals); and somatostatin (1638 Da; Bachem H-2260).

3.2.1.4 Reverse-phase high performance liquid chromatography

Following separation of proteins and peptides by gel filtration, fractions of these were further separated by reverse-phase HPLC. This technique, also integral to the research for this thesis, has similarly been described in section 3.1. Reverse-phase HPLC was performed using an Applied Biosystems (ABI) 140B solvent delivery system attached to an ABI 112A oven/injector and an ABI 785A UV absorbance detector. Two different reverse-phase columns were used during this research; a C18 and a C3 Jupiter column (both 250 mm x 5 μm, 300 Å; Phenomenex).

A number of different buffer combinations were used for reverse-phase HPLC during the course of this research. These are listed in Table 3.2.
Table 3.2. Reverse-phase HPLC buffers used in this research. (TFA = trifluoroacetic acid, MeCN = acetonitrile)

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08% TFA in MilliQ water</td>
<td>0.08% TFA, 80% MeCN in MilliQ water</td>
</tr>
<tr>
<td>1% TFA in MilliQ water</td>
<td>1% TFA, 80% MeCN in MilliQ water</td>
</tr>
<tr>
<td>0.08% TFA in MilliQ water</td>
<td>0.08% TFA, 30% Isopropanol, 70% MeCN</td>
</tr>
</tbody>
</table>

There are usually two components to the mobile phase in reverse-phase HPLC: an ion pairing reagent and an organic modifier or solvent component. The most commonly used buffers in the research described in this thesis, contained 0.08% trifluoroacetic acid (TFA) as the ion pairing reagent and 0-80% acetonitrile (MeCN) as organic phase. These were prepared as follows:

1. To make buffer A, 800 μl of TFA was added to 1 L of milli-Q water.
2. To make buffer B, 200 ml of milli-Q water and 800 ml MeCN were measured separately, then combined. Due to an endothermic reaction, the combined volume is slightly less than 1 L. Approximately 600 μl of TFA was added to this mixture.
3. The absorbance of each buffer at 214 nm by UV spectroscopy was measured, and small volumes of TFA were added to buffer B until the absorbance at 214 nm, of the two buffers was balanced.
4. Buffer A was filtered to 0.2 μm using a nitrocellulose filter (Sartorius), and buffer B was filtered to 0.45 μm using a regenerated cellulose filter (Sartorius). The buffers were protected from light by wrapping the bottles in tin foil.

The other buffer combinations in table 3.2 were prepared in a similar way using the various components listed. Buffers containing organic solvent were always filtered through regenerated cellulose filters as described. In addition to the 2 different reverse-phase columns and 3 different buffer combinations, a number of different organic solvent gradient profiles were used. These are shown in Table 3.3.
Table 3.3. Reverse-phase HPLC buffer gradient profiles. Light grey arrow represents opening of injection loop to the flow; t = 0 minutes. Dark grey arrow represents closure of loop.

<table>
<thead>
<tr>
<th>Program step</th>
<th>% Buffer B</th>
<th>Program 1 (minutes)</th>
<th>Program 2 (minutes)</th>
<th>Program 3 (minutes)</th>
<th>Program 4 (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Step 1</td>
<td>5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Step 1a</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Step 1b</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Step 2</td>
<td>80</td>
<td>48</td>
<td>55</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td>Step 3</td>
<td>100</td>
<td>49</td>
<td>56</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>Step 4</td>
<td>100</td>
<td>52</td>
<td>58</td>
<td>48</td>
<td>44</td>
</tr>
</tbody>
</table>

During the course of this research, more than 250 reverse-phase HPLC experiments were performed. Some resulted in peaks containing proteins that could be sequenced by N-terminal sequencing (described in section 3.2.6). Others resulted in peaks containing proteins that were able to produce a mass spectrum by MALDI-TOF mass spectroscopy (described in section 3.2.5). Many resulted in peaks containing proteins that were unable to be detected by N-terminal sequencing or mass spectroscopy. This may be due to N-terminal blocking in the former case, or an inability of the protein(s) to be ionised in a MALDI-TOF system in the latter case. The results of these experiments will be discussed in several subsequent sections, as reverse-phase HPLC was retained as a protein purification method throughout the entire project, despite modifications to (and often removal of) other purification steps. Optimisation of HPLC was achieved by the modification of various chromatographic conditions including mobile phase, organic solvent and gradient profile as described (Hubbard, 1993; Serwe & Meyer, 1994; Young & Wheat, 1990).

3.2.2 Modifications - Separation of nuclei

Neuronal inclusions have been described as intranuclear, perinuclear and within dystrophic neurites (Becher et al., 1998; DiFiglia et al., 1997). The Congo red-stained inclusions described in Chapter 2 were observed in all of these locations. It has been suggested that neuronal dysfunction could be a result of the nuclear localisation of a particular form of huntingtin which aggregates there (Sadou et al., 1998). In order to improve the likelihood of purifying these relatively small inclusions from blocks of tissue, and because of the possible toxic effects of the neuronal intranuclear inclusions
(NIIs), it was decided to isolate nuclei from tissue homogenates at the beginning of the purification procedure. Two different methods were used to separate nuclei from other cellular components during the course of this research. The first method that was attempted was taken from Davies et al. (1999) and is described in section 3.2.8.1. This method utilized a sucrose gradient and high-speed centrifugation to achieve a high degree of nuclear separation. Unfortunately however, the yield of nuclei was deemed too low for purification of inclusions as very little protein was retained following the subsequent purification steps. A second, somewhat cruder method utilized a low speed spin following cell lysis to pellet nuclei as well as large cellular debris. Although the nuclei were less pure than obtained using the previous method, the amount of protein retained at the end of all the processing was sufficient to observe on chromatograms during HPLC. This method is described in section 3.2.8.2.

To test these methods and to avoid wasting precious human brain tissue, rat brain obtained from an animal sacrificed for a different project, was used to determine success. Because the human tissue listed in table 3.1 was all processed according to the method of Cooper et al. (1987), new tissue was obtained for analysis according to the modified protocols. The details of this tissue are given in table 3.4.

Table 3.4. Brain number, type, region and HD grade of tissue processed using modifications to original protocol. PM = post-mortem. Control and HD brains were age and sex matched.

<table>
<thead>
<tr>
<th>Brain</th>
<th>Type</th>
<th>Sex / Age at Death</th>
<th>Region</th>
<th>Repeat # / (HD Grade)</th>
<th>PM Delay</th>
</tr>
</thead>
<tbody>
<tr>
<td>H113</td>
<td>Control</td>
<td>M/68</td>
<td>Middle-frontal gyrus</td>
<td>NA</td>
<td>19</td>
</tr>
<tr>
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<td>Control</td>
<td>M/78</td>
<td>Middle-frontal gyrus</td>
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<tr>
<td>HC81</td>
<td>HD</td>
<td>F/70</td>
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<td>19/41 (1)</td>
<td>8</td>
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<tr>
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<td>HD</td>
<td>F/57</td>
<td>Middle-frontal gyrus</td>
<td>17/44 (2)</td>
<td>19</td>
</tr>
<tr>
<td>HC91</td>
<td>HD</td>
<td>F/63</td>
<td>Middle-frontal gyrus</td>
<td>23/44 (3)</td>
<td>5.5</td>
</tr>
</tbody>
</table>

3.2.2.1 Sucrose gradient method (Davies et al., 1999)

1. Frozen brain tissue was thawed and homogenised in a teflon-glass homogenizer, in 0.5 ml/100 mg (homogenization buffer / tissue); homogenization buffer = 0.25 M sucrose in buffer A (50 mM triethanolamine; 25 mM KCl; 5 mM MgCl₂; 2.6 mM DTT; 0.5 mM PMSF) made fresh each use.

2. The homogenate was adjusted to 5 mM DTT and centrifuged at 800 x g for 15 minutes at 4 °C.
3. The supernatant was discarded and the pellet was re-homogenized in 3.7 ml of homogenization buffer.
4. The new homogenate was mixed gently with 7.4 ml of 2.3 M sucrose in buffer A.
5. The mixture was overlaid onto 1.9 ml of 2.3 M sucrose in buffer A, in SW-40Ti ultracentrifuge tubes (Beckman).
6. Tubes were centrifuged at 26 400 rpm for 120 minutes.
7. Nuclei partition to the interface of the 2.3 M sucrose ‘cushion’ and the mixed solution. The top layer was carefully pipetted off and the nuclear layer was gently pipetted into a siliconized Eppendorf tube (1.5 ml) for further processing.

3.2.2.2 Sucrose homogenate method (W. van Roon-Mom, personal communication)
1. Frozen brain tissue was thawed and homogenized in a teflon-glass homogenizer in 1 ml/100 mg (homogenization buffer / tissue), buffer = 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 17 μg/ml PMSF (added fresh), sonicated before use.
2. Homogenates were centrifuged at 800 x g for 15 minutes at 4 °C.
3. The supernatant was removed, nuclear pellets were resuspended in lysis buffer (400 mM KCl, 1% Triton X-100, 1mM PMSF) and stood on ice for 30 minutes.
4. Lysed nuclear fractions were then centrifuged at 13 000 rpm for 15 minutes and pellets were processed as previously described from step 6 in section 3.2.1.1.

3.2.2.3 Staining of nuclei
In order to determine the efficacy of the nuclear separation protocols, a method was developed to determine the yield and purity of nuclei in a sample using Mayer’s haematoxylin, a stain that stains nuclei purple.
1. Nuclei were pelleted by centrifugation at 800 x g for 5 minutes.
2. 100 μl of Mayer’s haematoxylin was added to pelleted nuclei and mixed by trituration.
3. Stained nuclei were pelleted by centrifugation at 800 x g for 5 minutes and washed twice in sucrose buffer (0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA).
4. Pelleted nuclei were resuspended in 25 μl of sucrose buffer and counted using a hemocytometer.
3.2.3 Other Modifications

In order to assist efforts to obtain peptide sequence from purified proteins, and to circumvent possible N-terminal blockage (to sequencing) of proteins, a number of different HPLC buffer combinations were used (as described in section 3.2.1.4) and cleavage of proteins into peptide fragments was performed. Two methods of cleavage were applied: enzymic (using trypsin) and chemical (using cyanogen bromide). These methods are described below. In order to increase the solubility of proteins targeted for trypsin cleavage, reduction and alkylation of cysteine residues was performed.

3.2.3.1 Reduction and Alkylation

Reduction and alkylation of peptides involves the S-carboxymethylation of cysteine residues within the protein. This is done using a reducing agent such as dithiothreitol (DTT) to break disulphide bonds between cysteine residues, and iodoacetic acid (IAA) to convert cysteine to S-carboxymethylcysteine. The chemical reaction was performed according to Fontana & Gross (1986) as follows:

1. 1-10 nmol of purified protein was solubilized in 1 ml (6 M GHCl; 3 mM NaEDTA; 0.2 M Tris-Cl, pH 8.0)
2. 6.2 mg DTT was added and dissolved to a final concentration of 40 mM.
3. Reducing solution was mixed gently by rotation at room temperature for 3 hours.
4. The solution was rapidly cooled on ice. 15 mg of IAA and 20 μl of fresh 4 M NaOH were added to the reduced peptide sample and incubated in the dark for 1 hour.
5. Reduced and alkylated proteins were re-purified by reverse-phase HPLC, using 1% TFA in the mobile phase, and collected for trypsinization based UV detection at 280 nm.

\[
\text{Cysteine} + \text{Iodoacetic acid} \rightarrow \text{S-carboxymethylcysteine}
\]

![Figure 3.1. Diagram illustrating the modification with iodoacetic acid, of a cysteine residue to S-carboxymethylcysteine.](image-url)
3.2.3.2 Trypsinization

Trypsin is one of the most specific proteolytic enzymes available. It is very widely used and is available from a variety of sources. However, because trypsin may be contaminated with small amounts of chymotrypsin, it is often treated with L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK) which inactivates chymotryptic activity (Wilkinson, 1986). For the research described in this chapter, TPCK-treated trypsin was used.

Trypsin specifically cleaves peptide bonds on the C-terminal side of lysine and arginine residues. Like all enzymes, trypsin is sensitive to temperature, pH, and enzyme-substrate ratio, and cleavage may fail or be incomplete if the conditions are not perfect. Protein and peptide cleavage was performed according to Wilkinson (1986) as follows:

1. 1 ml aliquots were dried by vacuum evaporation in siliconized Eppendorf tubes.
2. 1 ml of 100 mM NH₄CO₃ (aq), pH 8.0 was added to dried sample.
3. 50 μl of 10 mg/l (wt/vol) TPCK-treated trypsin (Boehringer Mannheim) in 0.01 M HCl was added and the mixture was incubated at 37 °C for 3 hours.
4. 10 μl of 2.5 M PMSF in ethanol was added to the mixture to a final concentration of 25 mM.
5. Trysinized samples were injected onto a reverse-phase HPLC column, separated with a mobile-phase containing 0.08% TFA, and peptides were collected based on UV detection at 214 nm.

3.2.3.3 Cyanogen Bromide cleavage

Cyanogen bromide (CnBr) reacts with the sulphur of the thioether side chain of methionine residues to yield a mixture of homoserine and homoserine lactone plus methylthiocyanate (Fontana and Gross, 1986). When applied to methionine-containing proteins, the bond involving the carboxyl-group of methionine is cleaved. As a more robust alternative to the enzymic cleavage by trypsin described above, chemical cleavage using CnBr was applied to the purified proteins that were difficult to characterise. CnBr cleavage was performed as follows:

1. Peptide samples were dried to near-dryness by vacuum evaporation, and re-solubilized in 50-200 μl 70% formic acid (in MilliQ water).
2. 10 μl of 0.5 mg/μl (500 mg/ml) cyanogen bromide in 70% formic acid was added.
3. Tubes were covered in foil and placed at 4°C overnight (18-20 hours).
4. 1 ml of distilled water was added to each sample, and samples were dried to near-dryness by vacuum evaporation.
5. Step 4 was repeated.
6. Cleavage products were taken up in 6 M GHCl (containing 3 mM NaEDTA; 0.2 M Tris-Cl, pH 8.0), injected onto reverse-phase HPLC column, separated with a mobile phase containing 0.08% TFA and collected based on UV detection at 214 nm.

3.2.4 Siliconization of tubes
Siliconized Eppendorf tubes (1.5 ml) were used for most of the processing and handling of samples from tissue homogenisation to HPLC. Tubes were siliconized as follows:
Uncoated tubes were dipped in a 2% solution of dichlorodimethylsilane (Acros) (2% (v/v) in distilled water) and inverted several times. Tubes were dried upside down on tissue paper for 2-3 hours, rinsed with distilled water and dried a further 2-3 hours before storage. The entire procedure of coating, drying and rinsing was performed in a fume cupboard. Only for the collection of peaks from reverse-phase HPLC that were to be submitted for N-terminal sequencing, were non-siliconized tubes used, as the siliconizing reagents can interfere with this form of peptide sequencing.

3.2.5 MALDI-TOF Mass Spectrometry
Matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry was performed using the following items of equipment:
- G2024A Sample Prep Accessory (Hewlett Packard)
- G2025A MALDI-TOF Mass Spectrometer (Hewlett Packard), equipped with:
  a) Nitrogen laser (337 nm emission, 150 μJ maximum output, 3 ns pulsewidth)
  b) 30 kV ion acceleration potential
  c) 1 meter flight tube
  d) 9350AM 500 MHz digital oscilloscope (Le Croy) G2030AA
- ChemStation loaded with Hewlett Packard MALDI-TOF software
MALDI-TOF mass spectrometry is undertaken by mixing sample (or standard) with a molar excess of a suitable matrix, applying this mixture to a probe tip, and vacuum drying to produce a layer of crystals of both sample and matrix components (Burlinghame et al., 1999; Siuzdak, 1996). The probe tip is irradiated by pulses of laser light within the MALDI-TOF mass spectrometer, under high vacuum. Laser energy is transferred from the matrix to enable ablation and ionisation of sample, and the ions that are produced are accelerated through an electric field and into the flight tube. Within the flight tube, ions are separated according to their unique mass to charge (m/z) ratio, being proportional to the ion’s time of flight. Small ions and highly charged ions reach a detector (at the opposite end of the flight tube) before large ions and weakly charged ions. The formula that described the relationship between these variables is: \( t = A(m/z)^{1/2} \), where \( t \) = time of flight, \( A \) = instrument calibration constant, \( m \) = mass, and \( z \) = charge. Standards of known molecular weight are used to calibrate the instrument prior to measurement of samples of unknown molecular weight. The mass spectra that are displayed in this thesis are averages of detector signals, from multiple laser shots (>50). This averaging enables an increase in signal to noise ratio.

### 3.2.5.1 Sample preparation

1. 0.5 µl of α-CHC (α-cyano-4-hydroxycinnamic acid; Hewlett Packard) matrix was applied to each probe tip mesa (sample positions), and vacuum crystallised in the G2024A Sample prep accessory. Crystal formation was monitored through a 25x lens. This step was repeated once.

2. 0.5 µl of a sample:matrix mixture (1:1) was applied to a mesa and vacuum crystallised as before. Crystallisation was monitored as before. This step was repeated three times.

3. The probe tip was inserted into the MALDI-TOF mass spectrometer for data acquisition

### 3.2.5.2 Data acquisition

After insertion of the probe tip into the MALDI-TOF, the mass spectrometer was left to reach a high vacuum (< 1 µTorr). Single and/or multiple shot modes within the MALDI-TOF software were used to acquire data. Molecular weight standards
consisted of recombinant human insulin (5808.66 Da; Novo Nordisk Pharmaceuticals) diluted in 50% acetonitrile and MilliQ water to 10 μM, and somatostatin (1638.91 Da; Bachem H-2260) diluted similarly. The masses given are ionised molecular weights: M + H+. Two additional molecular weight peaks were provided by these standards for calibration purposes: Insulin + 2H+ (2904.83 Da), and a sodium-adduct of somatostatin, M + Na+ (1660.90 Da).

The 0-10PS, 0-20PS and 0-100PS methods (programs within the MALDI-TOF software package) were used to acquire data in positive polarity mode, in the molecular weight range of 0-10, 0-20 or 0-100 kDa. Following external calibration with the standards discussed above, the MALDI-TOF software automatically calculates the molecular weights of unknowns within samples. A mass spectrum for the molecular weight range investigated is generated.

### 3.2.6 N-terminal Sequencing

N-terminal protein sequencing was performed by either Catriona Knight or Indu Anthony, on a Procise 492 protein sequencer (Applied Biosystems) equipped with an on-line PTH analyser. This is a core service within the School of Biological Sciences, University of Auckland. Protein samples were absorbed onto Biobrene treated glass fibre filters (Applied Biosystems) in microcartridges and analysed in pulsed-liquid mode. Separation of amino acids was performed by HPLC on a Spheri-5 PTH column (5 μm, 220 mm x 2.1 mm; Applied Biosystems). Peptide sequences of at least five amino acids were obtained if possible.
3.2.7 Progression of methodology

Separation of nuclei (1 000 x g) → Tissue homogenisation NaCl / Sucrose buffer → Homogenates pelleted at 10 000 x g → Collagenase treatment

Polarised-light microscopy → Congo red staining of homogenates → Solubilisation Formic Acid/ GHCl

Filtering to 0.2 μm → Gel filtration HPLC → Calibration

Reverse Phase HPLC → Reduction & Alkylation Trysinization, CNBr

0.08% TFA, 80% MeCN in B → 0.08% TFA, 70% MeCN, 30% IsoP in B → 1.0% TFA, 80% MeCN in B

Mass Spectrometry N-terminal Sequencing

Figure 3.2. Flow chart demonstrating a summary of the progression of methodology used for the research described in this chapter.
3.3 Results

3.3.1 Results by Cooper Method

As discussed above, early efforts to purify aggregated protein from HD brain followed methods used by G.J.S. Cooper (1987) to purify amylin from plaques within diabetic pancreases. The middle-frontal gyrus from two control brains and two HD brains were processed according to this method. The results of this approach are described below.

3.3.1.1 Congo red staining of tissue homogenates

An aliquot of each tissue homogenate was removed for Congo red staining to investigate the purity of amyloid-like particles, prior to solubilization (and consequent disruption of the peptide structure required for Congo red staining with birefringence). As shown in figure 3.3 (a & b) there is Congo red staining without any obvious birefringence in the control sample. In contrast, the HD tissue homogenate (figure 3.3 c & d) reveals Congo red staining with ample birefringent particles present. Tissue homogenates were further processed as described and proteins were separated by gel filtration.

3.3.1.2 Gel Filtration

Gel filtration resulted in a crude separation of proteins and peptides based on their size. Measured by ultra-violet spectroscopy at 280 nm, individual proteins and peptides were not able to be resolved, however the elution profile of the mixture was plotted and 1 ml aliquots were collected. The molecular mass range in each aliquot was estimated using molecular weight standards.

A representative calibration curve constructed from these standards is shown in figure 3.4. Characteristic gel filtration chromatograms for HD and normal brain are shown in figure 3.5. The x-axis in all chromatograms (including reverse-phase chromatograms) refers to the time (in minutes) following the opening of a valve allowing the sample to be pumped on to the particular column in use. Elution volume is then calculated as a function of time and flow rate.
Figure 3.3. Congo red staining of tissue homogenates. (a) Transmitted light image of Congo red stained, amyloid-enriched tissue homogenate from the middle frontal gyrus of a normal control brain, corresponding to the polarized light image in (b). (c) Transmitted light image of Congo red stained, amyloid-enriched tissue homogenate from the middle frontal gyrus of a Grade 2 (HC76) HD brain, corresponding to the polarized light image in (d). Tissue homogenates were prepared as described in section 3.2.1.1 and stained according to the protocol in section 3.2.1.2. Scale bar = 25 μm. The pattern of pink-red staining in (c) appears to be much more structured and ‘fibrillar’ than that in (a), and birefringence is apparent in (d) yet absent in (b). The apparent colour of birefringence is affected by the section/smear thickness; hence a light green colour is not necessarily required for a positive stain for amyloid.
Figure 3.4. Calibration curve of molecular weight standards used in gel filtration. \( R^2 = 0.9367; y = -0.0359x + 6.4926 \). Gel filtration HPLC was performed using the equipment and according to the methods described in section 3.2.1.3. Molecular weight standards include (from left): bovine serum albumin (66 430 Da; Serva), cytochrome C (12 360 Da; Sigma), recombinant human insulin (5808 Da; Novo Nordisk Pharmaceuticals) and somatostatin (1638 Da; Bachem). Blue dextran (2 000 000 Da) was used to calculate the column void volume \( (V_0) \). The calibration curve was used to estimate the molecular weight of proteins eluting during gel filtration HPLC of tissue homogenates.

The chromatograms show an increase in the absorbance maxima \( (A_{\text{max}}) \) at an elution volume of approximately 8.8 ml (8 minutes, 42 seconds elution time, \( R_f = 88 \text{ mm} \)) in HD samples relative to control samples (peak I). The position of this peak corresponds to an approximate molecular mass of approximately 2150 daltons according to the calibration curve (figure 3.4). UV detection was performed at a wavelength of 280 nm, which is absorbed by aromatic residues such as tyrosine, tryptophan and phenylalanine. The reason 280 nm was chosen over 214 nm, which is absorbed by peptide bonds, is the proximity of the extinction coefficient of guanidine to this latter wavelength. The shape and width of the peaks in the chromatograms, suggests that all samples contained a complex mixture of peptides and proteins. Although these were not resolved using this method, 1 ml fractions were collected and subjected to further purification by reverse-phase HPLC.
Figure 3.5. Gel filtration chromatograms of normal and HD middle-frontal gyrus following homogenisation and amyloid-enrichment. (a) Typical (n=2) gel filtration chromatogram obtained after 200 µl of tissue homogenate from a neurologically normal control brain (H111), subjected to amyloid-enrichment methods (section 3.2.1.1), was injected onto a Phenomenex SEC-2000 size exclusion chromatography column. The absence of amyloid particles was confirmed by Congo red staining of tissue homogenates prior to HPLC (figure 3.3a & b).

(b) Typical (n=2) gel filtration chromatogram obtained after 200 µl of tissue homogenate from a Grade 4 HD brain (HC87; 21/50 repeats), subjected to amyloid-enrichment methods (section 3.2.1.1), was injected onto a Phenomenex SEC-2000 size exclusion chromatography column. The presence of amyloid particles was confirmed by Congo red staining of tissue homogenates prior to HPLC (figure 3.3c & d).

Mobile phase = GnHCl, pumped at 1 ml/min. Detection = UV @ 280 nm. The equipment and methods used for gel filtration HPLC are described in section 3.2.1.3.
CHAPTER THREE – PURIFICATION AND CHARACTERISATION OF POORLY SOLUBLE PROTEIN FROM HUNTINGTON DISEASE BRAINS

(a) 

(b) 

Time (minutes)
3.3.1.3 Reverse-phase HPLC and N-terminal sequencing

Gel filtration fractions collected between 8-10 ml elution volume (peak I) were subjected to reverse-phase HPLC on a Jupiter (Phenomenex) C18 column. UV detection at 214 nm was used as this wavelength is much more sensitive for detecting proteins than 280 nm, and there were no interference problems from the mobile phase components. Preliminary reverse-phase HPLC experiments on these fractions resulted in good separation of proteins within control samples (figure 3.6a) and separation against a relatively high background in HD samples (figure 3.6b). This background was also observed in repeat experiments and was consistent for all HD samples. In all cases, 0.08% triflouroacetic acid was used as an ion-pairing reagent, 80% acetonitrile as the organic component of buffer B, and program 1 as the buffer gradient profile.

Despite the observed background, the most obvious difference between control and HD samples was the size of an absorbance peak eluting at approximately 50% buffer B (peak II, figure 3.6). This peak was consistently bigger in HD samples than control samples with an $A_{214}$ ratio of 2:1, although the high background in HD samples may have partly contributed to some of this difference. Due to this difference in peak size, the protein components of this peak were targeted as potential HD-associated proteins. This difference could have been due to an alteration in conformation affecting purification, an increase in protein expression or a decrease in protein breakdown. Initial attempts to characterise peak II by N-terminal sequencing were unsuccessful, probably due to the poor separation of proteins in HD samples. Despite the high background caused by contaminants, an impure protein sequence was obtained as follows in figure 3.7.

![Figure 3.7. Primary structure of unknown protein(s) obtained by N-terminal sequencing of peak II (figure 3.6a). Ambiguities representing a possible minor protein species in peak II are shown in brackets. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) found no identity for either major or minor primary sequences with known proteins.](image)

The initial yield of protein detected by the sequencer was estimated to be 5.92 picomoles and the repetitive yield was 94%. In repeat experiments, peak I was
collected manually to decrease the dilution of the protein(s) present that occurred when 1 ml aliquots were collected from the time of valve-opening. When this more concentrated sample was then injected on to a reverse-phase column, peak II was slightly higher indicating the presence of more protein, and the background had decreased slightly (figure 3.8a). In an effort to separate the protein(s) of interest from contaminants peak II was collected, vacuum-dried to remove the acetonitrile, TFA was added to an estimated final concentration of 5% (v/v), and the mixture was subjected to a second round of reverse-phase HPLC (figure 3.8b). Re-injection led to the collection of a sharp peak at 50% buffer B (peak III) with no background or obvious shoulder indicating that this peak was likely to consist of a single component. When submitted for N-terminal sequencing the result was once again not clear, this time due to the protein being present at very low level despite being relatively clean. The initial yield of protein detected by the sequencer was 2.33 picomoles, far lower than that expected based on the height of peak III, and less than half the yield of protein detected during sequencing of peak II. The repetitive yield was once again 94%. Despite the low level of protein, an estimate of the protein sequence was obtained as illustrated in figure 3.9.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Leu (Thr)</td>
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<td>(Lys)</td>
<td>Met</td>
<td>Ala</td>
<td>(Thr)</td>
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</tbody>
</table>

Figure 3.9. Primary structure of unknown protein obtained by N-terminal sequencing of peak III (figure 3.8b). Ambiguities (position 1 in this figure) and uncertain residues (positions 6, 9 and 10) are shown in brackets. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) found no identity with known proteins using either possibility for position 1.

This sequence was remarkably similar to the protein sequence illustrated in figure 3.7, however as before a BLAST search revealed no identity with known proteins. Due to the low level of protein detected by the sequencer, and the uncertainty of some of the residues, this data could not be relied on. A degree of protein 'stickiness' and the associated loss of protein to the sides of tubes had been anticipated. It is for this reason that Eppendorf tubes were siliconized (section 3.2.4).
Figure 3.6. Reverse-phase chromatograms of gel filtration fractions obtained between 8-9 ml elution volume, from normal and HD tissue. (a) 200 μl of the 8-9ml fraction (figure 3.5a) from control (H111) brain was injected onto a Phenomenex C18 Jupiter column. Buffer A= 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 1 (Table 3.3). Detection = UV @ 214 nm. A similar chromatogram was observed for the equivalent gel filtration fraction of a different control brain (H112; data not shown).

(b) 200 μl of the 8-9 ml fraction (figure 3.5b, labelled peak I) from HD (HC87; 21/50 repeats, Grade 4) brain was injected onto a Phenomenex C18 Jupiter column. Buffer A= 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 1. Detection = UV @ 214 nm. A similar chromatogram was observed for the equivalent gel filtration fraction of a different HD brain (HC76; 17/42 repeats, Grade 2; data not shown).

The equipment and methods used for reverse-phase HPLC are described in section 3.2.1.4.
CHAPTER THREE - PURIFICATION AND CHARACTERISATION OF POORLY SOLUBLE PROTEIN FROM HUNTINGTON DISEASE BRAINS

(a)

(b)
CHAPTER THREE – PURIFICATION AND CHARACTERISATION OF POORLY SOLUBLE PROTEIN FROM HUNTINGTON DISEASE BRAINS

Figure 3.8. Optimisation of protein recovery at 50% buffer B. (a) 200 μl of manually collected peak I from HD brain (HC76; 17/42 repeats, Grade 2) was injected onto a Phenomenex C18 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 1. Detection = UV @ 214 nm.

(b) Peak IIb (total volume ~60 μl, figure 3.8a) was re-injected onto a Phenomenex C18 Jupiter column following removal of acetonitrile and addition of TFA (~5% (v/v) final concentration) as described. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 1. Detection = UV @ 214 nm.

(c) Pooled peak IIb (total volume ~180 μl) was re-injected onto a Phenomenex C18 Jupiter column following removal of acetonitrile and addition of TFA (~5% (v/v) final concentration) as described. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 1. Detection = UV @ 214 nm. Protein sequence data and a mass spectrum were obtained for peak IIIb.
It was postulated that the apparent loss of protein from peak III between elution from HPLC and N-terminal sequencing may have been due to: i) the protein sticking to surfaces as described above, ii) N-terminal blockage of much of the protein in peak III, or iii) the formation of a precipitate by aggregation of purified monomer.

A subsequent experiment involved the pooling of peak II from three reverse-phase HPLC experiments, and re-injection of these for a second round of reverse-phase HPLC (figure 3.8c). The result of this was another sharp peak at 50% B (peak IIIb), which also appeared to consist of a single component, and was approximately two and a half times the amplitude of peak III. This peak was also submitted for N-terminal sequencing. The initial yield of protein detected by the sequencer was this time considerably higher, being 19.18 picomoles, allowing an unequivocal sequencing result. The repetitive yield was again approximately 94%. The sequence that was obtained is illustrated in figure 3.10.

<table>
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<td>Asp (Thr)</td>
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<td>Glu</td>
<td>Ile</td>
<td>Phe (Lys)</td>
<td>Met</td>
<td>Ala (Thr)</td>
<td>Val</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.10. Primary structure of the protein constituent of peak IIIb (figure 3.8c) obtained by N-terminal sequencing. Primary structures obtained from peaks II and III are repeated here for comparison. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) identified the protein as an internal fragment of glial fibrillary acidic protein (GFAP).

This peptide sequence provided a 100% match with an internal segment of glial fibrillary acidic protein (GFAP), a class III intermediate filament protein and cell-specific marker for astrocytes. Figure 3.11 illustrates the position of this segment within the full-length sequence of GFAP.
3.3.1.4 Mass Spectrometry

Mass spectrometry by MALDI-TOF did not result in the detection of any consistent peptide or protein mass for peak II despite two separate attempts using both positive and negative ion modes, and the use of three different matrices (Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid); DHB (2,5-dihydroxybenzoic acid); αCHC (α-cyano-4-hydroxycinnamic acid); all Hewlett Packard; the latter of these was used in all subsequent mass spectrometry experiments). This was almost certainly due to the low abundance of the peptide of interest (eventually characterised as an internal fragment of GFAP). The presence of a mixture of other peptides, as evidenced by the presence of a prominent shoulder on peak II and the impure N-terminal sequencing result (figure 3.7), may also have contributed to the lack of a result. Mass spectrometry of peaks III and IIIb (figures 3.8b & c; HD brain HC76) however, detected two peptide masses within each; a strong peak at 3210.2 Da and a much weaker peak at 3933.7 Da (figure 3.12a). The same two peaks were detected within the peak III and IIIb equivalents for the HD brain HC87.

When the much smaller peak II equivalent from control brain (figure 3.6a) was repurified and concentrated in the same way as peak II had been, mass spectrometry of this peak consistently detected peptides at 3207.7 Da and 3924.8 Da (figure 3.12b).
Figure 3.12. MALDI-TOF mass spectrometry. (a) Typical (n=2 cases) mass spectrum obtained for peaks III and IIIb, following both gel filtration and reverse-phase HPLC of HD brain homogenates (HC76 & HC87). The spectrum is the average of 50 single shots from the laser. (b) Typical (n=2 cases) mass spectrum for the peak III and IIIb equivalents from normal control brain homogenates (H111 & H112). The spectrum is the average of 30 single shots from the laser. Spectra were obtained in positive ion mode. The equipment and methods used to perform MALDI-TOF mass spectrometry are described in section 3.2.5. Human insulin and somatostatin were used to calibrate the mass spectrometer prior to sample analysis. The x-axis measurement m/z = mass to charge ratio.
Given the similarity in size between these two peaks and the two detected from mass spectrometry of peak III/IIIb, it is likely that they represent the same peptide fragments, one of which is an internal fragment of GFAP as confirmed by N-terminal sequencing. The very slight differences (±2.5 Da and ±8.9Da) are greater than the usual expected error margin for MALDI-TOF, however the experiments were performed on separate days and different solutions of the calibrants were used to calibrate the mass spectrometer in each experiment. It is therefore likely that the differences are due to the calibration and the peptides are identical. Because of this conclusion, the peak III equivalent from normal control brain was not submitted for N-terminal sequencing. Hypotheses regarding the nature of the second peptide entity detected in peak III/IIIb and the peak III equivalent from normal control brain are discussed in section 3.4.1.

Investigations into the possible role of the internal fragment of GFAP in the neuropathology of HD are described in Chapter 4.

In parallel with these investigations however, refinements were made to the original protocol of Cooper et al. (1987) and further efforts were made to identify potentially amyloidogenic, aggregating protein species in HD brain. These experiments are described below.

### 3.3.2 Results following nuclear separation

Following the set of experiments described above, modifications were made in an attempt to improve the resolution of HPLC peaks and to identify proteins associated with amyloid-like inclusions in HD. The first modification to the original protocol was the separation of nuclei from other cellular material early in the purification procedure. The results obtained following this modification are described below.

#### 3.3.2.1 Nuclear separation

As described in section 3.2.2, two different methods of nuclear separation were used. The first method was part of a method used by Davies et al. (1999) on R6/2 (described in section 1.9.1, Chapter 1) transgenic mouse brain tissue, to purify nuclear inclusions for their filter-trap assay. The remainder of their method for purification of nuclear inclusions, beyond the initial separation of nuclei, could not be followed due to their use of high concentrations of SDS, a detergent not compatible with the
chromatography columns used in this work. In addition, rat brain tissue was used as a control to determine nuclear yield and purity following nuclear purification and staining with Mayer's haematoxylin as described. The Davies method for nuclear purification resulted in approximately 200 000 nuclei per 100 mg of rat brain tissue. The nuclei often appeared to be broken or were present in clumps. This experiment was carried out in triplicate and a similar yield of nuclei was obtained in each case. When the alternative 'sucrose homogenate' method was applied (also in triplicate), there was an approximately ten-fold increase in yield of nuclei which appeared to be mostly intact, 6-8 μm in diameter, but surrounded by a considerable amount of unstained material.

Based on these experiments the sucrose homogenate method was applied to human brain tissue. Nuclear fractions from human brain were prepared as described in section 3.2.2.2. These fractions then underwent amyloid enrichment as described in section 3.2.1.1. Congo red staining and polarized light microscopy of aliquots removed after collagenase treatment revealed particulate amyloid in HD nuclear fractions as previously described (figure 3.3 c & d). No amyloid was observed in normal control nuclear fractions. The results of further analysis by HPLC, N-terminal sequencing and mass spectrometry are described below.

3.3.2.2 Gel filtration

Amyloid-enriched nuclear fractions from two normal control brains and three HD brains (see table 3.4) were separated by gel filtration HPLC. Gel filtration of nuclear fractions produced chromatograms (figure 3.13) that appeared dramatically different to those for total tissue homogenate (figure 3.5). Firstly, a large proportion of the background contributed to by non-nuclear components had been removed. HD chromatograms had considerably higher absorbance during elution and the elution profiles were more complex, than was observed for normal control brain chromatograms. As before, the resolution of peaks remained poor with gel filtration for all samples, so 1 ml aliquots (and later 0.5 ml aliquots) were collected and separated by reverse-phase HPLC. The two obvious peaks at the far right of the HD chromatograms (figure 3.13b) were consistent in all three HD samples but were not detected in normal control chromatograms (figure 3.13a). Their elution volume was consistent with masses of approximately 140 Da and 55 Da; the former being in the
range of a single amino acid, the latter being smaller than the smallest amino acid glycine (75 Da) suggesting that the peaks do not represent protein or peptides.

Three of the aliquots collected from gel filtration runs revealed differences in the reverse-phase chromatograms they produced, between HD and control samples. The position of these aliquots is indicated on the representative HD gel filtration chromatogram shown in figure 3.13b, by the bars labelled IV, V and VI. The expected molecular mass range based on the calibration curve, for each of these aliquots is approximately 40 to 90 kDa for IV, 7.5 to 17.5 kDa for V, and 3.3 to 7.5 kDa for VI.

3.3.2.3 Reverse-phase HPLC and N-terminal sequencing

When the gel filtration fraction V (HC88) was subjected to reverse-phase HPLC, a peak eluting at 60% buffer B was collected (peak VII) and submitted for sequencing (figure 3.14a). When the equivalent fraction from control brain was subjected to reverse-phase HPLC (chromatogram not shown), this peak was absent and very little absorbance was observed above baseline, consistent with the low absorbance observed during gel filtration. Due to the relatively high absorbance-unit-full-scale (AUFS) setting (0.4) in the chromatogram in figure 3.14a, peak VII appears small despite being slightly above 0.08 absorbance units. Furthermore, peak VII is somewhat broad suggesting the possible presence of more than one peptide.

N-terminal sequencing of the protein(s) within peak VII resulted in the following peptide sequence being obtained:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>Leu</td>
<td>Ile</td>
<td>Arg</td>
<td>Lys</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.15. Primary structure of the protein constituent of peak VII (figure 3.14a) obtained by N-terminal sequencing. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) identified the protein as an internal fragment of the histone protein H3 (calculated molecular weight 15.5 kDa).

The initial yield for this peptide was 8.4 picomoles, and the repetitive yield was 96.8%. The obtained peptide sequence provided a 100% match for the histone protein H3. It is likely that histone proteins were present in abundance in these nuclear preparations, and therefore possible that the purification and sequencing of one was artefactual (ie. co-purified rather than amyloid-associated).
Figure 3.13. Gel filtration chromatograms of normal and HD middle-frontal gyrus following homogenisation in sucrose buffer, nuclear purification and amyloid-enrichment. (a) Typical (n=2) gel filtration chromatogram obtained after 200 µl of the nuclear fraction from a neurologically normal control brain (H113), subjected to amyloid-enrichment methods (section 3.2.1.1), was injected onto a Phenomenex SEC-2000 size-exclusion chromatography column. The absence of amyloid particles was confirmed by Congo red staining of enriched nuclear fractions prior to HPLC.

(b) Typical (n=3) gel filtration chromatogram obtained after 200 µl of the nuclear fraction of Grade 2 HD brain (HC88; 17/44 repeats), subjected to amyloid-enrichment methods (section 3.2.1.1) was injected onto a Phenomenex SEC-2000 size-exclusion chromatography column. Similar chromatograms were observed for the equivalent gel filtration fractions of two other HD brains (HC81 - 19/41 repeats, Grade 1; HC91 – 23/44 repeats, Grade 3). The presence of amyloid particles was confirmed by Congo red staining of enriched nuclear fractions prior to HPLC. Mobile phase = GnHCl, pumped at 1 ml/min. Detection = UV @ 280 nm.
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(a) 

(b) 

Time (minutes)
Figure 3.14. Reverse-phase chromatograms for gel filtration fractions V (a) and IV (b) obtained following gel filtration of the amyloid-enriched nuclear fraction of a Grade 2 HD (HC88; 17/44 repeats) middle-frontal gyrus (figure 3.12b). (a) 200 µl of fraction V was injected onto a Phenomenex C18 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 1. Detection = UV @ 214 nm.

(b) 200 µl of fraction IV was injected onto a Phenomenex C5 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 1. Detection = UV @ 214 nm.
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(a)  

\[ A_{214} \]  

Time (minutes)  

73 %B  

VII (60% B)  

(b)  

\[ A_{214} \]  

Time (minutes)  

43 %B  

VIII (32% B)
CHAPTER THREE - PURIFICATION AND CHARACTERISATION OF POORLY SOLUBLE PROTEINS FROM HUNTINGTON DISEASE BRAINS

The non-detection of a similar peak containing histone H3 in identical fractions of normal control brains is likely to be due to a re-localisation or altered conformation of this protein in HD brain. It is also possible that a peptide of interest was present in peak VII in addition to the histone protein, and that the histone simply sequenced more favourably. The potential relevance of histone H3 to Huntington’s disease is discussed in section 3.4.2. Figure 3.16 illustrates the position of the histone fragment that was sequenced, within the full-length sequence of histone H3.

1 MARTKQTARK STGGKAPRKQ LATKVARSKA PATGGVKKPH RYRPGTVALR
2 EIRRYQKSTE LLIRKLFFQR LMREIAQDFK TDLRFQSSAV MALQECASEY
3 LVGLFEDTNL CVIHAKRVTI MPKDIQLARR IRGERA

Figure 3.16. Full-length sequence of histone H3, with single letter amino acid codes, illustrating the position of the amino acid residues that were sequenced (bold font, box) from peak VII (figure 3.14a). Genebank accession number: 1362815. The residue in position 1 of the purified fragment (Y; tyrosine) was not determined during sequencing.

It was noted during these experiments that numerous blank runs were required to achieve a flat baseline, indicating that certain peptides were strongly adsorbed on the column and did not elute during the reverse-phase run.

In an effort to avoid missing an important peptide, or losing a large proportion of an important peptide to the column, the C18 Jupiter (Phenomenex) column was replaced at this point with a C5 Jupiter (Phenomenex) column. This latter column displays lower retention of proteins and peptides due to having fewer carbon atoms in the hydrophobic alkyl groups, and as such a C5 column is better suited to the separation of hydrophobic samples. All other HPLC conditions remained the same.

When the gel filtration fraction IV (HC88) was subjected to reverse-phase HPLC on this new column, a very clean-looking peak was collected at 32%B (peak VIII, figure 3.14b). When peak VIII was submitted for sequencing, none was obtained due to a very low signal. Given the amplitude of peak VIII, this is most likely to be due to either N-terminal blockage of much of the peptide in this peak or loss of protein (as considered previously) between elution and sequencing.

The reverse-phase HPLC experiments described above were then repeated on fractions IV and V obtained from a second HD brain (HC91; 23/44 repeats, Grade 3), also using the C5 chromatography column.
Figure 3.17. Reverse-phase chromatograms of gel filtration fractions V (a) and IV (b) following gel filtration of a different HD brain tissue homogenate (HC91, Grade 3, 23/44 repeats; see figure 3.12b). 200 µl of each sample was injected onto a Phenomenex C5 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 1. Detection = UV @ 214 nm.
Figure 3.17a shows the reverse-phase chromatogram for fraction V. Peak IX, which eluted at 32% buffer B was collected and submitted for sequencing. The absence of this peak in the chromatogram displayed in figure 3.14a is most likely due to the decreased retention of the C5 column. The position of peak IX matched that of peak VIII described above. Reverse-phase chromatography of fraction IV from HD brain (figure 3.17b) resulted in the collection of two peaks: peak X (also at 32% buffer B) and peak XI (62%B). These were also submitted for sequencing. The absence of the latter peak in the chromatogram displayed in figure 3.14b is most likely due to the increased sensitivity of UV detection on the repeat experiments (using HC91).

As with peak VIII, no sequence could be obtained for peaks IX, X or XI, despite at least two repeat efforts. Mass spectrometry results are presented in section 3.3.2.4 and illustrated in figure 3.18.

Lastly, reverse-phase HPLC was also performed on fraction VI obtained from HC91 (chromatogram not shown). The resultant chromatogram was very similar in appearance to that obtained for fraction V from HC91 (figure 3.17a), having the same peak at 32% buffer B (peak IX). This peak could also not be sequenced, however a mass spectrum was obtained that mirrored the mass spectrum for peak IX. The results described here, and others, are summarised in Table 3.5 at the end of the results section (3.3).

3.3.2.4 Mass Spectrometry

Mass spectrometry consistently revealed a mass entity of 4285 ± 5 Da in peaks VIII, IX, X and XI, despite the latter of these peaks eluting at a completely different hydrophobicity to the other three. Figure 3.18 illustrates a typical mass spectrum obtained from these four samples (MS on peak IX). In addition to this mass, there was a small peak at 8572 ± 4 Da (exception at 8537.9 Da). This peak was usually, but not always, low in intensity relative to the former mass (for some samples the reverse was observed). A third peak, present at approximately 4800 Da was a component of the system being present in control samples and matrix-only controls also. A common phenomenon in mass spectrometry is the detection of either multiply charged isotopes (ie. doubly charged - M+2H), or peaks representing singly charged multimers (ie. 2M+H).
Figure 3.18. MALDI-TOF mass spectrometry. Typical mass spectrum obtained for peaks VIII-XI, following nuclear separation, gel filtration and reverse-phase HPLC of HD brain homogenates (n=2 cases). The spectrum illustrated (MS on peak IX, HC91; see figure 3.15a) is the average of 70 single shots from the laser. Spectra were obtained in positive ion mode. The equipment and methods used to perform MALDI-TOF mass spectrometry are described in section 3.2.5. Human insulin and somatostatin were used to calibrate the mass spectrometer prior to sample analysis. The x-axis measurement m/z = mass to charge ratio. Peaks at 4283.7 and 8570.4 Da are likely to be relevant. 4798.0 Da peak was present in control samples.
It is usually assumed that the higher peak represents the mono-isotropic mass. In the case of the mass spectra obtained for peaks VIII through XI however, the larger mass was close to but not precisely double the mass of the smaller, higher peak. Furthermore, there was no consistent relationship (ie. correlation coefficient) between the two masses, and no correlation could be found between their relative heights and either gel filtration fraction (ie. size range of sample) or elution volume by reverse-phase chromatography peak. The relevance of these observations is discussed in section 3.4.2.

3.3.3 Results following further modifications
Following separation of nuclei, the next modification to the original protocol was a suggested change in reverse-phase HPLC buffers to include isopropanol (D.L. Christie, personal communication). 0.08% TFA was maintained as the mobile phase, however the organic solvent strength was increased by using a mixture of isopropanol (30%) and acetonitrile (70%) for buffer B. As isopropanol is more viscous than acetonitrile, there was an associated increase in back-pressure during chromatography. Due to isopropanol’s greater eluting power, it was expected that peptides would elute earlier with the new buffers. In addition, a slightly shallower elution profile (program 2) was applied, followed later by a step-wise elution profile with a very shallow gradient (0.33% B per minute) between 25 and 28% buffer B (program 3). Other modifications included an increase in the concentration of the ion pairing reagent (to 1% TFA, G.J.S. Cooper, personal communication), reduction and alkylation to assist in subsequent protein cleavage by either enzymic (trypsin) or chemical (cyanogen bromide) methods (C. Knight, personal communication). Cleavage of the polypeptide chain into fragments is commonly used to enable characterisation of proteins that may be N-terminally blocked or otherwise unsuitable for N-terminal protein sequencing (Fontana & Gross, 1986). As no modifications were made to the nuclear purification through to gel filtration steps, and the results of these were essentially the same, the results of these will not be repeated here.

3.3.3.1 Increased Organic Solvent strength
The results of reverse-phase chromatography using isopropanol / acetonitrile (30:70 vol/vol) as the organic modifier are displayed in figure 3.19. When fraction IV
obtained from HC91 (see figure 3.12b) was subjected to reverse-phase chromatography a small peak (peak XII) was collected at 26% B (figure 3.19a). It was assumed due to its position and amplitude and the increased eluting strength of isopropanol, that peak XII was likely to be equivalent to the 32% B peak (VIII, IX and X) collected in previous experiments. This could not be confirmed as sequence data could not be obtained for either and a mass could not be obtained for peak XII. A prominent shoulder on this peak is apparent indicating the presence of more than one peptide, and these peptides could not be separated by repeated rounds of chromatography. Separation of peptides and proteins beyond 26% B was poor and the background rapidly climbed, as revealed by the chromatogram in figure 3.19a.

To achieve separation of the peptides responsible for peak XII, reverse-phase HPLC with program 3 was applied first to crude (ie. without prior gel filtration) HC91 extract resulting in peak XIII at 26% B (figure 3.19b). Reverse-phase HPLC with program 3 was then applied to this peak resulting in peak XIV that was spread between 26 and 27 %B (figure 3.19c). Two shoulders were now apparent, and separation of peptides was worse than under previous conditions. Due to the poor resolution of peaks that was achieved with isopropanol, neither mass spectrometry or peptide sequencing was performed on these peaks.

3.3.3.2 Increased TFA strength; reduction and alkylation; enzymatic and chemical cleavage.

In an effort to improve both the solubility and chances of characterising HD-associated proteins, a new strategy was developed. Firstly, the concentration of the ion-pairing reagent (TFA) was increased from 0.08% to 1%. TFA is a strongly eluting solvent with chaotropic properties.

It was thought that a higher concentration of TFA might improve the solubility of the peptides we were attempting to characterise, and prevent them from sticking to plastic Eppendorf tubes during subsequent reactions. Because of this increase, the pH of the HPLC buffers was lowered below the pH range of Jupiter columns (pH 1.5-10). Insulin was therefore used as a protein standard to ensure that the column was still functioning properly after use at this pH. In addition, 1% TFA was no longer transparent to ultraviolet detection at 214 nm, so detection at 280 nm was required when 1% TFA was used in the mobile phase.
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(a)

XII (26% B)

Time (minutes)

(b)

XIII (26% B)

Time (minutes)
Figure 3.19. Reverse-phase chromatography using increased organic solvent strength.

(a) Reverse-phase chromatogram of fraction IV, following gel filtration of HD brain tissue homogenate (HC91, Grade 3, 23/44 repeats; see figure 3.12b). Elution profile = program 2. (b) Reverse-phase chromatogram of crude (i.e. without gel filtration) HD brain tissue (HC91 middle-frontal gyrus) homogenate. Elution profile = program 3.

(c) Reverse-phase chromatogram following re-injection of peak XIII (figure 3.17b). Elution profile = program 3.

200 μl of each sample was injected onto a Phenomenex C5 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 70% MeCN, 30% isopropanol; Flow rate = 250 μl/min. Detection = UV @ 214 nm.
The increase in TFA concentration resulted in effective elution of peptides from the column demonstrated by flat baselines on blank runs. The resolution of hydrophobic peptides however was only marginally better than with isopropanol, being very poor above ~35% buffer B. Evidence for this observation is provided by the high background absorbance seen above ~35% buffer B concentration in the chromatograms illustrated in figures 3.19b and 3.20a. Regardless of this, 5 fractions were collected from reverse-phase chromatography of crude (ie. without prior gel filtration) HC88 extract: peaks XV (29% B), XVI (32% B), XVII (37% B), XVIII (40% B), and XIX (50% B) (figure 3.20a). All of these peaks were either absent or much reduced (in the case of peak XIX) in control brain extract. A peak resembling peak XVI was present in control brain extract at 31% B. Peaks XV-XIX were reduced and alkylated using DTT and iodoacetic acid as described in section 3.2.3.1, and subjected to a second round of reverse-phase HPLC with 1% TFA in the mobile phase. Small peaks were collected from samples XV and XVI, eluting at the same percent B as previously and termed XV R/A and XVI R/A (the latter of these is illustrated in figure 3.20b).

No peaks were collected for samples XVII-XIX, however the low sensitivity of UV detection at 280 nm may have caused these to be missed. Fractions XV R/A and XVI R/A were then subjected to another round of reverse-phase HPLC, this time with 0.08% TFA in the mobile phase and UV detection at 214 nm. Separation of the tryptic fragments of XVI R/A resulted in seven peptides of varying hydrophobicity (chromatogram not shown). None of these peptides could be characterised due to low signal levels detected by the sequencer. Separation of the tryptic fragments of XV R/A resulted in a single large peak at 34% B (peak XX, figure 3.20c) and potentially others that were below the detection threshold. Peak XX was submitted for N-terminal sequencing, however none was obtained due again to very low signal levels. Lastly, reverse-phase chromatography of crude HC88 extract was repeated (as shown in figure 3.20a), and the same fractions were collected. These were subjected to fragmentation by cyanogen bromide as described in section 3.2.3.3. As described earlier, this reagent cleaves proteins at methionine residues. The rationale behind this experiment was to circumvent possible N-terminal blockage of the sequencing reaction; a problem sometimes encountered during sequencing of non-fragmented proteins.
Figure 3.20 (over page). Reverse-phase chromatography using increased TFA strength, reduction and alkylation, and enzymatic cleavage. (a) Reverse-phase chromatogram of crude (ie. no gel filtration) Grade 2 HD brain tissue homogenate (HC88, 17/44 repeats). 200 µl of homogenate was injected onto a Phenomenex C18 Jupiter column. Buffer A = 1.0% TFA in milliQ water; Buffer B = 1.0% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 4. Detection = UV @ 280 nm.

(b) Reverse-phase chromatogram of peak XVI (figure 3.18a), following reduction and alkylation. 200 µl of peak XVI was reduced and alkylated as described in section 3.2.3.1 and injected onto a Phenomenex C18 Jupiter column. Buffer A = 1.0% TFA in milliQ water; Buffer B = 1.0% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 4. Detection = UV @ 280 nm. Peak XV R/A (not shown) was obtained in a similar manner following reduction, alkylation and re-chromatography of peak XV.

(c) Reverse-phase chromatogram of peak XV R/A following trypsinisation. 150 µl of peak XV RA was digested by trypsin as described in section 3.2.3.2 and injected onto a Phenomenex C18 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 4. Detection = UV @ 214 nm.
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(a)

(b)
It was hypothesised that the low signal levels detected by the sequencer for several of the peaks described in this chapter might be due to N-terminal blockage of the peptide constituent(s) of these peaks. The cyanogen bromide fragments were then separated by reverse-phase HPLC with 0.08% TFA in the mobile phase, using UV detection at 214 nm and the C5 Jupiter column. The chromatogram in figure 3.21 illustrates the two fragments that were collected (peaks XXI at 26% B and XXII at 30% B) following cyanogen bromide cleavage of peak XVIII.

The same two fragments were observed following cyanogen bromide cleavage of peak XIX. Again, neither peak could be characterised using N-terminal sequencing due to a low signal level. MALDI-TOF mass spectrometry of peak XXI revealed mass entities at 4300.3 Da and 8598.8 Da, the latter of which was slightly higher in intensity.

Table 3.5 summarises the HPLC, mass spectrometry and N-terminal sequencing results described in this chapter.
Figure 3.21. Reverse-phase chromatogram of peak XVI (figure 3.18a), following cyanogen bromide cleavage. 200 μl of peak XVI was fragmented with cyanogen bromide as described in section 3.2.3.3 and injected onto a Phenomenex C5 Jupiter. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 μl/min. Elution profile = program 4. Detection = UV @ 214 nm.
Table 3.5. Summary of HPLC, mass spectrometry and N-terminal sequencing results obtained in this chapter. NB/ Mass spectrometry and N-terminal sequencing are not compatible with gel filtration fractions; those boxes are left blank. R/A/T = reduced, alkylated and trypsinised; CnBr = cleaved with cyanogen bromide. *Neither mass spectrometry or N-terminal sequencing of peaks XII to XIV was performed due to the poor resolution of peaks that was obtained using isopropanol as an organic modifier. †Neither mass spectrometry or N-terminal sequencing of peaks XV to XIX was performed as these were intermediate peaks.

<table>
<thead>
<tr>
<th>Fraction/Peak #</th>
<th>Source</th>
<th>%B @ A_max</th>
<th>Mass Spectrometry (Da)</th>
<th>N-terminal sequencing</th>
</tr>
</thead>
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<tr>
<td>I</td>
<td>HC87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Fraction I</td>
<td>50</td>
<td>Not performed</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>III</td>
<td>Peak II</td>
<td>50</td>
<td>3210.2</td>
<td>3933.4</td>
</tr>
<tr>
<td>IIIb</td>
<td>III x 3</td>
<td>50</td>
<td>3210.2</td>
<td>3933.7</td>
</tr>
<tr>
<td>IV</td>
<td>HC88 &amp; HC91</td>
<td></td>
<td></td>
<td>iGFAP</td>
</tr>
<tr>
<td>V</td>
<td>HC88 &amp; HC91</td>
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</tr>
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<td>HC88 &amp; HC91</td>
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<tr>
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<td>Fraction IV – HC88</td>
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<td>8569.2</td>
</tr>
<tr>
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<td>4283.7</td>
<td>8570.4</td>
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<tr>
<td>IXb (chromatogram not shown)</td>
<td>Fraction VI – HC91</td>
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<td>8575.7</td>
</tr>
<tr>
<td>X</td>
<td>Fraction IV – HC91</td>
<td>32</td>
<td>4287.4</td>
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<td>Not performed *</td>
</tr>
<tr>
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<td>Not performed *</td>
</tr>
<tr>
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<td>Not performed *</td>
</tr>
<tr>
<td>XV</td>
<td>HC88</td>
<td>29</td>
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<td>Not performed †</td>
</tr>
<tr>
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<td>Low signal</td>
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<tr>
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<td>30</td>
<td>No detectable mass</td>
<td>Low signal</td>
</tr>
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</table>
3.4 Discussion

3.4.1 Cooper Method

One peptide was successfully purified and characterised by the original method of Cooper et al. (1987). That peptide was the fragment of GFAP illustrated in figure 3.10. Mass spectrometry revealed two mass entities within the peak that was submitted for sequencing: a large peak at 3210.2 Da and a smaller peak at 3933.4 Da. It is not possible to say which of these masses corresponds to the internal fragment of GFAP, however it is likely to be the higher of the two at 3210.2 Da due to its greater relative abundance in the sample that was sequenced. It is possible that both represent fragments of GFAP; one being approximately six or seven residues longer than the other. Another possibility is that one peak corresponds to GFAP, in this case a potential artefact (see Chapter 4), whilst the other peak corresponds to a peptide component of inclusions (and therefore a peptide of interest) which was not sequenced. In order to test this possibility, the known amino acid sequence of GFAP was subtracted from the ambiguous sequences obtained for peaks II and III. The resultant amino acid sequence that was obtained is illustrated in figure 3.22 below.

<table>
<thead>
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<tbody>
<tr>
<td>Leu (Thr)</td>
<td>X</td>
<td>Asp</td>
<td>Ile</td>
<td>Phe</td>
<td>Leu</td>
<td>Met</td>
<td>Leu</td>
<td>Thr</td>
<td>X</td>
<td>X</td>
<td>Lys</td>
<td>X</td>
<td>Lys</td>
</tr>
</tbody>
</table>

Figure 3.22. Primary structure of unknown protein obtained by subtraction of the known amino acid sequence of the GFAP fragment described, from the N-terminal sequencing result for peaks II & III displayed in figures 3.7 and 3.9 respectively. Ambiguities are shown in brackets. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) found 100% homology for residues 4-9 (inclusive; bar) with a C-terminal region of hypothetical protein FLJ20623 (Genebank accession number: 8923575). No homology was found in flanking regions.

A BLAST search was performed using both the full-length amino acid sequence displayed in figure 3.22, and the hexapeptide representing residues four through nine (containing the least ambiguities). A 100% match with the hexapeptide sequence was found for hypothetical protein FLJ20623 (Genebank accession number 8923575), described by the NEDO human cDNA sequencing project. This 524 amino acid protein is hypothetical being based on a cDNA sequence; the mRNA record is
supported by experimental evidence, however the coding sequence is predicted. This hypothetical protein possessed the highest degree of homology to the full-length sequence displayed in figure 3.22, however no homology was found in the regions flanking the hexapeptide. There is no information regarding the expression pattern or potential functional role for this protein and no putative conserved domains were detected. The predicted protein sequence was confirmed by a simulated translation of the mRNA sequence, with the two alternative reading frames containing multiple stop codons. It is possible that this protein or a related protein was present in peaks II and III along with a fragment of GFAP, however no further experiments were performed to determine its relevance. Figure 3.23 illustrates the position within the predicted full-length sequence for this hypothetical protein, of the hexapeptide that possessed homology with the unknown protein illustrated in figure 3.22.

1 MKVLGRSFFW VLFPVLPWAV QAVEHEEVAQ RVIKLRHGRG VAAMQRSQWV
51 RDSCRLSLGL LRPKANAFLNLK LTATAGAVEK DVGLSDKDEKL FQVHTFEIFQ
101 KELNESENSV FQAVYGLQRA LGQDVLDVNN MKESSQRQLE ALREAAIKEE
151 TEYMELLAAE KHQVEALKMN QHNFQQLSML DILEEDVRKA ADRLDEEYEE
201 HAFDDNKSVK GVNFEAVLRY EEEANSKQN TIKREVEDDL GLSLMLDSQN
251 NQYLSTKPRD STIPRAHHF IKDIVTIGML SLPCGWLCAT IGLPTMFYI
301 ICGVLLGPGS LNSIKSIVQV ETILGFVGVFF TLFLVGEFEPS PELKRVWKI
351 SLQGPCYMTL LMIAFGMLWG HLLRJKPTQS VFISTCLSL QSTPLVSEFLM
401 GSARGDKEGD IDYSTVLLGM LVTQDVQLGL FMAVPMTLQ AGASTSSSTV
451 EVVLRILVLQ GQILFSLAAV FLCLIVKYY LIGPYRKHL MELSGKNEEL
501 ILGISAP^IFL MLY^VILKLCV IYVI

Figure 3.23. Full-length sequence of hypothetical protein FLJ20623, with single letter amino acid codes, illustrating the position of the hexapeptide (bold font, box) with 100% homology to an unknown protein (figure 3.22) that was sequenced along with GFAP (see figure 3.10 for ambiguities).

The relevance of the finding of a novel fragment of GFAP in tissue homogenates from HD brain was uncertain at the time of sequencing. In Huntington disease, like numerous other neurological disorders and insults to the brain, astrocytes proliferate in a process known as astrocytosis. That a structural protein from these cells was found to be specifically increased in poorly soluble fractions of HD brain is therefore not altogether surprising. However, the fact that a short, novel fragment of GFAP was purified warranted further investigation of this novel fragment. Moreover, a number
of reports describe the association of various other fragments of GFAP with neurological disorders, and additional studies describe interactions between huntingtin and proteins closely related to GFAP. These studies and experiments designed to investigate the role of the novel fragment of GFAP that we purified from HD brain are described in Chapter 4.

The Cooper method had been used successfully to purify aggregated amylin from large and abundant amyloid plaques in the diabetic pancreas (Cooper et al., 1987). It was clear from the start that the HD-associated amyloid was a lot less abundant and that the method without modification may not enable the isolation of sufficient material for protein characterisation. This proved to be the case and it was decided that the purification procedure needed to be refined to further enrich tissue homogenates in amyloid-like inclusions prior to the HPLC steps.

**3.4.2 Nuclear separation**

By fractionation of nuclei prior to processing and analysis as before, the background within chromatograms was greatly reduced, and HD and control samples appeared dramatically different. Following gel filtration, two peaks consistent with masses of approximately 140 Da and 55 Da were repeatedly detected in HD samples but were absent in control samples. These peaks fell outside the range of the calibration curve, so their approximate masses were extrapolated rather than interpolated. As such, their calculated masses are inaccurate. It is possible that these peaks represent individual aromatic residues (e.g. Phenylalanine, 147.2 Da) or short peptides outside the size-range of the protein standards used. It is also possible that these peaks represent small proteins or peptides that were able to interact in some way with the column packing, thereby delaying their elution. A final possibility is that the peaks represent non-proteinaceous material. No further attempts were made to characterise these peaks.

A number of protein- or peptide-containing peaks were purified from the three gel filtration fractions (IV, V and VI) that were subjected to reverse-phase HPLC. These peaks eluted at 60% buffer B in the case of histone H3 (peak VII), 32% buffer B in the case of peaks VIII, IX and X, and 62%B in the case of peak XI. All of these peaks stood out as being absent in the equivalent gel filtration fraction obtained from control brain preparations.
The relevance of histone H3 to Huntington disease is uncertain. Goebel et al. (1978) observed high levels of three histone-like proteins in the microsomal fraction of purified neurons from both juvenile and adult HD cases, using SDS-polyacrylamide gel electrophoresis. Cooper et al. (2000) demonstrated that histone H1 is an excellent lysyl substrate of tissue transglutaminase and suggested that transglutaminase may cross link histone proteins to glutaminyl donors (such as polyglutamine domains) in nuclear chromatin. The possible involvement of tissue transglutaminase in the formation of nuclear aggregates has been discussed in section 1.10.4, Chapter 1. Cooper et al. (1999) also suggested that a build up of polyglutamine domains together with increased transglutaminase activity may lead to stripping of histones from the nucleus which could in turn lead to increased DNase activity, DNA laddering and cell death. These authors were unable to demonstrate localisation of histone H1 to nuclear aggregates. Likewise, we have been unable to show localisation to nuclear aggregates of histone H3, or double labelling of this protein and ubiquitin (data not shown). This may be due to masking of the epitope following aggregate formation, or due to the acetylation-state of the histones - the antibody used (Chemicon #AB3050) recognises acetylated histone H3 isoforms. A recent study demonstrated a decrease in the acetyltransferase activity of CREB-binding protein (CBP) in the presence of polyglutamine-expanded huntingtin, which led to a decrease in the levels of acetylated histones H3 and H4 (Steffan et al., 2001). Furthermore, the same study demonstrated that the administration of histone deacetylase inhibitors arrested neurodegeneration in two Drosophila models of HD. Hence, although there is no direct evidence of a role for histone proteins in HD pathology, the possibility can not be ruled out.

Peaks VIII-XI were all considerably 'sticky' as revealed by the observation that they would often be eluted during blank runs, even on the C5 Jupiter column. Unfortunately, none of these peaks could be sequenced by N-terminal protein sequencing methods. However, the mass spectrometry data suggested that peptides of the same mass were eluting at quite different hydrophobicities (32%B and 62%B). In addition, although the supposed monoisotopic mass (~4285 Da) was within the expected mass range of fraction VI from the gel filtration experiment, it also appeared in peaks collected following reverse-phase chromatography of fractions IV and V.

These phenomena could be explained by a monomer of molecular weight ~4285 Da, which is undergoing dynamic conformational and multimeric changes during the purification process. In this scenario, it is likely that dimers and/or trimers are present
in fraction V, and that larger oligomers are present in fraction IV. Of course, given the relatively poor resolution of gel filtration there is likely to be some overlap of these entities. Sonication prior to reverse-phase chromatography, and/or the presence of trifluoroacetic acid and acetonitrile could then lead to the breakdown of these oligomers into monomer and some dimer. Conformational changes of the monomer and/or dimer between the hydrophilic (32% B peaks) and hydrophobic (62% B peaks) states would then explain the appearance of very similar mass spectra for peaks VIII-XI. Such a model fits well with our current understanding of amyloid-forming proteins. Contributing to this understanding is recent evidence showing that β-amyloid fibrillogensis involves conformational changes leading to the formation of extended β-pleated sheets, possibly via an oligomeric, α-helix-containing intermediate (Kirkitadze, Condron & Teplow, 2001). In addition to β-amyloid, evidence for this model of fibrillogensis has also been found for prion protein (Jackson, et al., 1999), huntingtin (demonstrating nucleation-dependent polymerization; Scherzinger et al., 1999; reviewed by Wanker, 2000), and a synthetic amyloid peptide (Orpiszewski & Benson, 1999). If the protein component of peaks VIII-XI is undergoing dynamic conformational changes as hypothesised above, it may represent either a fibrillogenic fragment of huntingtin, or a different protein involved in amyloid formation in HD.

The inability of N-terminal sequencing methods to provide sequence information for this monomer was frustrating and may represent an incompatibility of this method with the monomer of interest in HD. Edman degradation is not a suitable method for sequence determination when the N-terminus of a protein is blocked. N-terminal blockage may be natural for a protein of interest, or may occur during a purification procedure. It has been reported that the majority (~80%) of soluble proteins have blocked N-termini (Brown & Roberts, 1976). There are a number of ways in which this can occur, including acetylation or formylation of the amino group, cyclization of N-terminal glutamine to form pyroglutamyl residues, and migration of the O-acyl groups of serine or threonine residues to the N-terminus (Casagranda & Wilshire, 1996). Tarr and Crab (1983) report that exposure to formic acid appears to esterify serine and threonine residues. It is possible that the formic acid treatment of amyloid-enriched protein extracts described in this chapter may have lead to N-terminal formylation of these proteins.
If the protein component of peaks VIII-XI is a peptide fragment of huntingtin with its N-terminus within the polyglutamine stretch, it is likely that cyclization of the terminal glutamine residue to pyroglutamic acid would occur (figure 3.24). This reaction is accelerated by the presence of phosphate ions (Khandke et al., 1989), which were included in the 6M guanidine-HCl solution used during protein purification and gel filtration. Through the work of Melville (cited in Blomback, 1967), it became apparent that cyclization of N-terminal glutamine would be expected during lengthy preparations of proteins and peptides, especially at increased temperature. Theoretically, such transformations might also occur at glutamine residues positioned within proteins and peptides. The protein purification procedure described in this thesis took two to three days to complete, and the temperature was increased at a number of stages including prior to and during collagenase digestion, and during sonication. The presence of pyroglutamic acid in proteins and peptides imposes considerable difficulties in their structural analysis, most seriously the inability for sequential degradation from the N-terminal end to be performed (Blomback, 1967).

Methods have been developed for the removal of N-terminal pyroglutamic acid (Walker & Sweeney, 1996) and for the removal of N-terminal acetyl or formyl groups (Miyatake et al., 1993), however in our experience these methods have not enabled a suspected N-terminally blocked protein to be sequenced (C. Knight, personal communication). Because of this, these methods were not applied during the research described in this thesis. Instead, we applied both enzymatic and chemical fragmentation of purified protein (as recommended by Shannon, Beggerly & Fox, Biomolecular Research Laboratory, University of Virginia, VA, http://hsc.virginia.edu/research/biomolec/seqguid4.htm) and sequencing of the
resultant peptides, as a means to overcome N-terminal blockage of protein sequencing. The results of these refinements are discussed in the following section.

3.4.3 Other modifications

The ‘stickiness’ of the potentially amyloid-associated peptide peaks (VIII-XI) was demonstrated by retention on a C5 Jupiter column with elution during blank runs, and is suggested by the loss of peptide between elution and sequencing. In an effort to improve the solubility, and therefore the chances of further characterisation of these peaks, a number of further modifications were made to the purification protocols.

Firstly, isopropanol (in 30% in acetonitrile) was used to increase organic solvent and therefore elution strength. The addition of isopropanol caused peptides to be eluted earlier and at a lower percent of buffer B than expected due to the greater eluting strength of this solvent. However, the separation of peptides was poor even with a very shallow gradient (0.375% B per minute, figure 3.19c). Because of this poor resolution, no protein or peptide peaks were subjected to mass spectrometry or N-terminal sequencing. Although the use of isopropanol did achieve a more complete elution of proteins and peptides from the column (evidenced by the absence of peptide peaks in blank runs), this organic solvent was not used in any future chromatography experiments due to the poor resolution of peaks described.

Secondly, the concentration of the mobile phase trifluoroacetic acid was increased from 0.08% to 1%. Trifluoroacetic acid is an ion-pairing reagent that complexes with the positively charged groups present in all basic residues and amino-terminus at low pH. It is a strongly eluting solvent with chaotropic properties and it was thought that it might prevent loss of peptides (through stickiness) by improving their solubility. Unfortunately, the resolution of protein fractions was poor above ~35% B and the loss of protein between elution and sequencing continued. Fractions collected under the 1% TFA conditions were subjected to either reduction, alkylation and trypsinisation, or cyanogen bromide cleavage; reactions during which protein and/or peptide could have been lost. None of these protein modifications, enzymic or chemical means of cleavage were able to provide a peptide fragment that could be characterised.
3.4.4 Conclusions

Although three potentially amyloid-associated proteins were characterised by N-terminal protein sequencing (GFAP, histone H3 and hypothetical protein FLJ20623), a number of protein fractions with amyloid-like properties were isolated but not characterised (e.g. peaks VIII to XI). The inability to fully characterise any of these suggests that the characterisation methods used (mass spectroscopy and particularly N-terminal sequencing), were not sensitive or powerful enough to resolve the sticky, aggregating, and potentially N-terminally blocked proteins we targeted. It is possible that modifications used to tailor the purification protocols to amyloid-associated proteins enabled purification of the proteins of interest (such as in peaks VIII-XI). However, this cannot be confirmed due to either loss of protein prior to sequencing or N-terminal blockage of the proteins that were submitted for sequencing. Following a number of modifications to the original protocol, the only protein that was successfully purified and characterised was the histone protein H3. This protein was purified from amyloid-enriched tissue extracts and was characterised on the basis of its absence in equivalent extracts from control brain tissue.

Other techniques were considered for the purification of amyloid-associated proteins in HD such as affinity chromatography using antibodies to the N-terminus of huntingtin, a peptide that has been shown to aggregate in HD. However, the strongly chaotropic, denaturing solvents that were used to solubilise these proteins, would not have been compatible with such a technique and would most likely result in the stripping of these antibodies from the support being used.

If a peptide of interest had been purified within peaks VIII-XI, it is possible that Q-TOF MS/MS (tandem-quadrupole-time of flight mass spectrometry/mass spectrometry) would have enabled characterisation of this peptide. This technique is capable of providing sequence data on a very small quantity of sample (femtomole range) by fragmenting peptides within a vacuum chamber and detecting individual amino acids as they are cleaved from the peptide. Unfortunately, this equipment is not yet available at the University of Auckland. Samples that were prepared later in the research, and will be described in chapters 5, 6 and 7, were sent to APAF (Australian Proteome Analysis Facility) at Macquarie University, Sydney, for this type of analysis.
Chapter 4

Investigations into the role of iGFAP in Huntington Disease

4.1 Overview

This chapter describes the research undertaken to investigate the role of the internal fragment of glial fibrillary acidic protein (GFAP) that was purified from amyloid-enriched HD brain extracts as described in the previous chapter. In order to avoid confusion between this fragment of GFAP and the full-length protein, the internal fragment we purified will be termed iGFAP.

GFAP is a 49.88 kDa, astrocyte-specific, type III intermediate filament protein. Intermediate filaments, along with microtubules and microfilaments, make up the cytoskeleton of most eukaryotic cells (Chen & Liem, 1994). The principal function of most intermediate filaments is to provide mechanical support to the cell and its nucleus (Alberts et al., 1989). There are at least five types of intermediate filament based on amino acid sequence homology and intron splice sites (Bloemendel & Pieper, 1989). These are specifically expressed in restricted tissues, with the exception of type V lamins, which are expressed in the nucleus of all eukaryotic cells (Chen & Liem, 1994). The type III family of which GFAP is a member also includes vimentin and desmin, additional cytoskeletal components. All intermediate filament proteins consist of an α-helical rod domain flanked by non-helical N-terminal head and C-terminal tail domains (Geisler & Weber, 1982; Ralton et al., 1994). The rod domain possesses a heptad repeat pattern of the form (a-b-c-d-e-f-g)n, where positions a and d are usually occupied by apolar residues, favouring the formation of coiled-coils between two α-helices. The rod domain by itself can form only dimers and tetramers; further assembly of these oligomers into higher-order structures requires the...
contribution of the non-helical end domains. The structural domains of GFAP are illustrated in schematic form in figure 4.1.

GFAP is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells including microglia and oligodendrocytes (Reeves et al., 1989). Astrocytes are the most numerous class of glial cell, possessing irregularly shaped cell bodies and often long processes (Kandel, Schwartz & Jessell, 1991). Following brain injury, and in numerous neurodegenerative diseases including HD, astrocytes proliferate in the affected brain region(s) in a process known as reactive gliosis (Cajal, 1928, cited in Xu et al., 1999). They are thought to have a nutritive role (toward neurons), as well as a role inducing endothelial cells to form the blood-brain barrier. Additional functions include the uptake of certain neurotransmitters in synaptic regions, the removal of neuronal debris, and sealing off damaged brain tissue following injury (Kandel et al., 1991). The role of GFAP in this latter function is demonstrated in a study showing that GFAP is necessary for mature astrocytes to react to β-amyloid (Xu et al., 1999). The results of this study suggest that GFAP may be essential to constrain certain types of inflammatory lesions in the brain.
Recent research suggests that glial cells are intimately involved in the active control of neuronal activity and synaptic neurotransmission (reviewed by Araque et al., 1999). For example, Ca$^{2+}$-dependent glutamate release was demonstrated in cultured astrocytes (Bezzi et al., 1998; Parpura et al., 1994). In addition, it was shown that stimulation of cultured astrocytes can lead to neuronal responses (Parpura et al., 1994). These findings and others demonstrate that astrocytes are able to modulate synaptic neurotransmission, leading to the proposal that astrocytes should be considered as an integral modulatory component of the synapse (Araque et al., 1999).

The internal fragment of GFAP (iGFAP) that was purified and characterised by N-terminal sequencing as described in Chapter 3 begins at residue 106 of the parent protein. Residue 106 is further likely to be the first residue of iGFAP as it is adjacent to a protease cleavage site (Arg-X). Based on the mass of this fragment, as determined by MALDI-TOF mass spectrometry (3210.2 Da), the termination residue is likely to be either 132 or 133. This uncertainty is due to the detected mass falling between the sum of the mass of all residues from 106-132 and the sum of the mass of all residues from 106-133. The discrepancy between the calculated and detected masses for iGFAP is likely to be due to modifications to the peptide. For example, 3-chlorination of the two tyrosine residues in this fragment would cause the mass of the fragment 106-132 to be raised to 3209.61 Da (Krishna & Wold, 1998). This type of modification occurs at Cl:tyrosine mole ratios greater than 1, at acidic pH (Nickelsen et al., 1991). Following collagenase digestion during amyloid enrichment, extracts were washed with 150 mM NaCl prior to the addition of 70% (v/v) formic acid. These steps may have enabled the type of modification described above to occur.

Peptide fragments of GFAP have been described on several occasions in the literature. GFAP isolated from normal and gliosed human brain separates into multiple bands ranging in molecular weight from 40 500 to 54 000 Da (Dahl & Bignami, 1975). The lower molecular weight bands (40 500 to 45 000) were demonstrated to be more stable to degeneration and still present after prolonged autolysis. More specifically to Huntington’s disease, Selkoe et al. (1982b) report immunostaining of 45 kDa, 43 kDa and 40 kDa proteins with an antiserum to GFAP, in HD striatum but not in control striatum. These authors also report a 3- to 8-fold increase of full-length GFAP, in HD striatum relative to control. Neither study describes the presumably small (ie. <10 kDa) truncated peptides that are lost from full-length GFAP to generate these fragments.
Further evidence of GFAP fragmentation is provided by Newcombe, Woodroffe & Cuzner (1986) who describe 11 GFAP polypeptide bands of molecular weight 37-49 kDa in gliosed white matter from multiple sclerosis plaques and cerebral infarcts. Other fragments of GFAP have been reported in the literature as being associated with certain neurodegenerative disorders. In the spinal cords of patients with amyotrophic lateral sclerosis (ALS), increased levels of fragmented GFAP were found (Fujita et al., 1998a). Compared with protein extracts from control spinal cord, ALS-spinal cord had decreased GFAP-immunoreactivity for full-length protein (49.88 kDa) and a 45 kDa product, and increased immunoreactivity for 36 kDa and 37 kDa bands. In addition, Fujita et al. (1998a) describe massive degeneration of motor neurons was associated with GFAP-stained astrocytes in the shrunken ventral horn. The 36 kDa and 37 kDa bands were demonstrated by amino acid sequence analysis to be GFAP devoid of 40 and 59 amino acids from the N-terminus respectively. Once again, it is not clear what happens to the N-terminal peptides as the gel illustrating the GFAP fragments described did not extend to molecular weights below ~30 kDa.

Similar sized fragments of GFAP were observed to be increased in the spinal cord of a motor neuron degeneration mouse model (Fujita et al., 1998b; Nagata et al., 1998). Alternative cleavage products of GFAP and the other type III intermediate filament proteins vimentin and desmin are also generated by human immunodeficiency virus type 1 protease (HIV-1 PR) (Shoeman et al., 1990).

Mutations in GFAP are associated with a rare disorder of the central nervous system, known as Alexander disease (Brenner et al., 2001). The pathological hallmark in this disease is the presence of Rosenthal fibres, cytoplasmic inclusions in astrocytes that contain GFAP in association with small heat-shock proteins. Alexander disease is the first reported primary genetic disorder of astrocytes.

Neurofilament proteins, which are closely related to GFAP (being type IV intermediate filaments), are associated with aggregative plaques of various neurodegenerative diseases including Pick’s disease, Parkinson’s disease and Alzheimer’s disease (Singhrao et al., 1998; Strittmatter et al., 1996). Neurofilaments have also been shown to interact with huntingtin containing expanded polyglutamines (Nagai et al., 1999). Additionally, the intermediate filament protein vimentin was demonstrated to coimmunoprecipitate with long polyglutamine fusion proteins (56Q-GFP and 80Q-GFP) expressed in COS-7 cells, but not with short polyglutamine fusion proteins (19Q-GFP) (Onodera et al., 1997).
In investigating the role of iGFAP in HD, an antiserum was raised in rabbit against the first 25 amino acids of iGFAP (Chapter 3). SDS-PAGE and Western blotting were performed to investigate the specificity of the iGFAP-antiserum and it was decided not to further purify specific antibodies from serum. Immunohistochemical studies using this antiserum and a commercial antibody to GFAP (DAKO) were performed to compare the intracellular distribution of GFAP and/or iGFAP within HD and normal brain. Specifically, it was of interest to determine if either GFAP or iGFAP localise to inclusion bodies in HD. Lastly, various steps in the purification procedure (described in section 3.2.1, chapter 3) were analysed for a possible role in the generation of iGFAP.

4.2 Materials and Methods

4.2.1 iGFAP-antiserum

A synthetic peptide identical in sequence to residues 106-130 (inclusive) of GFAP, with the addition of an N-terminal cysteine residue (required for conjugation), was manufactured by Auspep Pty. Ltd. (Parkville, Australia). This peptide has a molecular weight of 3072 daltons. This molecular weight was confirmed by the manufacturers by mass spectrometry and the product purity was determined to be >70% (mass spectrum and high performance liquid chromatogram included in Appendix 1). The synthetic peptide had the following sequence: CAKEPTKLADVYQAELRELRLRLDQL. The underlined portion represents the peptide fragment of GFAP that was purified and sequenced as described in Chapter 3. Peptides smaller than 5 kDa do not generally elicit an immunogenic response and must therefore be conjugated to a large carrier protein to increase their immunogenicity (Harlow & Lane, 1988). Approximately half of the synthetic peptide (by mass) was conjugated with glutaraldehyde to keyhole limpet haemocyanin (KLH; MW = 6x10^6 Da) by Auspep, via a C-terminal cysteine residue, added to the peptide for the purposes of immunisation of a rabbit. Amino acid analysis (also performed by the manufacturers) enabled an estimation of the amount of the synthetic peptide (7 mg) present in the peptide conjugate (32 mg; 21%). Immunisation was very kindly carried out by Ms Louise Moffat (Department of Molecular Medicine, University of Auckland). Conjugated synthetic peptide was
reconstituted at a concentration of 10 mg/ml and diluted ten-fold for the immunisation mix to 1 mg/ml. 100 µl of immunisation mix was added to 900 µl of phosphate-buffered saline (PBS), and 1 ml of Incomplete Freund's Adjuvant. The resulting emulsion was injected subcutaneously at 6-8 sites on a single rabbit. Ethical approval for immunisation was obtained from the University of Auckland's Animal Ethics Committee. Three immunisations were performed over a period of one month, according to the schedule in table 4.1.

Table 4.1. Immunisation schedule of rabbit with KLH-[peptide] conjugate.

<table>
<thead>
<tr>
<th>Day</th>
<th>Date</th>
<th>Procedure</th>
<th>Serum volume</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>27/8/99</td>
<td>Pre-bleed</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>0</td>
<td>27/8/99</td>
<td>First immunisation</td>
<td></td>
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<tr>
<td>7</td>
<td>3/9/99</td>
<td>Second immunisation</td>
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<td>19</td>
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<tr>
<td>34</td>
<td>30/9/99</td>
<td>Sacrifice, harvest serum</td>
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</tbody>
</table>

An ELISA (enzyme-linked immunosorbent assay) was also performed by Ms Louise Moffat on serum obtained at the test bleed 19 days following the first immunisation. The results of this assay are shown in section 4.3.1. Serum obtained at sacrifice, 34 days after the first immunisation was frozen and used at a range of dilutions (listed where used) in immunohistochemistry studies and Western blotting studies described below. Antibodies were not further purified from the serum.

4.2.2 Characterisation of iGFAP-antiserum

Before immunohistochemistry was performed using the iGFAP-antiserum, the specificity of the serum was investigated by SDS-urea-polyacrylamide gel electrophoresis (PAGE) and Western blotting. These techniques were also used to determine the optimal serum dilutions. The methods used for these experiments are described below.

4.2.2.1 Polyacrylamide gel electrophoresis

Proteins were separated on 8% (w/v acrylamide) polyacrylamide gels containing 0.1% SDS and 12 M Urea as denaturing agents. A molecular weight ladder containing protein standards between 10 and 220 kDa (Benchmark™ protein ladder, Life
Technologies) was loaded in one lane. The remainder of the gel forming one very wide line was loaded with 60 µl of a human control brain (H111) tissue homogenate (tissue homogenised in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 µg/ml PMSF (prepared fresh)). Gels were prepared and run as follows:

1. Glass plates and plastic spacers and combs were washed in ethanol followed by milliQ water, assembled on the gel casting stand and checked for leaks.

2. 10 ml of polyacrylamide gel solution was made from the following reagents:

   - 50% acrylamide mix: 1.6 ml
   - Urea: 3.6 g
   - 1.0 M Tris-HCl (pH 8.8): 3.75 ml
   - 10% SDS: 100 µl
   - 10% ammonium persulphate (SERVA, made fresh): 34 µl
   - TEMED (BDH): 15 µl
   - Milli-Q water: 2.0 ml

3. The polyacrylamide gel solution (resolving gel) was pipetted into the space between the glass plates ensuring no air bubbles were present and overlaid with butanol to exclude oxygen, thus facilitating polymerisation at room temperature.

4. Once the gel solution had polymerised (about 15-20 minutes, visualised by the checking the remainder of gel solution), the butanol was poured off and the top of the gel was rinsed with Milli-Q water. A 1 ml stacking gel solution was prepared from the following reagents to aid in the movement of proteins into the gel:

   - 50% acrylamide solution: 510 µl
   - Urea: 1.8 g
   - 1.0 M Tris-HCl (pH 6.8): 650 µl
   - 10% SDS: 50 µl
   - 10% ammonium persulphate: 32 µl
   - TEMED: 8.0 µl
   - Milli-Q water: 3.0 ml

5. A plastic gel comb containing the desired number of teeth (wells) was inserted into the space between the glass plates (leaving a small space for pipetting) and
the stacking gel was pipetted on top of the resolving gel ensuring no air bubbles were present. The comb was removed following polymerisation and the wells were thoroughly rinsed with Milli-Q water.

6. Samples were mixed 1:1 with 2x Laemmli sample buffer (section 6.2.4.1, Chapter 6) and denatured in a boiling water bath for 5 minutes.

7. Electrophoresis of samples was performed in running buffer in a Mini-Protean II electrophoresis cell (BioRad), at 50 V through the stacking gel, increasing to 150 V through the resolving gel.

4.2.2.2 Protein transfer and Western bloting

The technique of Western bloting will be described in section 6.2.5, Chapter 6, as this technique was used extensively in that chapter. Because the method of Western bloting used in this chapter is slightly different to that used in Chapter 6, the materials and methods that were used for the research described in this chapter are described here. Transfer and detection of proteins was performed as follows:

1. Proteins resolved by SDS-urea-PAGE were transferred and immobilised onto Immobilon-P polyvinylidenfluoride (PVDF; Millipore), in a semi-dry transfer unit (BioRad Trans-Blot SD).

2. Regions of PVDF with no protein bound were blocked by incubating in a 5% milk solution for 1 hour with shaking at room temperature.

3. The PVDF membranes were incubated for 2 hours in iGFAP-antiserum at a range of dilutions in sealed strip-wells contacting the membrane (1:1000, 1:5000, 1:10 000, 1:20 000, 1:50 000, 1:100 000 and 1:500 000).

4. The membranes were thoroughly washed in two changes of TBST, then incubated in 3 changes of TBST for 5 minutes each.

5. The membranes were incubated for 1 hour in a solution of anti-rabbit secondary antibody (conjugated to horseradish peroxidase) at a dilution of 1:3000 or 1:6000.


7. The membranes were incubated for 5 minutes in ECL Plus reagent (Amersham) according to the manufacturer's instructions.

8. Membranes were exposed to X-ray film (Hyperfilm ECL, Pharmacia Biotech Ltd.) prior to developing in an Kodak M35 X-OMAT Developer.
Additionally, the affinity of both the iGFAP-antiserum and the commercial rabbit anti-GFAP antibody (DAKO) for the synthetic peptide (Auspep), was assessed by running synthetic peptide on 12% (w/v acrylamide) SDS-urea-PAGE gels. These were prepared as described above (section 4.2.2.1) with adjustment for the percentage of acrylamide. Western blots were detected by the method described above using iGFAP-antiserum at a dilution of 1:5000 and rabbit anti-GFAP at a dilution of 1:10 000. Secondary antibody was used at a dilution of 1:3000. At a later stage, after immunohistochemistry was complete, further PAGE experiments were performed on a range of samples designed to investigate the possible role of the purification procedure in the generation of iGFAP. The preparation of these samples is described in section 4.2.4.

4.2.3 Immunohistochemistry

As before, the human brain tissue was obtained from the New Zealand Neurological Foundation Human Brain Bank (School of Medicine, University of Auckland), and ethical approval for use of this tissue was obtained from the University of Auckland’s Human Subjects Ethics Committee. Immunohistochemistry was performed on floating sections (50 μm) of perfused tissue that had been processed as previously described (section 2.2.7, Chapter 2). For consistency with much of the other work described in this thesis, middle-frontal gyrus tissue from two control brains and two HD brains was used.

Two different primary antibodies were used: rabbit polyclonal anti-GFAP (DAKO) at a dilution of 1:5000, and iGFAP-antiserum (described above), at dilutions of 1:50, 1:100, 1:250, 1:500 and 1:5000. Sections were incubated in 0.2% triton in PBS (PBS-triton) at 4°C overnight, transferred to 1% H₂O₂ in 50% methanol for 20 minutes, washed in PBS-triton (3 x 10 minutes), and then incubated overnight at 4°C in primary antibodies diluted in 1% goat serum in PBS with 0.2% triton and 0.4% merthiolate. Control incubations included no primary antibody (goat serum only), and iGFAP-antiserum mixed with the synthetic peptide antigen. All antibody incubations were performed in triplicate. The sections were washed with PBS-triton (3 x 10 minutes) and then incubated with goat anti-rabbit Ig biotinylated secondary antibody (1:1000, Sigma) overnight at room temperature. The sections were washed with PBS-triton, incubated in Streptavidin-biotinylated horseradish peroxidase complex (1:1000,
Amersham, Life Science) for 3 hours and washed again in PBS-triton. The antigen was visualised with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H$_2$O$_2$ to produce a brown reaction product. Floating sections were mounted on gelatin-coated slides and air-dried. Finally, all sections were then counter-stained in cresyl violet (~20 minutes), dehydrated and coverslipped. Sections were examined on a Leica DMR microscope and images were captured using a ProgRes 3008 digital camera (Zeiss).

4.2.4 Sample preparation for analysis of purification steps

Samples were prepared as described in section 3.2.1.1, Chapter 3, with alterations as described below:

1. Samples prepared according to the Cooper method, but resuspended in [8 M Urea, 2% SDS, 0.2 M Tris-Cl] for electrophoresis (rather than 6M GHCl), were labelled FCS having been treated with 70% (v/v) formic acid, collagenase and sonication.

2. Collagenase control samples had the collagenase digestion step and subsequent washes left out. Samples were resuspended in [8 M Urea, 2% SDS, 0.2 M Tris-Cl] following the removal of formic acid, for electrophoresis. These samples were labelled FS as they were still sonicated in 70% (v/v) formic acid.

3. A collagenase and sonication “combined” control had both of these steps removed from the protocol. Pelleted, saline-extracted samples were resuspended in 70% (v/v) formic acid, shaken overnight and resuspended in [8 M Urea, 2% SDS, 0.2 M Tris-Cl] for electrophoresis following the removal of formic acid. These samples were labelled F.

4. Lastly, a minimal treatment control was also prepared by resuspending the pelleted, saline-extracted samples in [8 M Urea, 2% SDS, 0.2 M Tris-Cl] and shaking overnight before electrophoresis. These samples were labelled P as they represent resuspended pellet.

Tissue from a single HD brain (HC76; Grade 2, 17/42 repeats) was used to generate these samples, however two additional P samples were also visualised. These were saline extracts from a Grade 4 HD brain (HC87; 21/50 repeats) and a normal control brain (H111). Electrophoresis and Western blotting of these samples was then kindly
performed by Miss Suzanne Reid (Department of Molecular Medicine, University of Auckland) according to standard protocols (Laemmli, 1970; Hames & Rickwood, 1990). Samples were separated on either 16% SDS-PAGE gels or 12% SDS-urea-PAGE gels, transferred to PVDF, and probed with either the commercial GFAP antibody (DAKO) at 1:10 000 or the iGFAP-antiserum at 1:5000. Bound primary antibody was visualised according to the method described in section 4.2.2.2, with anti-rabbit secondary antibody used at a dilution of 1:3000.
4.3 Results

4.3.1 Characterisation of antiserum

The ELISA experiment, performed by Ms Louise Moffat following the test bleed (see table 4.1), demonstrated a titre value of 312 500 for the KLH-[peptide] antiserum. This is illustrated in figure 4.2.

![ELISA: KLH-[peptide] serum 15/9/99](image)

**Figure 4.2.** ELISA results demonstrating a titre value of 312 500 for the test bleed serum of immunised rabbit (collected 19 days post-immunisation) compared with pre-bleed serum (collected prior to immunisation). This ELISA experiment was kindly performed by Ms Louise Moffat who also performed the immunisation.

The result of electrophoresis and Western blotting of human control brain homogenate, using an 8% SDS-urea-PAGE gel and iGFAP-antiserum, is shown in figure 4.3. Detection of the synthetic peptide on 12% SDS-urea-PAGE gels by iGFAP-antiserum and rabbit anti-GFAP (DAKO) is illustrated in figure 4.4. Full length GFAP (~49 kDa) can be clearly seen in the first two lanes (a, b) of figure 4.3a and in the first lane (a) of figure 4.3b, accompanied by varying degrees of high and low molecular weight smearing. The source of high molecular weight smearing is unknown but this could represent aggregated GFAP or GFAP-containing complexes. The low molecular weight smearing could represent the small N-terminal fragments of GFAP that when cleaved produce the 34-45 kDa fragments discussed previously.
Figure 4.3. Western blots following separation of protein extract from control brain (H111) middle-frontal gyrus on an 8% SDS-urea-PAGE gel. Tissue was homogenised in [0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 μg/ml PMSF] and mixed 1:1 with Laemmli sample buffer prior to loading. Detection of GFAP by iGFAP-antiserum was performed at various antiserum dilutions (a = 1:1000, b = 1:5000, c = 1:10 000, d = 1:20 000, e = 1:50 000, f = 1:100 000, g = 1:500 000). (A) Secondary antibody used at 1:3000, (B) secondary antibody used at 1:6000. Electrophoresis and Western blotting were performed as described in section 4.2.2. Molecular weights shown at side were determined by electrophoresis of Benchmark (Life Technologies) protein standards.

Figure 4.4. Western blots showing affinity of rabbit anti-GFAP (1:10 000; DAKO) and iGFAP-antiserum (1:5000) for the synthetic peptide fragment of GFAP (Auspep) against which the antiserum was raised. The secondary antibody was used at a dilution of 1:3000. Approximately 5 μg of peptide was run on 12% SDS-urea-PAGE gels and detected by Western blotting as described in section 4.2.2. Molecular weights shown at side were determined by electrophoresis of Benchmark (Life Technologies) protein standards.
Electrophoresis and Western blotting of the synthetic peptide antigen revealed a strongly detected band between ~3-9 kDa with the commercial rabbit anti-GFAP antibody (figure 4.4a), and a very weak band between ~3-6 kDa with the iGFAP antiserum (figure 4.4b). A small spot at ~25 kDa, detected by the commercial antibody may represent a small amount of aggregated peptide. The results of these experiments demonstrate that whilst the iGFAP-antiserum can detect both full-length GFAP and synthetic iGFAP, the commercial antibody is capable of detecting the iGFAP epitope and is markedly more sensitive than the antiserum.

### 4.3.2 Immunohistochemistry

Staining of human middle-frontal gyrus brain tissue with iGFAP-antiserum and an antibody against GFAP revealed a similar pattern of immunogenicity, allowing for variability due to antibody concentration. Middle-frontal gyrus was investigated for the sake of consistency with much of the other work described in this thesis, and because this region of the brain of HD patients was found to be relatively abundant in inclusions. Figure 4.5 demonstrates the staining pattern observed with each of these antibodies.

**Figure 4.5.** Immunohistochemistry of control and HD brain middle-frontal gyrus with rabbit polyclonal anti-GFAP antibody (DAKO; 1:5000) (left) or iGFAP-antiserum (1:250) (right) raised in rabbit as described. 50 μm floating sections were labelled with anti-GFAP antibody at a dilution of 1:5000 (A, C, E), or with iGFAP-antiserum at a dilution of 1:250 (B, D, F).

(A) Control brain (H112), (B) Control brain (H104). Areas containing activated astrocytes were selected for the micrographs in (A) and (B), however macroscopically these cells were far less abundant in control brain than HD brain. (C&D) Grade 3 HD brain (HC75; 18/52 repeats). (E&F) Grade 3 HD brain (HC87; 21/50 repeats).

Antibodies were detected using biotinylated secondary antibodies, streptavidin-conjugated horseradish peroxidase, and DAB to produce a brown reaction product. Scale bars = 10 μm (a-e), 30 μm (f).
Control Brain

Control Brain

HD Brain

HD Brain

HD Brain

HD Brain

Rabbit anti-GFAP (DAKO) 1:5000

iGFAP-antiserum 1:250

137
Clear and abundant activated (ie. expressing GFAP) astrocytes were observed in HD tissue using the commercial antibody against GFAP (figure 4.5c & e). Activated astrocytes were also observed in control brain sections with this antibody, albeit at a lower abundance (the micrograph in figure 4.5a is centred on an area containing these cells). Using the iGFAP-antiserum at a dilution of 1:250 (figure 4.5b, d & f), numerous activated astrocytes were also observed and the background appeared to be stained lightly. No intranuclear or perinuclear inclusions, or dystrophic neurites were detected with either iGFAP-antiserum or the anti-GFAP antibody, suggesting that neither full-length GFAP nor the fragment iGFAP are localised to these. However, it may be that the epitopes detected by the antibody and/or antiserum would be unavailable if either GFAP or iGFAP were localised to these structures. Interestingly, the iGFAP-antiserum appeared to label predominantly astrocyte cell bodies with very little staining of processes, whilst the commercial GFAP antibody labelled both cell bodies and processes equally well. Tissue sections labelled without primary antibody had no DAB reactivity, confirming the specificity of the secondary antibodies used.

4.3.3 Analysis of purification protocols
SDS-PAGE and Western analysis of samples prepared according to the methods described in section 3.2.1.1 (Chapter 3), with various steps removed, revealed interesting step-dependent fragmentation of GFAP. Western blots typical of those observed (the electrophoresis experiments were performed a minimum of twice each) are displayed in figure 4.6. Both 16% (w/v acrylamide) SDS-PAGE gels (figure 4.6a & b) and 12% (w/v acrylamide) SDS-urea-PAGE gels (figure 4.6c) were used. Western blots were performed using both the commercial GFAP antibody and the iGFAP-antiserum. Each of the four sample types described in section 4.2.4 was generated from the same amount (400 mg) of starting tissue, and equal volumes of these samples were loaded onto SDS-PAGE gels. Consequently, the amount of protein loaded in each well is not equivalent, but the effect of the various purification steps can be seen on retention/removal of protein as well as fragmentation of protein. All of the Coomassie-stained SDS-PAGE gels (including the SDS-urea-PAGE gels) revealed a marked reduction (to almost nil) of detectable protein in samples that had been subjected to the entire Cooper purification method (labelled FCS).
Figure 4.6. Analysis of the role of various steps of the Cooper purification procedure (section 3.2.1.1, Chapter 3) in the fragmentation of GFAP, by SDS-polyacrylamide gel electrophoresis and Western blotting. (A & B) 16% (w/v acrylamide) SDS-PAGE gels. (C & D) 12% (w/v acrylamide) SDS-urea-PAGE gels. Western blots were detected by either a commercial anti-GFAP antibody (DAKO, 1: 10 000; A & C) or the iGFAP-antiserum (1:5000; B & D). Molecular weights shown at side were determined by electrophoresis of Benchmark (Life Technologies) protein standards.

Sample treatments are described in section 4.2.4. In summary: 'F, C, S' (Formic acid treated, Collagenase treated and Sonicated) refers to samples prepared according to the Cooper method, except resuspended in [8M Urea, 2% SDS, 0.2 M Tris-Cl] for electrophoresis, rather than 6M guanidine-HCl as described in that method. 'F, S' samples were not collagenase digested and subsequent wash steps were removed. 'F' samples had collagenase and sonication steps removed — effectively these represent the saline-extracted pellets (10 000 x g) resuspended in formic acid. The minimally treated 'P' samples had formic acid also removed from the protocol - effectively these represent the saline-extracted pellets (10 000 x g) resuspended in [8M Urea, 2% SDS, 0.2 M Tris-Cl] for electrophoresis.

Each sample was generated from the same amount (400 mg) of starting tissue, and an equal volume of each sample was loaded. The Coomassie-stained gels reveal a marked reduction in the amount of detectable protein in samples subjected to collagenase digestion. All other samples appeared to possess approximately equivalent amounts of protein and the appearance of the Coomassie-stained bands in these samples suggests normal electrophoresis occurred. The 'white' smears between -15-75 kDa in the non-collagenase treated samples in (A) represent an overexposure of the X-ray film to the chemiluminescent ECL reagent, even after 1 second. This signifies an abundance of GFAP-immunoreactivity in these lanes.

HC76 = Grade 2 HD brain, 17/42 repeats. HC87 = Grade 4 HD brain, 21/50 repeats. H111 = normal control brain.
CHAPTER FOUR – INVESTIGATIONS INTO THE ROLE OF iGFAP IN HUNTINGTON DISEASE

A

B

C

D

DAKO Anti-GFAP (1:10 000)

iGFAP (1:5000)

HC76

HC76

HC76

HC76
There was considerably more detectable protein in all of the other samples (FS, S and P), suggesting that the collagenase digestion step is responsible for removing a large amount of protein from the tissue homogenates. The collagenase preparation that was used (Sigma, Cat.# C9891) was considered crude due to the presence of probable contaminating enzymes clostripain and trypsin. This preparation was chosen because it was more successful than purified collagenase for the purification of amylin from diabetic pancreas (G. Cooper, personal communication.) Additionally, the presence in the collagenase buffer of the nonionic detergent Nonidet P-40 is likely to have assisted in the solubilisation and subsequent removal of some protein.

When the 16% (w/v acrylamide) SDS-PAGE gels were blotted with the commercial GFAP antibody, a wide range of GFAP-immunoreactivity was observed in all samples (figure 4.6a). Despite there being no Coomassie-stained bands following separation of the full treatment sample (FCS), GFAP-immunoreactivity was clearly seen in this sample in smears of low molecular weight (less than ~10 kDa) and of ~26-80 kDa. This result may suggest an enrichment of samples prepared according to the Cooper purification method in GFAP moieties, a suggestion further supported by the purification of iGFAP from 'amyloid-enriched' protein extracts prepared using this method (Chapter 3). Numerous Coomassie bands were observed in the other samples (FS, F and P), indicating that electrophoresis was normal, however these all had GFAP-immunoreactive smears ranging from ~9 kDa to >100 kDa, with particularly intense immunogenicity between ~15-80 kDa (figure 4.6a). The low molecular weight smear (below ~10 kDa) was absent in all of these samples, suggesting that collagenase (or possibly the contaminating enzymes) is responsible for the generation of low molecular weight (>10 kDa) fragments of GFAP such as iGFAP. There appeared to be no significant effect on GFAP fragmentation or the amount of protein present, due to sonication or formic acid steps.

When identical gels were blotted with the less sensitive iGFAP-antiserum, considerably less GFAP-immunoreactivity was observed as expected (figure 4.6b). No iGFAP-positive bands were detected in the FCS sample, although this is likely to be due to the low amount of protein in this sample as revealed by Coomassie staining (described above). A broad, strongly detected band centred on ~38 kDa and a weaker band of ~22 kDa were detected in FS, F and P samples. In addition, an approximately 16 kDa band was detected in FS and F samples but absent in P samples. Neither full-
length GFAP nor small (>10 kDa) fragments of GFAP were detected in these samples.

Interestingly, when the same FCS sample was separated on a 12% (w/v acrylamide) SDS-urea-PAGE gel and blotted with the commercial GFAP antibody, the high molecular weight smear was no longer detected. Instead a low molecular weight smear (~3-22 kDa) was observed (figure 4.6c). This result further supports a role for collagenase in generating low molecular weight fragments of GFAP. The high molecular weight smear in the FCS sample in figure 4.6a could represent aggregated small fragments of GFAP that are better separated in the urea-containing gel. Minimally treated samples (P) from HD (HC87) and control (H111) brains, separated on SDS-urea-PAGE gels, revealed a GFAP-immunoreactive smear between ~14 kDa and >100 kDa similar to the same samples separated on urea-free gels. The less sensitive iGFAP-antiserum again did not detect GFAP products in the FCS sample when this was separated on SDS-urea-PAGE gels (data not shown). However, smears of ~51-64 kDa were detected in the minimally treated (P) samples of HD (HC87) and control (H111) brains (figure 4.6d). The two bands (38 and 22 kDa) detected by iGFAP-antiserum in the HC76 P sample, were not detected in the P samples from HC87 and H111 separated on urea-containing gels.

Table 4.2 lists the various combinations of the three steps in the purification procedure that were altered to generate samples for electrophoresis and Western analysis, and the resulting molecular weight species of GFAP that were detected.

4.4 Discussion

The work described in this chapter served to investigate the pathological relevance of an internal fragment of GFAP (termed iGFAP), and its possible role in HD. An antiserum was raised in rabbit against the portion of GFAP that was purified from amyloid-enriched HD brain protein extracts and characterised (described in Chapter 3). The antiserum was shown by Western analysis to specifically detect full-length GFAP from human brain tissue homogenates, and the synthetic peptide antigen. A commercial anti-GFAP antibody (DAKO) demonstrated greater immunoreactivity for the synthetic peptide than iGFAP-antiserum.
Table 4.2. Step-dependent fragmentation of GFAP. + indicates step was used according to original protocol (section 3.2.1.1, Chapter 3). – indicates the step was left out. HC76 is a Grade 2 HD brain, HC87 is a Grade 4 HD brain, H111 is a control brain. 400 mg of middle-frontal gyrus tissue was used in the preparation of each sample. The Western blots summarised here are displayed in figure 4.6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Brain #</th>
<th>Sample Label</th>
<th>Collagenase</th>
<th>Sonication</th>
<th>Formic Acid</th>
<th>GFAP-positive bands (kDa)</th>
<th>iGFAP-positive bands (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16% (w/v acrylamide) SDS-PAGE gels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>HC76</td>
<td>FCS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3-9, 26-80</td>
<td>NONE</td>
</tr>
<tr>
<td>2</td>
<td>HC76</td>
<td>FS</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>9 - &gt;100, intense between 15-80</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>HC76</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>9 - &gt;100, intense between 15-80</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>HC76</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9 - &gt;100, intense between 16-80</td>
<td>38</td>
</tr>
</tbody>
</table>

| 12% (w/v acrylamide) SDS-urea-PAGE gels |
| 5 | HC76 | FCS | + | + | + | 3-22 | NONE |
| 6 | HC87 | P | - | - | - | 14 - >100 | 51-64 |
| 7 | H111 | P | - | - | - | 14 - >100 | 51-60 |

In addition to detecting full-length GFAP, some high and low molecular weight smearing was more weakly detected in homogenates with the iGFAP-antiserum. This smearing may represent GFAP fragments of various length or non-specific immunoreactivity of the antiserum.

Immunohistochemistry using both iGFAP-antiserum and a commercial antibody to GFAP demonstrate reactive gliosis in HD brain. Reactive gliosis is a term used to describe the massive proliferation of astrocytes (for which GFAP is a cell-specific marker), a process known to occur in head injury and neurodegenerative diseases (Cajal, 1928, cited in Xu et al., 1999). The pattern of labelling using either antiserum or antibody was slightly different, with the most recognisable difference being the paucity of process labelling using the antiserum. Processes were strongly labelled using the commercial GFAP antibody. This difference could be due to epitope availability - it is conceivable that the internal epitope of GFAP detected by the iGFAP-antiserum is inaccessible in processes, where the rod domains of GFAP...
molecules are likely to be complexed. In the cell body, where GFAP is manufactured and transported, this internal epitope may be more readily accessible. The commercial GFAP antibody, being polyclonal, would be capable of detecting head and tail domains of GFAP molecules in astrocytic processes. However, because the polyclonal GFAP antibody is in a purified form, and the iGFAP-antiserum represents a complex mixture of molecules, it is to be expected that the former would work better than the latter.

No intranuclear or perinuclear inclusions were detected by either antibody or antiserum, indicating that the co-purification of iGFAP and amyloid components of HD brain may not be due to the localisation of GFAP to these inclusions in brain tissue. However, it is also possible that the epitopes recognised by antibody and antiserum would not be accessible if GFAP was localised to these. This finding suggests that the internal fragment of GFAP that was purified and described in Chapter 3 may have been enriched in the amyloid-containing fraction of brain tissue, enabling its purification and characterisation. Whether this enrichment is due to a localisation of iGFAP to amyloid inclusions, an interaction of iGFAP with amyloid components, or the molecular properties of iGFAP remains uncertain.

GFAP is a well-characterised molecule and antibodies against GFAP are routinely used to identify activated astrocytes (Dahl et al., 1985). The results of immunohistochemistry suggested that Huntington disease is not responsible for an alteration in the intracellular localisation of GFAP or a fragment thereof. Given this, we wished to determine what process had led to the generation of iGFAP, which was purified and characterised based on its apparent increased abundance and insolubility in HD brain.

An investigation of the role of various steps of the purification procedure in generating GFAP fragments revealed a major effect due to collagenase digestion and essentially no effect due to formic acid treatment or sonication (table 4.2). Treatment of tissue homogenates with a collagenase preparation, including the potential contaminating enzymes trypsin and clostripain, resulted in the generation of low molecular weight (<10 kDa) products detectable by the GFAP antibody. When collagenase treatment was removed from the extraction process, fragments smaller than ~9 kDa were no longer observed. High molecular weight smearing that was also detected by the GFAP antibody in collagenase treated samples separated on SDS-PAGE gels, is likely to be due to aggregates composed of these low molecular weight
products as this smear is not detected on blots from urea-containing gels. In contrast, the wide range (~9->100 kDa) of GFAP products detected in non-collagenase treated samples is not affected by urea content providing further evidence that no fragments <9 kDa are present in these samples.

It is possible that iGFAP, demonstrated by mass spectrometry to be 3210.2 Da (Chapter 3), was one of the small fragments of GFAP that was generated by collagenase digestion. However, the iGFAP-antiserum detected no bands in collagenase-treated samples although this is likely to be due to the poor sensitivity of the antiserum and the low level of protein in the FCS sample (as shown by Coomassie staining). The iGFAP-antiserum did detect at least three GFAP fragments between 16-38 kDa, in samples that had not been treated with collagenase. The largest of these fragments is reminiscent of the 36 kDa and 37 kDa bands described by Fujita et al. (1998), that were increased in abundance in amyotrophic lateral sclerosis (ALS) spinal cord. A study on bovine and rat GFAP reports that the protein is extremely susceptible to proteolysis when the brain is not frozen rapidly, and that full-length GFAP degrades into two smaller components of approximately 40.5 kDa and 45 kDa when incubated at room temperature for up to 48 hours (Dahl, 1976). These products were more resistant to proteolysis and were still present after prolonged periods of tissue autolysis. GFAP degradation products of the same size have also been identified in post-mortem human tissue (Dahl & Bignami, 1975; Newcombe, Woodrooffe & Cuzner, 1986) including HD striatum (Selkoe et al., 1982b). Smaller products in the range 18-37 kDa, also seemingly resistant to proteolysis, have been reported more recently (Dahl et al., 1984; Fujita et al., 1998). Given that the post-mortem delay for HC76 was 16 hours, it is therefore likely that the bands detected by iGFAP-antiserum in non-collagenase treated samples, and the smears detected by the commercial antibody, may represent the degradation products described in the literature. The non-detection of 38 and 22 kDa bands in the P samples of HC87 and H111 separated on urea-containing gels could reflect a difference between individual brains and/or sample preparation. Post-mortem delay is unlikely to be responsible for this difference being 18 hours for HC87 and 10 hours for H111.

Using iGFAP-antiserum, the only apparent effect on GFAP fragmentation due formic acid treatment or sonication, was the presence of a 16 kDa band in FS and F samples that was absent in the minimally treated P samples. This band is likely to be a
degradation product of GFAP that is augmented by formic acid treatment. No effect for formic acid or sonication was observed using the commercial antibody.

The detection of a wider range of degradation products with the commercial GFAP antibody, compared with the iGFAP-antiserum, is most likely due to its greater sensitivity and the detection of more than a single epitope being polyclonal.

The high molecular weight smearing (i.e., greater than full-length GFAP - 49 kDa) that was observed with the commercial antibody may be due to the aggregation and/or aberrant migration of some of these degradation products. GFAP naturally forms multimers in vivo (Shaw & Hawkins, 1992) and certain fragments of GFAP are likely to retain this tendency following purification (Ralton et al., 1994).

The abundance of GFAP (due to reactive gliosis) in protein extracts from HD brains is most likely what led to its purification based on a much lower relative peak from control brain extracts (see Chapter 3). As discussed in the introduction, Selkoe et al. (1982b) describe a 3- to 8-fold increase of full-length GFAP and GFAP degradation products, in unfractionated homogenates of HD striatum. A closer inspection of the GFAP polypeptide sequence using a protease simulator (CUTTER program at PROLYSIS, http://delphi.phys.univ-tours.fr/Prolysis/) reveals multiple cleavage sites within the GFAP sequence for trypsin and clostripain (both potential contaminating enzymes in the crude collagenase preparation). Simulated cleavage of GFAP produced over 200 predicted fragments following theoretical digestion with these two enzymes. The largest of these is 2760.16 daltons, and the fragment beginning at residue 106 (identical N-terminus to iGFAP) proceeds to residue 121, having a molecular weight of 1833.09 daltons. Collagenase was not one of the enzymes available in this program for simulated protein digestion.

The obvious discrepancy between the molecular weight detected by mass spectrometry (3210.2 Da), and the molecular weights generated in silico, can be explained by a number of feasible scenarios including incomplete cleavage, aggregation of fragments, or post-translational modifications. The discovery of a novel fragment of GFAP in amyloid-enriched samples from HD brain (Chapter 3), together with reports in the literature describing the presence of GFAP fragments in a number of neurodegenerative diseases, led to interest in a possible role for iGFAP in HD. However, the work described in this chapter demonstrates that small fragments of GFAP (i.e., 3-9 kDa) are present only in collagenase-treated samples. This result and the presence of cleavage sites in GFAP for potential contaminating enzymes in the
collagenase preparation suggest that cleavage by either these enzymes or collagenase, rather than the disease process in HD, may be responsible for the generation of iGFAP.

The results described in this chapter imply that iGFAP is unlikely to be a pathologically relevant peptide with a role in HD. Full-length GFAP on the other hand does have a role in HD, even if this is only due to the proliferation of astrocytes. It is conceivable that GFAP or fragments thereof could be fibrillogenic and possibly amyloidogenic. This could be confirmed by in vitro protein assays.

It is also possible that GFAP could interact with amyloid component(s) of HD brain and this interaction may be important in HD pathogenesis. The possibility of such a molecular interaction is suggested by the co-purification of GFAP and an amyloidogenic protein or peptide (which may or may not include expanded polyglutamine) from HD brain, as described in Chapter 3. Furthermore, molecular interactions have previously been demonstrated for expanded polyglutamine tracts with the intermediate filament proteins vimentin (Onodera et al., 1997) and 68 kDa neurofilament protein (Nagai et al., 1999). As the expression of huntingtin is not limited to neuronal cells an interaction between polyglutamine and GFAP might be predicted to occur in activated astrocytes. Such an interaction could be confirmed by the expression of polyglutamine-GFP fusion proteins in a glial-derived cell line, and demonstration of this proposed interaction might implicate a role for astrocytes in Huntington disease pathogenesis.
Chapter 5

Purification and Characterisation of Expressed-Polyglutamine Protein

5.1 Overview

This chapter describes experiments designed to purify and characterise aggregated proteins from a cell culture model of polyglutamine generated in our laboratory. The purpose of this work was to determine the usefulness of the chromatography techniques used for the work described in Chapter 3, for purifying aggregated protein. High performance liquid chromatography (HPLC) was an integral part of the method that was successfully used to purify amylin from amyloid plaques in diabetic pancreases (Cooper et al., 1987). HPLC was also an important part of the method that was successfully applied in the purification of β-amyloid from amyloid plaques in Alzheimer’s disease (AD) brains (Glenner & Wong, 1984). As has already been pointed out in section 3.4, Chapter 3, the amyloid-like aggregates that were observed in Huntington’s disease (HD) (described in Chapter 2) are much smaller and much less frequent than the amyloid plaques of both diabetes and AD. This observation led to refinements being made to the original protocol with the aim of further enriching samples in amyloid-like inclusions.

As a model to test these methods, Neuro-2A cells (a mouse neuroblastoma-derived cell line; ATCC number CCL-131) were transiently transfected by plasmid vectors to express a fusion protein consisting of GFP- (green fluorescent protein) linked to 0, 30 or 100 glutamine (Q) residues. Details of the vector used and transfection are provided in section 5.2.1. Protein expression in these cells can be easily visualised by
fluorescence microscopy, and aggregation of the expressed protein is seen in cells expressing 30Q-GFP and 100Q-GFP. Similar models demonstrating polyglutamine aggregation and disruption of intermediate filament networks are described in the literature, using COS7 cells (a monkey kidney-derived cell line; Onodera et al., 1997), and TR1 cells (a murine neuronal cell line; Nagai et al., 1999).

Prior to chromatography, poorly soluble proteins were extracted directly into formic acid, which is thought to dissociate aggregating proteins (Hazeki et al., 1999). Congo red staining, which is dependent on the linearity of the dye molecule (through aggregation of a monomer) is therefore made redundant as this conformation is destroyed by exposure to formic acid (Masters et al., 1985). Due to the abundance of aggregates observed by fluorescence in the Neuro-2A cell lines, it was felt that these cells would provide an excellent model for testing the methods we had applied to the purification of aggregated proteins in HD tissue. In addition, because these cells are grown in a monoculture without contaminating cell types, extracellular matrix, blood vessels, etc., it was felt that the expressed polyglutamine-containing protein would be more easily detected during purification.

Finally, because SDS-PAGE was being considered as a possible means of separating and identifying proteins of interest prior to chromatography and characterisation (see Chapter 6), protein extracts from Neuro-2A cells were also separated by SDS-PAGE and examined by Western analysis using the antibody 1C2. This monoclonal antibody was raised against the homopolymeric glutamine stretch of TBP. On Western blot analysis it has been shown to recognise expanded polyglutamine tracts (within TBP and other proteins) of greater than ~38 residues (Trottier et al., 1995). It was therefore used to detect the expressed protein in 100Q-GFP cell lines.

Aggregation of full-length TATA-binding protein (TBP) containing an expanded polyglutamine tract (with 90 glutamine residues), has also been demonstrated in Neuro-2A cells by immunohistochemistry (S. Reid, personal communication). Protein extracts from cells transfected with this TBP-containing plasmid expression vector were used in these SDS-PAGE experiments as a positive control for the antibody 1C2. The aim of these experiments, peripheral to the rest of the work described in this chapter, was to determine the usefulness of one-dimensional polyacrylamide electrophoresis in isolating soluble, N-terminal fragments of huntingtin from human brain tissue, for further purification and characterisation.
5.2 Materials and Methods

Because most of the methods that were used for the research described in this chapter have been described in detail elsewhere, they will be cross-referenced here and only explained further where there are differences.

5.2.1 Cell Culture

Neuro-2A cells transfected with plasmids expressing various lengths of polyglutamine, were very kindly supplied by Miss Suzanne Reid (Department of Molecular Medicine, University of Auckland). A mouse neuroblastoma cell line was used (ATCC number CCL-131) and designated Neuro-2A. This cell line is an adherant cell line that shows neuronal and amoeboid stem cell morphology. Cells were maintained in 150 ml flasks at 37°C in 5% CO₂ in 20 ml of an RPMI (Gibco BRL) based media. Cells were split twice weekly, once confluent, and 0.05% trypsin was used to detach the cells. Cells were split 24 hours prior to plating for optimum health, and then plated in six-well plates (35 mm diameter wells).

100Q-GFP clones were a kind gift from Dr Don Love (School of Biological Sciences, University of Auckland), and were constructed as follows. 100(CAG) oligonucleotides were generated by PCR using CAG₂₀ and CTG₂₀ oligonucleotides as both primers and template. Consecutive cycles of annealing and extension generated a population of oligonucleotides of different lengths. A 100(CAG) oligonucleotide was selected and blunt cloned into the EcoRV site of the pEGF-N1 HDTag plasmid. This plasmid includes a his tag-containing linker, which was inserted between the Bgl II/Bam H1 sites of the pEGFP-N1 vector (Clontech). A 30(CAG) oligonucleotide was generated by PCR using CAG₂₀ and CTG₂₀ oligonucleotides as primers and DNA from a 100Q-GFP clone as template. 30Q-GFP clones were then constructed by the method described above.

The resulting amino acid sequence of these clones was: MVSSH₆SSVLDQ₆ILVRGRSR-GFP. The Neuro-2A cell line for expression of 0Q-GFP was transfected with pEGFP-N1 vector (Clontech) only.

Fugene transfection reagent (Boehringer Mannheim) was used to transf ect cells according to the manufacturer’s instructions. Cells were transfected at 24 hours after plating, at approximately 80% confluency. Each well was transfected with a total of 4.5 µg DNA using 9 µl of Fugene and 300 µl of Optimem (serum free media (Gibco
Evidence of expression was provided by the presence of a green fluorescent signal, which was disperse in 0Q-GFP clones, but formed punctate aggregates in 30Q-GFP and 100Q-GFP clones.

Neuro-2A cells for the expression of TATA-binding protein (positive control for Western studies with 1C2) were transfected with full-length TBP (accession #M55654) containing 90 glutamines, cloned into EcoRI and Xba1 sites of pcDNA3.1(+) (Invitrogen). Seven wells were used for each construct and cells from these were pooled in PBS after 46 hours.

5.2.2 Protein Extraction
For extraction of aggregated protein from these cells, cells were pelleted (800 x g for 5 minutes) and PBS was removed. 200 µl of lysis buffer (400 mM KCl, 1% TritonX100, 1mM PMSF) was added to the pelleted cells, which were mixed and left to stand on ice for 30 minutes. The cell mixture was then centrifuged at 13 000 rpm for 30 minutes and the supernatant was discarded. 100 µl of 100% (v/v) formic acid was added to the cell pellet, and sonicated using a Soniprep 150 probe-sonicator (MSE). Formic acid was removed by vacuum evaporation to near dryness.

Samples for separation by HPLC were taken up in 6 M GHCl, filtered to 0.2 µm using cellulose acetate filters (Sartorius) and injected directly onto a SEC-2000 size exclusion chromatography column (Phenomenex). Samples for separation by polyacrylamide gel electrophoresis were washed several times to remove formic acid. 1 ml of Milli-Q water was added to the near-dry sample, the mixture was triturated and dried again by vacuum evaporation. This wash step was repeated and the near-dry sample was taken up in [6 M Urea, 2% SDS, 0.2 M Tris-Cl] for electrophoresis.

5.2.3 High Performance Liquid Chromatography (HPLC)
Gel filtration and reverse-phase HPLC of 0Q-, 30Q- and 100Q-expressing Neuro-2A cells were performed according to the methods described in sections 3.2.1.3 and 3.2.1.4, Chapter 3. For the reverse-phase experiments, the concentration of TFA used was 0.08% (v/v) and the organic component of buffer B consisted of 80% acetonitrile in Milli-Q water. A C5 Jupiter (Phenomenex) column (250 mm x 5 µm, 300 Å) was used in conjunction with the program 1 elution profile. Samples were pumped at 250 µl/min, and peptides were detected by UV spectroscopy at a wavelength of 214 nm.
5.2.4 Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

SDS-PAGE and Western blotting of cell protein extracts were performed according to the methods described in sections 6.2.4 and 6.2.5, Chapter 6. 12-well Criterion (BioRad) gels were used according to the manufacturer’s instructions. The primary antibody used in Western analysis was 1C2, specific for expanded polyglutamine tracts (Trottier et al., 1995).

5.2.5 Mass Spectrometry

Matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry was performed as described in section 3.2.5, Chapter 3.

5.2.6 N-terminal protein sequencing

N-terminal sequencing of proteins was performed according to the methods described in section 3.2.6, Chapter 3.

5.3 Results

5.3.1 High Performance Liquid Chromatography (HPLC)

Gel filtration HPLC of protein extracts from Neuro-2A cells resulted in markedly different chromatograms for the three types of construct that were used (figure 5.1). As in Chapter 3, 1 ml fractions were collected from the time of injection loop opening. Also as before, a calibration curve using molecular weight standards was constructed to allow an estimation of the mass range of the collected fractions (figure 5.2).
Figure 5.1. Gel filtration chromatograms of protein extracts from cells expressing polyglutamine-green fluorescent protein (GFP) fusion proteins. (a) Typical (n=2) chromatogram obtained from 0Q-GFP-expressing cells. (b) Typical (n=2) chromatogram obtained from 30Q-GFP-expressing cells. (c) Typical (n=2) chromatogram obtained from 100Q-GFP-expressing cells. In each case, 200 μl of protein extract (prepared according to the method described in section 5.2.2) was injected onto a Phenomenex SEC-2000 size exclusion chromatography column. Mobile phase = GnHCl, pumped at 1 ml/min. Detection = UV @ 280 nm. The methods and equipment used for gel filtration HPLC are described in section 3.2.1.3, Chapter 3.
(a) 0Q-GFP

(b) 30Q-GFP

(c) 100Q-GFP
Log MW vs Rf

Figure 5.2. Calibration curve of molecular weight standards used in gel filtration. ($R^2 = 0.9664; y = -0.0418x + 6.7899$). Gel filtration was performed using the equipment and according to the methods described in section 3.2.1.3, Chapter 3. Molecular weight standards include (from left to right): bovine serum albumin (Serva; 66 430 Da), Cytochrome C (Sigma; 12 360 Da), recombinant human insulin (Novo Nordisk Pharmaceuticals; 5808 Da), and somatostatin (Bachem; 1638 Da). Blue dextran (2 000 000 Da) was used to calculate the column void volume ($V_v$). The calibration curve was used to estimate the molecular weight range of fractions collected during gel filtration HPLC of tissue homogenates.

Certain gel filtration fractions of the cultured cell protein extracts were deemed to be of particular interest. These included fraction I, with a maximum absorbance of 0.05 AU, collected from 0Q-expressing cells between 6-7 minutes elution time (figure 5.1a). The absorbance for the equivalent fraction (II) from 30Q-expressing cells is slightly higher (0.075 AU), and significantly higher (>0.2 AU) for the equivalent fraction (III) from 100Q-expressing cells (figure 5.1b & c). Fractions I-III, eluting between 6-7 minutes elution time (Rf = 60-70 mm), are all estimated to have a mass range of approximately 7.5 kDa to 19.5 kDa. The calculated molecular weight of one hundred glutamine residues plus the linker peptide is 15.73 kDa, which is within this range. Thirty glutamines plus linker peptide would have a molecular weight of approximately 6.8 kDa.

Fractions I-III were injected onto a reverse-phase chromatography column for further separation of the peptides contained in these fractions. Figure 5.3 shows typical chromatograms that were obtained for fraction I (0Q-GFP, n=2; figure 5.3a) and fraction III (100Q-GFP, n=4; figure 5.3b). The chromatogram displayed in figure 5.3b is also typical of those obtained for fraction II (30Q-GFP, n=3). Several peaks
between 55% and 65% buffer B were observed in the reverse-phase chromatograms from all three fractions I-III.

In addition, there were two peaks present in fractions II and III that were absent in fraction I. These peaks eluted at 35% buffer B (peak IV, figure 5.3b) and 77% buffer B (peak V, figure 5.3b). These peaks were slightly higher from fraction III than fraction II, however they were always absent in reverse-phase chromatograms of fraction I.

5.3.2 Mass Spectrometry

MALDI-TOF mass spectrometry of peak IV was not able to detect any mass entities, possibly due to low level of peptide. Mass spectrometry of the peak V however, revealed two very low mass peaks at 4289.5 Da and 8588.6 Da. These are shown in figure 5.4. The larger of these is approximately 10 daltons bigger than double the mass of the smaller peak. It is therefore uncertain if the peaks represent the same peptide, with one peak representing either 2M + H (double the mass) or M + 2H (half the mass). As described in Chapter 3, it is possible that an error of 10 daltons could be due to the very low level of peptide present. It is also conceivable that the peaks could represent different peptides.

Because the estimated molecular weight range of gel filtration fraction III is 7.5-19.5 kDa (based on the calibration curve in figure 5.2), considerably less than the fusion protein (42 kDa), it was thought that the purified peaks could represent cleavage products of the fusion protein. No signal was detected at 12.81 kDa, the expected mass for 100 glutamine residues, 15.73 kDa, the expected mass for 100 glutamines plus linker, or 42.02 kDa, the expected mass for 100 glutamines plus linker and GFP. Curiously, the two masses described here are very close to two masses that were detected in peaks VIII to XI described in Chapter 3 (figure 3.11). In fact the smaller peak (4289.5 Da) is within the range of 4283.7 Da ±10 Da that was observed between different samples. The larger peak (8588.6 Da) also fitted into the range of masses observed for the larger mass entity in peaks VIII-IX.
Figure 5.3. Reverse-phase chromatograms of gel filtration fractions obtained between 6-7 ml elution volume, from polyglutamine-GFP fusion protein-expressing cells.

(a) Typical (n=2) reverse-phase chromatogram obtained after injection of 200 µl of peak I (from gel filtration of protein extract of 0Q-GFP-expressing cells; see figure 5.1a) onto a Phenomenex C5 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 4 (Table 3.3, Chapter 3). Detection = UV @ 214 nm.

(b) Typical (n=4) reverse-phase chromatogram obtained after injection of 200 µl of peak III (from gel filtration of protein extract of 100Q-GFP-expressing cells; see figure 5.1c) onto a Phenomenex C5 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 4. Detection = UV @ 214 nm. The chromatogram displayed is also typical (n=3) of reverse-phase chromatograms obtained after injection of peak II (30Q-GFP; figure 5.1b)

The equipment and methods used for reverse-phase HPLC are described in section 3.2.1.4, Chapter 3, with specific conditions described in section 5.2.3.
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(a) [Graph showing A_{214} and %B vs. time in minutes for 0Q-GFP]

(b) [Graph showing A_{214} and %B vs. time in minutes for 100Q-GFP]
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Figure 5.4. MALDI-TOF mass spectrum obtained for peak V. Peak V was collected following formic acid extraction of protein from 100Q-GFP-expressing cells, gel filtration and reverse-phase HPLC. The spectrum illustrated is the average of 53 single shots from the laser. Spectra were obtained in positive ion mode. The equipment and methods used to perform MALDI-TOF mass spectrometry are described in section 3.2.5, Chapter 3. Human insulin and somatostatin were used to calibrate the mass spectrometer prior to sample analysis. The x-axis measurement m/z = mass to charge ratio.

5.3.3 N-terminal sequencing

N-terminal protein sequencing was unable to provide peptide sequence for either peak IV or peak V despite repeated efforts, due once again to very low levels of signal. For a different approach to peptide sequencing, samples of peaks IV and V were sent to APAF (Australian Proteomics Analysis Facility) for Q-TOF MS/MS (tandem quadrupole-time of flight mass spectrometry/mass spectrometry) peptide sequencing. This method is much more sensitive than N-terminal protein sequencing and is describe in section 7.1, Chapter 7. Unfortunately, this method was also not able to provide peptide sequence for these peaks due to trace amounts of a polymer which interfered with the method. The mass spectrum generated by the polymer was consistent with triton-X100, and this detergent was present in the lysis buffer used at the beginning of the protein extraction protocol. It is therefore likely that the retention of a very small amount of triton-X100 during protein extraction and HPLC phases led to the problems encountered during tandem mass spectrometry.
5.3.4 Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE of protein extracts from cultured cells was performed to determine the usefulness of this method for the separation and identification of proteins of interest prior to further purification by in-gel tryptic digestion and HPLC. Despite washing the protein extracts twice with Milli-Q water following formic acid treatment, the samples were still clearly acidic as revealed by the addition of sample buffer, whereon the indicator bromophenol blue turned yellow. This colour returned to blue as the samples entered the buffered SDS-PAGE gels that were used to separate them. The acidity of the samples, however, had the effect of some sideways spreading of lanes and an uneven dye-front, similar to the ‘smile’ phenomenon generated on gels that heat unevenly whilst running.

Western analysis of SDS-PAGE separated protein extracts, revealed the presence of 1C2-positive bands in extracts from both 100Q-GFP-expressing cells and TBP90-expressing cells (figure 5.5).

Figure 5.5. Western blot of protein extracts from Neuro-2A cells expressing GFP fused to either 0, 30 or 100 glutamine residues, or TATA-binding protein (TBP) containing 90 glutamine residues. Formic acid protein extracts were vacuum dried to near dryness and resuspended in 30 μl of Laemmli sample buffer. Proteins were separated on 12-well (45 μl capacity), 4-15% Criterion (BioRad) SDS-PAGE gels, transferred to PVDF and immuno-botted with 1C2, specific for expanded polyglutamines and raised against TBP (positive control; Trottier et al., 1995). Note: The appearance of spots rather than bands in the 100Q sample, is most likely due to the low pH of the samples which caused a ‘smile’ effect with a narrowing of internal lanes, and sideways spreading of outside lanes. Electrophoresis and Western blotting were performed as described in sections 6.2.5 and 6.2.6, Chapter 6, respectively. Molecular weights shown at the side were determined by electrophoresis of Precision Protein Standards (BioRad; 10-250 kDa).
Not surprisingly, no 1C2-positive bands were observed in protein extracts from either 0Q-GFP- or 30Q-GFP-expressing cells. Extracts from TBP90-expressing cells were immunopositive for a band at approximately 70-75 kDa. Extracts from 100Q-expressing cells contained two 1C2-immunoreactive species at approximately 85-95 kDa and 65 kDa. These bands are larger than the estimated molecular weight of the fusion protein (42 kDa), however the aberrant migration of expanded polyglutamine-containing proteins would account for the larger apparent molecular weight (Huang et al., 1998). In the SDS-PAGE experiments described in the following chapter, in-gel tryptic digestion is used to remove the proteins of interest (ie. N-terminal fragments of huntingtin) from the polyacrylamide gel. This was performed to allow a complete characterisation of these fragments. In-gel tryptic digestion was not applied to the 1C2-positive bands described here for two major reasons. Firstly, because the sequence of the polyglutamine-GFP fusion proteins was already known, characterisation was deemed not necessary. The polyglutamine stretch is located between the start codon and the GFP gene sequence; therefore the appearance of fluorescence in these cells provides evidence that the polyglutamine tracts are expressed. Secondly, whilst the aim of most of the work described in this thesis was to test the purification and characterisation methods applied in Chapter 3, the purpose of the work described in this section was somewhat different. As discussed in the introduction, the purpose of the experiments described in this section was to determine the usefulness of a different technique, SDS-PAGE, for isolating soluble, N-terminal fragments of huntingtin from human brain.

5.4 Discussion

The work described in this chapter was designed to investigate the efficacy of the purification and characterisation techniques that were used for the research described in Chapter 3. The purification methods included two types of high performance liquid chromatography (HPLC): gel filtration and reverse-phase HPLC. The protein characterisation methods that were used included matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry, and N-terminal protein sequencing. The research in Chapter 3 describes the purification of a number of peptide or protein ‘peaks’ from HD brain protein extracts based on their absence or lower level in control brain protein extracts. For many of these, a mass was
obtained by mass spectrometry, however only two peptide sequences were obtained for proteins that were present in greater abundance in HD versus control brain – GFAP and histone H3. The first of these proteins is the subject of Chapter 4. Because of the low abundance and very small size of the protein aggregates in HD, it was thought that the methods that were used might not have been sensitive enough to either purify or characterise them from blocks of tissue. In order to determine if the reason for non-characterisation was due to low concentration of aggregates or a problem in the methodology, these same techniques were applied to polyglutamine-expressing cells that had previously been shown to be rich in protein aggregates (S. Reid, personal communication). In addition to testing the same methods that had been used for the research in Chapter 3, two new methods were introduced. Firstly, polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein extracts from the same cells, to determine if it could be used as a preparative step with a means of positive identification, prior to HPLC. Secondly, samples were sent to the Australian Proteomics Analysis Facility (APAF) for analysis by Q-TOF MS/MS (tandem quadrupole-time of flight mass spectrometry/mass spectrometry), a more sensitive method capable of obtaining protein sequences.

Gel filtration of Neuro-2A cells expressing 0, 30 or 100 glutamine residues in a fusion protein, resulted in quite different chromatograms. A fraction collected between 6-7 minutes elution time was selected as being interesting because the UV absorbance signal was significantly higher in 100Q-expressing cells compared with 0Q-expressing cells. 30Q-expressing cells were intermediate. It is interesting that the mass range for this fraction, estimated from the calibration curve shown in figure 5.1, is 7.5-19.5 kDa. The mass of 100-glutamine residues plus linker peptide is within this range at 15.73 kDa. Re-injection of this fraction from all three different expression models resulted in the identification of two peaks that were present in 30Q- (data not shown) and 100Q-expressing cells, but absent in 0Q-expressing cells. These peaks termed IV and V, eluted at 35% buffer B and 77% buffer B respectively. A mass spectrum was obtained for the 77% peak from 100Q-expressing cells, with two mass peaks at 4289.5 daltons and 8588.6 daltons. This result was surprising, firstly because the smaller mass is outside the range of the gel filtration fraction, and secondly because both masses are almost identical to masses that were observed from four HPLC peaks (VIII-XI) from HD tissue extracts (figure 3.11, Chapter 3). The HPLC peaks VIII-XI eluted at 32% buffer B and 62% buffer B, and all had very similar mass
spectra. As discussed in Chapter 3, it was felt that the different peaks might represent different multimeric states of the same molecule. The same case could apply in the context of cultured cell extracts, if it were found that the peptides present in peaks VIII-XI consisted mostly of stretches of glutamine residues. Another possibility is that all of these peaks represent a novel protein expressed in both HD middle-frontal gyrus and Neuro-2A cells that is 'switched on' where proteins containing expanded polyglutamine tracts are expressed. Alternatively, such a protein may be permanently 'switched on' regardless of polyglutamine length, but in the presence of expanded polyglutamine is recruited into poorly soluble protein aggregates.

Further characterisation in the form of peptide sequence for peaks VIII-XI in Chapter 3, and peaks IV and V in the present chapter, could not be obtained. This consistent problem may indicate N-terminal blockage of a novel protein or an inherent problem of N-terminal protein sequencing methods when applied to expanded polyglutamine-containing proteins, possibly due to an altered protein conformation or oligomerization. Partial blockage to Edman degradation is reported when glutamine residues are N-terminal, as these residues can undergo cyclization under acidic conditions to pyroglutamyl residues (Allen, 1986). This reaction, described in more detail in section 3.4.2, Chapter 3, makes the determination of the sequences of peptides with N-terminal glutamine very difficult. Although methods have been developed for the removal of N-terminal blocking groups (Walker & Sweeney, 1996; Miyatake et al., 1993), these were not applied during the research described in this thesis as in our experience they have not enabled the sequencing of suspected-blocked proteins (Catriona Knight, personal communication).

As an alternative to N-terminal protein sequencing, Q-TOF MS/MS was performed on peaks IV and V, however the presence of trace amounts of Triton-X100 may have interfered with the method and no peptide sequence could be obtained. The method was used again for work that will be described in Chapters 6 and 7. Given the expense of both N-terminal sequencing, and the Q-TOF MS/MS analysis, protein sequencing efforts were not continued. Pooling of multiple peak IV fractions and/or multiple peak V fractions, followed by chemical or enzymatic fragmentation (as described in Chapter 3) and either N-terminal sequencing or Q-TOF MS/MS analysis, might enable further characterisation of this interesting 4289 Da peptide.

Finally, SDS-PAGE and Western blotting of protein extracts from Neuro-2A cells enabled the identification of 1C2-positive proteins in 100Q- and TBP90-expressing
cells. Extracts from the 100Q-GFP-expressing cells contained two 1C2-positive species with apparent molecular masses of approximately 65 kDa and 85-95 kDa, substantially larger than the calculated mass of the fusion protein, which was 42 kDa. This aberration was not surprising, as reduced electrophoretic mobility of expanded-polyglutamine due to the disproportionate impact of polyglutamine length on gel migration, has been previously described (Huang et al., 1998). SDS-PAGE and Western blot analysis of polyglutamine-GFP fusion proteins, using an anti-GFP antibody, demonstrate an apparent molecular mass of ~56 kDa for 80Q-GFP proteins (Nagai et al., 1999; Onodera, 1997), so an apparent molecular mass of ~65 kDa for 100Q-GFP seems to be consistent with the observations of others. The larger (~85-95 kDa apparent mass) 1C2-positive species may represent the presence of 100Q-GFP fusion protein in a complex either with itself, or with other interacting molecules. Alternatively, the 1C2 antibody could be detecting two pools of 100Q-GFP fusion protein of different conformation and therefore different electrophoretic mobility characteristics.

The unusual appearance of the ‘bands’ we observe in the 100Q-GFP lane was, however, inconsistent with the Western blots of expanded polyglutamine-GFP fusion proteins of other workers (Nagai et al., 1999; Onodera, 1997). The unusual appearance of ‘bands’ was reflected in an unusual protein distribution (‘smile’ effect) as visualised on the Coomassie blue-stained gel. This is likely to be due to the acidity of the samples lowering the pH in parts of the gel and leading to uneven migration in different lanes. Internal lanes (30Q-GFP and 100Q-GFP) became narrowly focussed, whilst outside lanes (0Q-GFP and TBP90) curved further outwards. Interestingly, the molecular weight standards that were loaded in lanes flanking samples also migrated in an outward curve away from the acidic samples. The lowering of pH in certain regions of the gel is likely to have had an effect on electrophoretic mobility.

In-gel tryptic digestion of 1C2-positive bands, followed by purification and characterisation of fragments, was not performed for reasons that have been described. In-gel tryptic digestion and subsequent purification of the resulting peptides are however applied in the next chapter, following SDS-PAGE of sub-cellular fractions of HD tissue homogenates. The detection of the expanded polyglutamine-containing fusion proteins from both 100Q-GFP expressing cells and TBP90-expressing cells, by SDS-PAGE and Western blotting, suggests that these methods might comprise a suitable first-step in the purification of expanded
polyglutamine-containing fragments of huntingtin from human brain. The positive identification of proteins enabled by Western blotting overcomes the major limitation of the methods described in Chapter 3: the lack of an assay following solubilisation in first formic acid, then 6 M guanidine-HCl, which destroys the protein conformation necessary for a positive Congo red stain.
6.1 Overview

Polyacrylamide gel electrophoresis (PAGE) was used as an alternative technique to high performance liquid chromatography as a means of visualising proteins extracted from Huntington Disease (HD) and control brains. Electrophoretic separation techniques have a wide range of different applications and the range of new methods and developments is diverse. The overriding principle of all electrophoretic techniques is the migration of charged molecules and particles in the direction of an electrode bearing the opposite charge (Westermeier, 1997). When the anionic detergent SDS is included within gels and samples, proteins are separated according to molecular weight with very high resolution (Westermeier, 1997). Advantages of this technique include solubilization of most proteins, high mobility (due to high charge), migration in one direction (due to uniform negative charge), high resolution and ease of staining due to protein unfolding. During the research described in this thesis, vertical 1-dimensional SDS-PAGE gels and horizontal 2-dimensional SDS-PAGE gels were used for the resolution of proteins. 2D-PAGE had previously been used to visualise and compare proteins from nuclear preparations of HD and control brain tissue in our laboratory, by Professor P. Ramasamay (University of Malaysia). Nuclear preparations, prepared according to the methods of Scherzinger et al. (1997), were used in an effort to increase the relative
amount of intranuclear inclusions present in samples. Only proteins that were soluble in a lysis solution [9 M urea, 2% Triton X-100, 2% v/v pharmalyte 3-10, 0.13 M DTT, 8 mM PMSF] were separated by 2D-PAGE; insoluble proteins were pelleted and discarded. In this project, excellent separation of soluble proteins was achieved by 2D-PAGE (P. Ramasamay, personal communication). Although consistent differences in the proteomes of HD and control samples were observed, these differences could represent downstream effects of the disease process on various pathways. There was no report of the presence or absence of amyloid in the samples analysed by Ramasamay, however it is likely that amyloid components were excluded from these during centrifugation of the lysis solution. None of the protein spots that differed between HD and control samples on these gels were characterised.

During the work for this thesis 2D-PAGE was performed on amyloid-enriched samples from HD brain, and samples from control brain that had been subjected to the same enrichment process. Methods and results are discussed in subsequent sections. In addition to this technique, following on from the SDS-PAGE experiments described in the previous chapter, 1-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was applied to sub-cellular fractions of tissue homogenates in an effort to identify the cellular compartment(s) where N-terminal and internal regions of huntingtin are localised in HD and control brain. The sub-cellular localisation of huntingtin has been previously described in human, mouse and rat brain (Wood et al., 1996). This study showed that in both normal and HD-affected human brain, huntingtin was membrane-bound with a distribution essentially the same as that of synaptophysin (a synaptic vesicle protein). The antibody used to detect huntingtin in these experiments was raised against a synthetic peptide corresponding to an internal region of the protein. Other studies have attempted to characterise the localisation of huntingtin by immunofluorescence (discussed in section 1.4, Chapter 1). Some of these have demonstrated the nuclear localisation of a truncated N-terminal fragment of huntingtin of unknown length, and aggregation of this fragment from both mutant and normal huntingtin (Becher et al., 1998; DiFiglia et al., 1997; Gutekunst et al., 1999).

Because we were interested in purifying and characterising this fragment, we used an antibody raised against the N-terminus of huntingtin to identify the sub-cellular fraction(s) where N-terminal huntingtin is localised. In addition to just identifying those fractions, we looked for a correlation with the presence of amyloid as identified
by Congo red staining. Furthermore, the identification within a polyacrylamide gel of a truncated N-terminal fragment of huntingtin would be expected to assist greatly in the purification and characterisation of such a fragment. The fragment could easily be excised and purified from the gel, subjected to reverse-phase HPLC (as described in Chapter 3) for further purification, and characterised by N-terminal protein sequencing and mass spectrometry. In this way, gel electrophoresis could provide a useful step for the initial purification and positive identification of a protein of interest prior to the steps described in Chapter 3, which are in a sense 'blind' due to the lack of an identification assay. This chapter describes these 1- and 2-dimensional PAGE experiments.

6.2 Methods

As in previous chapters, human tissue samples were obtained from the Neurological Foundation (NZ) Human Brain Bank, School of Medicine, University of Auckland. Ethical approval for use of this tissue was obtained from the University of Auckland’s Human Subjects Ethics Committee. Brains were perfused and blocked as described in section 2.2.1, Chapter 2. Middle-frontal gyrus from three control brains (H111, H113, H123) and four HD brains (HC76, HC81, HC88, HC91) were used for the research described in this chapter. Post-mortem delays, CAG-repeat number, and HD grades are provided in Tables 3.1 and 3.4, Chapter 3.

6.2.1 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE combines two different electrophoretic methods. Firstly, proteins are separated by isoelectric focusing on the basis of their pI (isoelectric point), and secondly they are separated by SDS-PAGE on the basis of their molecular weight. The result of this separation is a pattern of spots that can be visualised after staining. These two-dimensional protein maps afford the highest resolution of all separation methods currently known (Rabilloud, 2000). The methods used for the 2D-PAGE experiments described in this thesis are outlined below.
6.2.1.1 Equipment and solutions for 2D-PAGE

The following items were all supplied by Pharmacia Biotech Ltd:

- Multiphor II electrophoresis unit
- Electrophoresis power supply, EPS 3500 XL
- MultiTemp III refrigerated bath circulator, 220-240 V
- Pharmalyte 3-10 Immobiline DryStrip kit, pH 3-10, 18 cm
- IEF strip reswelling tray
- Immobiline DryStrip aligner and tray
- ExcelGel XL SDS 12-14 (12-14% polyacrylamide gradient)
- ExcelGel SDS buffer strips
- Urea

In addition to these SDS, DTT, Glycerol, Triton X-100, bromophenol Blue (BDH Chemicals) and Tris (Boehringer Mannheim) were required for 2D-PAGE. Analytical grade ethanol, methanol, glacial acetic acid, formaldehyde, silver nitrate, sodium carbonate, sodium thiosulphate, EDTA (BDH Chemicals) and glutardialdehyde (Merck Sharp and Dohme) were also required for silver staining of gels. Paraffin oil was supplied by Shell (NZ).

6.2.1.2 Protocol for 2D-PAGE

Tissue was processed as described in section 3.2.1.1 (Chapter 3) up until step 8. Following evaporation of formic acid by vacuum evaporation, dried samples were taken up in 50 μl of sample buffer (9 M urea, 0.06 M DTT, 2% v/v Pharmalyte 3-10, 0.5% v/v Triton X-100, 0.1% w/v bromophenol blue). 10 μl aliquots of each sample were taken for protein quantification (described in section 7.2.2). Following this assay, approximately 120 μg protein was loaded per rehydrated IEF (isoelectric focusing) strip. Rehydration of strips, sample loading and isoelectric focusing was performed as follows:

1. Immobiline DryStrips (18 cm, pH 3-10) were rehydrated for 16-20 hours at room temperature in a reswelling tray. 350 μl of rehydration solution (8 M urea, 0.5% v/v Triton X-100, 1% v/v Pharmalyte 3-10, 0.01 M DTT, 0.1% w/v bromophenol...
blue) was pipetted into slots within the tray and strips were laid gel side down ensuring they were fully wetted. Each strip was overlaid with 2-3 ml of paraffin oil, and a lid was put in place.

2. Strips were rinsed and blotted on filter paper before being placed in the grooves of a plastic Immobiline strip aligner, gel side up with acidic ends towards the anode. The strip tray was placed on a cooling plate at 20 °C, and paraffin oil was used to ensure good contact.

3. Moistened electrode strips were placed across the strips at either end, partially touching the gel surfaces. Electrodes were placed on the electrode strips and the sample cup bar placed below the anode.

4. Sample cups were positioned over the gel strips and pressed down to form a seal. Strips are covered in oil, which is excluded from sample cups.

5. 120 μg of protein was loaded into each sample cup and electrophoresis was performed following the parameters in Table 6.1.

Table 6.1. Electrophoretic parameters for the first dimension (isoelectric focusing) of 2D-PAGE experiments. (NB/ The current was doubled when two beds were being run).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Voltage (V)</th>
<th>Current (mA)</th>
<th>Power (W)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>1</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3500</td>
<td>1</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

Following isoelectric focusing, the strips were equilibrated in an equilibration solution (5 M urea, 0.05 M tris-HCl pH 6.8, 0.3 M glycerol, 0.03 M SDS, 0.05 M DTT) for 10 min at room temperature. The strips were then re-equilibrated in equilibration solution without DTT, containing 0.2 M iodoacetamide and 0.1% w/v bromophenol blue for a further 10 minutes at room temperature, prior to second dimension electrophoresis.

The second dimension of electrophoresis was performed as follows:

1. The gel bed was cooled to 15 °C.
2. Paraffin oil was spread over the gel bed and an ExcelGel precast (12-14% acrylamide) SDS-gradient gel was placed on top gel-side up, ensuring that there was good contact and no air bubbles.
3. Anodic and cathodic buffer strips were placed on the appropriate sides of the gel in straight lines and bubbles were removed.

4. An equilibrated, focussed gel strip was placed gel side down on the ExcelGel immediately below the cathodic strip and gently massaged to ensure good contact and the further removal of air bubbles.

5. Electrodes were placed over the buffer strips and a lid was put in place. Electrophoresis was carried out according to the parameters in Table 6.2. At the end of phase 1 the gel strip was removed. Following phase 2, the cathodic buffer strip was moved to the position the gel strip had occupied.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Voltage (V)</th>
<th>Current (mA)</th>
<th>Power (W)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>20</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>40</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>40</td>
<td>40</td>
<td>160</td>
</tr>
</tbody>
</table>

**Table 6.2. Electrophoretic parameters for the second dimension (SDS-PAGE) of 2D-PAGE experiments. (NB/ The current was doubled when two beds were being run).**

6.2.1.3 Silver Staining

Following the second dimension of electrophoresis, 2D-gels were silver-stained to visualise resolved proteins. Silver staining was performed as follows:

1. Gels were fixed for 30 minutes (40% v/v ethanol in milliQ H₂O, 0.1 M glacial acetic acid)
2. Gels were then sensitised for a further 30 minutes (30% v/v ethanol, 0.125% v/v glutardialdehyde (added immediately before use), 8 mM sodium thiosulphate, 0.5 M sodium acetate).
3. Following this step, gels were rinsed thoroughly in distilled water, and reacted for 20 minutes with silver solution (0.01 M silver nitrate, 5 mM formaldehyde (added immediately before use)).
4. Gels were then rinsed again and developed for 2-5 minutes (0.2 M sodium carbonate, 2.5 mM formaldehyde (added immediately before use)) which is stopped in EDTA (0.04 M) for 10 minutes.
5. Finally, gels were rinsed once more in distilled water before being preserved in glycerol (0.1 M) for 20 minutes, before being dried overnight at room temperature under cellophane.

Silver stained gels were scanned using ScanWizard PPC 3.2.4 (Microtek) software and a Microtek ScanMaker IIsp scanner.

6.2.2 Protein quantification assay

As described in section 6.2.1.2, 10 μl aliquots of samples that had been solubilized in SDS sample buffer, were removed for protein quantification. The method described below (J. Douglas, personal communication) is unaffected by the presence of SDS, urea, glycerol, β-mercaptoethanol, ammonium sulphate and 1% CHAPS. However, 1% triton X-100 or trace amounts of phenol do interfere with the assay. The assay is based on the fact that treatment of tyrosine with nitric acid produces 3-nitrotyrosine, which is distinguished by an absorbance peak at 358 nm. Protein quantification was performed in a 96-well plate as follows:

1. BSA standards were prepared in a 1:5 dilution of sample buffer (Final: 1.6 M urea, 0.1% v/v triton X-100, 0.2% v/v pharmalyte 3-10, 2 mM DTT, 0.02% bromophenol blue) at 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml. A blank of the diluted sample buffer was included.
2. 1:5 dilutions of the unknown samples (already in sample buffer) were made using milliQ water.
3. 10 μl aliquots were pipetted into each well. All samples and standards were assayed in triplicate.
4. 140 μl of 70% nitric acid was added to each well.
5. The plate was incubated at room temperature for 2 hours.
6. Softmax Pro software and a Spectra Max 340 plate reader were used to read the plate at a wavelength of 358 nm.
7. A standard curve of the standards was produced, and the results of the unknown samples was compared with this to give an estimate of the protein concentration in these wells.
6.2.3 Sub-cellular fractionation

In an effort to determine the intracellular localisation in normal and HD brain of various forms of huntingtin and the amyloid component we observed (section 3.3.1), sub-cellular fractionation was performed. These were then analysed by Congo red staining, one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and Western blotting of the various fractions. Although sub-cellular fractionation and 1D-PAGE with Western blotting had been reported using normal and HD brain (De Rooij et al., 1996; Wood et al., 1996), it was not applied for purifying aggregated protein, or to determine the localisation of the Congo red-stained component. Furthermore, these studies used antibodies to internal epitopes of huntingtin that would not detect N-terminal fragments.

The methods used for PAGE and Western blotting are described in the following two sections. The methods used for sub-cellular fractionation (from Wood et al., 1996) are as follows:

1. Tissue was homogenized on ice, in 10-20 volumes of buffer (5 mM HEPES (pH 7.4), 0.32 M sucrose, 1 Complete (Roche) protease inhibitor cocktail tablet per 50 ml homogenization buffer).

2. Homogenates were spun at 1000 x g for 5 minutes at 4°C.

3. The supernatant was put aside. The pellet was resuspended in buffer (above) and centrifugation was repeated. Pellet = nuclear fraction (P1).

4. The supernatants were combined and centrifuged at 10 000 x g for 20 minutes at 4°C.

5. The supernatant was put aside. The pellet was resuspended in buffer (above) and centrifugation was repeated. Pellet includes synaptosomes and mitochondria (P2).

6. The supernatants were combined and centrifuged at 105 000 x g for 1 hour at 4°C.

7. The supernatant was put aside. The pellet was resuspended in buffer (above) and centrifugation was repeated. Pellet = microsomal fraction (P3).

8. The supernatants were combined = cytosolic fraction (S).

6.2.4 Congo red staining

Congo red staining of sub-cellular fractions was performed according to the protocol described in section 3.2.1.2, Chapter 3.
6.2.5 One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was used for the separation of proteins within different sub-cellular fractions. Two different types of 1D-PAGE gels were used: 6% (w/v acrylamide) SDS-PAGE mini-gels were initially used and to improve protein resolution and consistency, 4-15% (w/v acrylamide) gradient, Criterion precast gels (BioRad) were used. The associated method is outlined below.

6.2.5.1 Equipment and solutions for 1D-PAGE

- Mini-PROTEAN II Electrophoresis Cell (BioRad)
- Gel-casting stand, Glass plates, plastic spacers, plastic gel combs
- Criterion Electrophoresis Cell (BioRad)
- Power Pac 3000 (BioRad)
- Tris-HCl Criterion (BioRad) precast 4-15% (w/v acrylamide) linear gradient gels
- 2x Laemmli sample buffer (8 ml):

\[
\begin{align*}
\text{Milli-Q H}_2\text{O} & : 2.9 \text{ ml} \\
0.5 \text{ M Tris-HCl, pH 6.8} & : 1.0 \text{ ml} \\
\text{Glycerol} & : 2.0 \text{ ml} \\
10\% (\text{w/v}) \text{ SDS} & : 1.6 \text{ ml} \\
\beta\text{-mercaptoethanol} & : 0.4 \text{ ml} \\
1\% (\text{w/v}) \text{ bromophenol blue} & : 0.1 \text{ ml}
\end{align*}
\]

All reagents were AnalaR grade and were purchased from BDH chemicals with the exception of the \(\beta\)-mercaptoethanol, which was purchased from Riedel-de Haen.

- Running Buffer, pH 8.3, 5x stock made to 1 L with Milli-Q water:

\[
\begin{align*}
\text{Tris (BDH, AnalaR)} & : 15 \text{ g} \\
\text{Glycine (BDH, AnalaR)} & : 72 \text{ g} \\
\text{SDS (BDH, AnalaR)} & : 5 \text{ g}
\end{align*}
\]

- 30% acrylamide mix, made up to 500 ml with Milli-Q water:

\[
\begin{align*}
\text{Acrylamide (BDH)} & : 58.4 \text{ g} \\
\text{Bis-Acrylamide (30\%T, 2.6\%C, BDH)} & : 1.6 \text{ g}
\end{align*}
\]
6.2.5.2 Protocol for PAGE

This method is different to the method outlined in section 4.2.2.1, Chapter 4, which describes SDS-urea-PAGE gels. 6% (w/v acrylamide) mini-gels were prepared and run as follows:

1. Glass plates and plastic spacers and combs were washed in ethanol followed by milliQ water, assembled on the gel casting stand and checked for leaks.

2. 5 ml polyacrylamide gel solution (per gel) was made from the following reagents:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide mix</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 μl</td>
</tr>
<tr>
<td>10% ammonium persulphate (SERVA, made fresh)</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED (BDH)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>2.7 ml</td>
</tr>
</tbody>
</table>

3. The polyacrylamide gel solution (resolving gel) was pipetted into the space between the glass plates ensuring no air bubbles were present and overlaid with butanol to exclude oxygen, thus facilitating polymerisation at room temperature.

4. Once the gel solution had polymerised (about 15-20 minutes, visualised by the checking the remainder of gel solution), the butanol was poured off and the top of the gel was rinsed with Milli-Q water. A 1 ml stacking gel solution was prepared from the following reagents to aid in the movement of proteins into the gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide solution</td>
<td>170 μl</td>
</tr>
<tr>
<td>1.0 M Tris-HCl (pH 6.8)</td>
<td>130 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 μl</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>10 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>1 μl</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>680 μl</td>
</tr>
</tbody>
</table>

5. A plastic gel comb containing the desired number of teeth (wells) was inserted into the space between the glass plates (leaving a small space for pipetting) and the stacking gel was pipetted on top of the resolving gel ensuring no air bubbles were present. The comb was removed following polymerisation and the wells were thoroughly rinsed with Milli-Q water.

6. Samples were mixed 1:1 with 2x Laemmli sample buffer and denatured in a boiling water bath for 5 minutes.
7. Electrophoresis of samples was performed in running buffer in the Mini-Protean II electrophoresis cell, at 70 V through the stacking gel, increasing to 120 V through the resolving gel. 

Alternatively, Criterion (BioRad) precast 4-15% (w/v acrylamide) linear gradient gels were run using a Criterion Electrophoresis Cell (BioRad) according to the manufacturers instructions. Samples were prepared as in step 6 above. Electrophoresis was performed at 100 V initially, increasing to 200 V when samples had entered the gel.

### 6.2.6 Western blotting

Western blotting is a powerful technique for the detection of specific proteins (Towbin, Staehlin & Gorden, 1979). Proteins resolved by polyacrylamide gel electrophoresis are transferred and immobilised onto polyvinylidenfluoride (PVDF), an inert membrane. Using antibodies, PVDF membranes are probed for target proteins. Three primary antibodies were used: 1C2 (Chemicon), a mouse monoclonal antibody specific for expanded polyglutamines (described in Chapter 5), HN1 (courtesy of Dr L. Jones, Cardiff), a rabbit polyclonal antibody raised against the N-terminal 17 amino acids of huntingtin, and HN2 (also courtesy of Dr L. Jones, Cardiff), a rabbit polyclonal antibody raised against residues 2110-2121 of huntingtin.

The secondary antibodies that were used were goat anti-mouse or goat anti-rabbit (depending on the primary antibody used) IgG conjugated to horseradish peroxidase (POD) that converts Lumi-Light™ substrate to emit light in the chemiluminescent reaction used to detect blotted antigens (Boehringer Mannheim).

#### 6.2.6.1 Equipment and solutions for Western blotting

- Mini-PROTEAN II Blotting Cell (BioRad)
- Power Pack 3000 (BioRad)
- PVDF membrane (ABI) (wet in methanol and rinsed in Milli-Q water prior to use)
- CAPS (transfer) buffer 10X stock: Dissolve 22.13 g CAPS (AppliChem) and 6.31 g NaOH (BDH, AnalaR) in Milli-Q water and bring to 1 L. To make 1X CAPS:
- Ponceau S stain solution (Boehringer Mannheim)
- Tris-buffered saline (TBS) 5X stock, pH 7.6:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.1 g</td>
</tr>
<tr>
<td>NaCl (BDH, AnalR grade)</td>
<td>40 g</td>
</tr>
<tr>
<td>HCl</td>
<td>to pH 7.6</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

- TBS with Tween (TBST): 1 ml Tween-20 (BDH, AnalR grade), 200 ml TBS, diluted to 1 L with Milli-Q water.
- Blocking solution. A 5% (w/v) solution of either bovine serum albumin (BSA, SERVA) or non-fat milk powder was used.
- Lumi-Light™ substrate (Boehringer Mannheim)

### 6.2.6.2 Protocol for Western blotting

This method is different to the method outlined in section 4.2.2.2, Chapter 4, which describes semi-dry protein transfer.

1. Transfer of proteins from polyacrylamide gels to PVDF was performed according to the manufacturer’s instructions using a Mini-PROTEAN II blotting cell (BioRad) and CAPS buffer. Transfer was assessed by Ponceau S staining prior to Western blotting.
2. Regions of PVDF with no protein bound were blocked by incubating in either a 5% milk solution for 1 hour with shaking at room temperature, or a 5% BSA solution (filtered) overnight at 4 °C.
3. The PVDF membrane was incubated for 1 hour in a solution of primary antibody (1C2 at 1:3000, HN1 at 1:4000, HN2 at 1:600) raised against the protein of interest.
4. The membrane was thoroughly washed in two changes of TBST, then incubated in 3 changes of TBST for 5 minutes each.
5. The membrane was incubated for 30 minutes in a solution of the appropriate secondary antibody (anti-mouse at 1:1000, anti-rabbit at 1:5000).
6. Same as step 3.
7. The membrane was incubated for 5 minutes in Lumi-Light substrate (prepared according to the manufacturer’s instructions).

8. Membranes were exposed on X-ray film (Hyperfilm ECL, Pharmacia Biotech Ltd.) for 30 seconds, 1 minute and 2 minutes prior to developing in an Agfa Curix 60 Developer.

6.2.7 Tryptic In-gel digestion

In an effort to obtain peptide sequence for the proteins present within bands detected by Western blotting, in-gel digestion and reverse-phase HPLC were performed. In-gel digestion of Coomassie-stained proteins was based on the method of Rosenfeld et al. (1992), and was performed as follows:

1. Coomassie-stained bands of interest were cut out of polyacrylamide gels using a scalpel blade, and the gel pieces were placed in labelled microcentrifuge tubes.

2. Gel pieces were washed twice in 200 μl of wash buffer (50% (v/v) acetonitrile in 100 mM Tris-Cl, pH 8.0) at 30 °C for 20 minutes with agitation. The supernatant was discarded after each wash.

3. Gel pieces were dried for 5 minutes at 37 °C (within open tubes), before the addition of 5 μl of digestion buffer (100 mM Tris-Cl, pH 8.0, 10% (v/v) acetonitrile, 0.02% Thesite). Rehydration was allowed for 3 minutes before the addition of trypsin (modified, sequencing grade, Roche) to a final concentration of 1.7 μM, and the mixture was incubated at room temperature for 5 minutes.

4. More digestion buffer was added to cover the gel pieces, the mixture was incubated at 37 °C for 15 minutes, briefly vortexed and incubated at 37 °C overnight (approximately 16 hours).

5. Following digestion, the peptides were extracted twice with 1.5 μl of neat TFA and 100 μl of extraction buffer (60% (v/v) acetonitrile, 0.1% (v/v) TFA, 0.02% (v/v) Thesite) at 37°C for 20 minutes with agitation. The supernatants were pooled and reduced in volume by vacuum to 40 μl.

6. The solution was brought to 5% (v/v) TFA and 10% (v/v) acetonitrile and peptides were separated by reverse-phase HPLC.
6.2.8 High Performance Liquid Chromatography (HPLC), Mass Spectrometry and N-terminal peptide sequencing

Reverse-phase HPLC was performed according to the methods described in section 3.2.1.4, Chapter 3. The concentration of TFA used was 0.08% (v/v), and the organic component of buffer B consisted of 80% acetonitrile in Milli-Q water. A C18 Jupiter (Phenomenex) column (250 mm x 5 μm, 300 Å) was used in conjunction with the program 1 elution profile. Samples were pumped at 250 μl/min, and peptides were detected by UV spectroscopy at a wavelength of 214 nm.

Mass spectrometry was performed according to the methods described in section 3.2.5, Chapter 3, and N-terminal peptide sequencing was performed according to the methods described in section 3.2.6, Chapter 3.

6.3 Results

6.3.1 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE of poorly soluble proteins extracted from HD tissue resulted in very poor resolution of these proteins (figure 6.1a). The resolution of poorly soluble proteins extracted from control brain was marginally better (figure 6.1b). Although some areas of the gels were relatively well resolved and spots could be visualised, other areas revealed a high degree of protein smearing. Despite boiling samples prior to loading, and despite a degree of reproducibility visualised by eye, the separation of proteins was not sufficient for a direct comparison of gels loaded with control and HD samples. 2D-PAGE of soluble protein extracts from HD and control brain tissue could possibly allow the identification of changes in protein expression that are important in the pathogenesis of HD. However, the purpose of the work described in this thesis was to purify and characterise poorly soluble, aggregating protein from HD brain. For this reason, high performance liquid chromatography (HPLC) was maintained as the major technique for the separation of poorly soluble proteins from brain tissue (Chapter 3).
6.3.2 Sub-cellular fractionation and Congo red staining

Two HD brains (HC81, Grade1, 19/41 repeats; and HC88, Grade 2, 17/44 repeats) and two normal control brains (H111 and H113) were fractionated as described in section 6.2.3, for Congo red staining and 1D-PAGE. Sub-cellular fractions of HD and normal tissue stained with Congo red and examined by polarised-light microscopy. A degree of particulate red-pink staining and birefringence was observed in the nuclear fractions (P1) of HD tissue as previously described and as illustrated for tissue extracts (figure 3.3, Chapter 3). The red-stained non-birefringent background observed in the tissue extracts was decreased in the nuclear fractions. Amyloid particles were not detected in any other sub-cellular fraction of HD tissue, nor was any amyloid observed in control brain tissue.

6.3.3 One-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and Western blotting

1D-PAGE and Western blotting of sub-cellular fractions enabled the identification of the various huntingtin fragments present in these. There was a small degree of variability between different samples and between repeat experiments on the same sample. This variability was probably due to differential post-mortem proteolysis of tissue, and small differences in the times/conditions for protein transfer and Western blotting. A representative set of data is shown.

Western blotting of SDS-PAGE-separated sub-cellular fractions with 1C2 detected no antigen in either control or HD tissue samples. 1C2 is an antibody that specifically recognises expanded polyglutamine stretches (Trottier et al., 1995), and was therefore expected to detect the expanded polyglutamine-containing mutant huntingtin within HD samples. The 1C2-blotting of cell culture extracts described in Chapter 5 was performed on the same day as one effort to perform Western blotting of sub-cellular fractions of tissue with 1C2. This positive result suggests that the lack of detection was not due to a problem with antibody or reagents. It may be that huntingtin moieties containing an expanded polyglutamine tract were excluded from the gel. It is unlikely that the lack of detection by 1C2 is a conformational effect due to epitope unavailability, as 1C2-positive bands were detected within extracts from polyglutamine-GFP fusion protein-expressing cells. It is however also possible that protein conformation played a role in detection/non-detection, with the 1C2-epitope being unavailable when in the context of the huntingtin protein.
Figure 6.1. Two-dimensional gel electrophoresis of HD and control brain extracts. (a) Two dimensional, silver-stained gel electrophoretogram of protein extract from the middle-frontal gyrus of a Grade 2 HD brain (HC76; 17/42 repeats). (b) Two dimensional, silver-stained gel electrophoretogram of protein extract from the middle-frontal gyrus of a control brain (H111). Protein extracts were prepared according to the protocol described in section 3.2.1.1 (Chapter 3) as far as step 8. Following formic acid evaporation, dried samples were taken up in 50 µl of sample buffer (9M urea, 0.06 M DTT, 2% v/v Pharmalyte 3-10, 0.5% Triton X-100, 0.1% bromophenol blue). Approximately 120 µm of protein was loaded on each gel. 2D-PAGE was performed using the equipment and according to the protocols described in section 7.2.1. The molecular weights at the side were determined by electrophoresis of Benchmark (Life Technologies) protein standards. The isoelectric points of protein spots range from 10.0 on the left-hand edge to 3.0 on the right-hand edge.
Western blotting of SDS-PAGE-separated sub-cellular fractions with HN1, raised against the N-terminus of huntingtin, detected protein bands of two different sizes (~20 kDa and ~50 kDa) in all fractions (figure 6.2). Full-length huntingtin (348 kDa) was not observed in any fraction of either control or HD brain tissue, where samples were boiled prior to electrophoresis, however a band of this size was very weakly detected in unboiled samples (described later). A strongly detected 50 kDa band was observed in the cytosolic fraction (S) of both control and HD. A 20 kDa band was observed in the nuclear fractions (P1) and in the mitochondrial/synaptosomal fractions (P2) of both HD and control tissue. The intensity of immunoreactivity in both P1 and P2 appeared to be markedly decreased in control relative to HD tissue.

For the membrane-enriched microsomal fractions (P3), bands of 20 kDa and 50 kDa were variably detected in both HD and control brain. In different experiments, one or other, neither, or both of these bands were detected in P3 samples. It is possible that the 50 kDa band may represent the incomplete removal of cytosolic fraction (S) from the microsomal fraction (P3) during fractionation. No difference between control and HD samples could be determined for the P3 fractions.

![Figure 6.2](image-url)

Figure 6.2. Typical (n=5) Western blots of sub-cellular tissue fractions from Grade 2 HD (HC88; 17/44 repeats; a) and control (H123; b) brains. Proteins were separated on 4-15% polyacrylamide gels (BioRad Criterion), transferred to PVDF and blotted with HN1, against the N-terminal 17 amino acids of huntingtin. P1 = nuclear fraction, P2 = mitochondria/synaptosome-enriched fraction, P3 = membrane-enriched microsomal fraction, S = soluble cytosolic fraction. SDS-PAGE and Western blotting were performed using the equipment and according to the protocols described in sections 6.2.5 and 6.2.6 respectively. The molecular weights at the side were determined by electrophoresis of Precision Protein Standards (BioRad; MW 10-250 kDa). Total protein loadings are approximately 60 µg for P1 and P2 fractions, 20 µg for P3 fractions and 10 µg for S fractions.
When the Western result was compared with Coomassie-stained polyacrylamide gels, the 20 kDa bands in P1 and P2 fractions of HD tissue were clear and abundant. The 50 kDa band that was present in cytosolic fractions from control and HD tissue, was not detected by Coomassie. The wave-like appearance of the 20 kDa bands may be due to these being in the non-linear region of the gel, less able to resolve small proteins, and the Coomassie-stained gels support this. It is therefore possible that these bands with an apparent molecular weight of 20 kDa represent proteins that are smaller than this, and that the apparent abundance on Coomassie-stained gels is due to the presence of other proteins of 20 kDa or less. Separating these fractions on Tris-Tricine gels might provide better resolution of these bands. The specificity of the goat anti-rabbit secondary antibody was confirmed by a control Western blot experiment that had the primary antibody left out.

Western blotting of the same four SDS-PAGE-separated, sub-cellular fractions with HN2, raised against an internal epitope of huntingtin, detected an approximately 20 kDa band in P1, P2 and P3 fractions from both control and HD brain. No bands were seen in the cytosolic (S) fraction of either. No difference in intensity was observed between fractions or between brain type (ie. control versus HD). Full-length huntingtin was once again not observed. Figure 6.3 illustrates the pattern of immunodetection that was observed using HN2.

![Figure 6.3](image_url)

Figure 6.3. Typical (n=2) Western blots of sub-cellular tissue fractions from HD (HC88; a) and control (H123; b) brains. Proteins were separated on 4-15% polyacrylamide gels (Biorad Criterion), transferred to PVDF and blotted with HN2, against an internal epitope of huntingtin (amino acids 2110-2121). Fraction labels are as for figure 6.2. SDS-PAGE and Western blotting were performed using the equipment and according to the protocols described in sections 6.2.5 and 6.2.6 respectively. The molecular weights at the side were determined by electrophoresis of Precision Protein Standards (BioRad; MW 10-250 kDa). Total protein loadings are approximately 60 μg for P1 and P2 fractions, 20 μg for P3 fractions and 10 μg for S fractions.
Although the 20 kDa bands in figure 6.3 are also in the non-linear region of the gels, the appearance of these bands is normal. Given that the epitope of huntingtin detected by HN2 (amino acids 2110-2121) is a long distance along the protein from the epitope detected by HN1 (amino acids 1-17), and that a 20 kDa protein is likely to comprise roughly 180 amino acids, it is certain that the ~20 kDa bands detected by these two antibodies represent different proteins. The Coomassie-stained gels corresponding to the blots in figure 6.3 provide further support for the specificity of the goat anti-rabbit secondary antibody. These gels (figure 6.4) reveal an abundance of protein in the P1 and P2 fractions and considerably less in P3 and S fractions, however the HN2-positive, 20 kDa bands shown in figure 6.3 are the same intensity in P1, P2 and P3.

![Figure 6.4. Coomassie-stained gels corresponding to the blots illustrated in figure 6.3. Proteins were separated on 4-15% polyacrylamide gels (Biorad Criterion) and stained with Coomassie blue. Fraction labels are as for figure 6.2. SDS-PAGE was performed using the equipment and according to the protocols described in section 6.2.5. The molecular weights at the side were determined by electrophoresis of Precision Protein Standards (BioRad; MW 10-250 kDa). Total protein loadings are approximately 60 μg for P1 and P2 fractions, 20 μg for P3 fractions and 10 μg for S fractions.](image)

Lastly, on the suggestion that boiling of samples prior to electrophoresis may in fact assist in the aggregation of huntingtin, or huntingtin fragments (Dr. L. Jones, personal communication), boiled and unboiled samples were separated on different gels and Western blotting was performed. Aliquots of P1, P2 and S fractions from H113 (control) and HC91 (Grade 3 HD, 23/44 repeats) were boiled, and aliquots were kept unboiled. These samples were separated by SDS-PAGE as before and Western blotting was performed with HN1. Interestingly, a band of ~350 kDa was very faintly detectable in the unboiled P1 fraction of HC91 and in the unboiled P2 fraction of
H113. These bands were absent from the equivalent boiled fractions and are too faint to display in a figure. The 20 kDa and 50 kDa bands described previously for boiled samples, were also present in unboiled samples.

6.3.4 Tryptic in-gel digestion

The 20 kDa HN1-positive bands from the P1 and P2 fractions, and the vicinity of the 50 kDa HN1-positive band from the S-fraction of HD brain (HC88), were excised from the polyacrylamide gel and subjected to in-gel trypsin digestion as described in section 6.2.7. Peptide fragments were separated by reverse-phase HPLC and collected. As expected, fewer and smaller peaks were seen for the 50 kDa band (relative to the 20 kDa bands) due to the lower abundance of this protein revealed by a lack of Coomassie staining. In all, 12 peptide fragments were observed following digestion of the 20 kDa P1 band, 10 for the P2 band of the same size, and 7 for the 50 kDa band from the soluble fraction. In addition to these peptides, remaining Coomassie dye was eluted in each case at 62% buffer B. Figure 6.6 illustrates the chromatograms that were obtained following separation of the peptide fragments of the 20 kDa bands from fractions P1 and P2, and the 50 kDa band from the S fraction of HD brain tissue (HC88). Several of the peptides that were collected were submitted for N-terminal sequencing. Peaks that were well resolved from other peaks were chosen, and care was taken to choose peaks over a wide range of elution times and therefore a wide range of hydrophobicities. Due to the expense of N-terminal sequencing it was not practical to sequence all of the peptides that were collected, especially given the likelihood that several of these would have the same protein of origin. Sequencing of peak III and peak V (figure 6.5a) produced the peptide sequences displayed in figure 6.5.

<table>
<thead>
<tr>
<th>Peak</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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</tbody>
</table>

Figure 6.5. Primary structure of the peptide components of peak III and peak V (figure 6.6a) obtained following in-gel tryptic digestion of the nuclear fraction (P1) of a Grade 2 HD brain (HC88; 17/44 repeats). A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) found 100% identity for both fragments with myelin basic protein (MBP).
Figure 6.6. Reverse-phase chromatograms obtained for tryptic digests of HN1-positive, Coomassie blue-stained bands from HD brain fractions. (a) An ~20 kDa Coomassie blue-stained band corresponding to the HN1 signal of the same molecular weight in the nuclear (P1) fraction of HC88 (figure 6.2a) was excised and digested with trypsin (according to the protocol described in section 6.2.7).
(b) An ~20 kDa Coomassie blue-stained band corresponding to the HN1 signal of the same molecular weight in the mitochondrial/synaptosomal (P2) fraction of HC88 (figure 6.2a) was also excised and digested with trypsin.
(c) An ~50 kDa Coomassie blue-stained band corresponding to the HN1 signal of the same molecular weight in the cytosolic (S) fraction of HC88 (figure 6.2a) was excised and trypsinised as described.
All chromatograms shown were obtained following the injection of ~50 µl of the resultant digest solutions onto a Phenomenex C18 Jupiter column. Buffer A = 0.08% TFA in milliQ water; Buffer B = 0.08% TFA in 80% MeCN and milliQ water; Flow rate = 250 µl/min. Elution profile = program 1. Detection = UV @ 214 nm. The equipment and methods used for reverse-phase HPLC are described in section 3.2.1.4, Chapter 3. COO = Coomassie-blue dye remaining following in-gel digestion.
(b)  

(c)
Both of these sequences provided a 100% match with a major class of proteins known as the myelin basic proteins. Figure 6.7 illustrates the position of these fragments within the full-length amino acid sequence of MBP.

Myelin basic proteins (MBP) are thought to maintain the proper structure of myelin and are found on the cytoplasmic side of myelin molecules that form an insulating sheath around axons (Baldwin & Carnegie, 1971; Ridsdale et al., 1997). There are seven exons and at least seven related proteins produced from a single MBP gene by alternative RNA splicing, ranging in size from 14-21.5 kDa (Schwartz & Westbrook, 2000). An 18.5 kDa isoform is the most abundant isoform in the adult human, with 14, 17.3 and 21.5 kDa isoforms also expressed at varying levels in adulthood. Three isoforms are expressed only in the fetus. Several charge isoforms are also generated by alternative post-translational modification such as phosphorylation, deamidation and methylation (Ridsdale et al., 1997). It is likely that an isoform of MBP (probably 18.5 or 21.5 kDa) migrated to approximately the same place as the N-terminal fragment of huntingtin in the polyacrylamide gel, and that both proteins were included in the gel piece that was digested. 2D-PAGE may have enabled better resolution of MBP and the N-terminal huntingtin fragment, by the additional separation of proteins by isoelectric focussing. As the sequences that were obtained both represent internal regions of the peptide sequence for myelin basic protein it is likely that several of the other peptide fragments on the chromatogram are fragments of this protein.

Sequencing of peak XV resulted in the peptide sequence displayed in figure 6.8.
Figure 6.8. Primary structure of the peptide component of peak XV (figure 6.6b) obtained following in-gel tryptic digestion of the synaptosomal fraction (P2) of a Grade 2 HD brain (HC88; 17/44 repeats). Ambiguities are shown in brackets. A protein BLAST search of non-redundant databases (www.ncbi.nlm.gov) found no significant match with any known proteins.

Due to the presence of more than one peptide in this sample, an accurate primary structure could not be determined for either. MALDI-TOF mass spectrometry of peak XV revealed a major peak at 1829.7 Da, and a minor peak of 1046.4 Da (figure 6.10). A simulated tryptic digest of myelin basic protein (CUTTER program at PROLYSIS, http://delphi.phys.univ-tours.fr/Prolysis/) resulted in the generation twenty-nine predicted fragments, ranging in size from 175.21 Da (single Arginine residue) to 1829.97 Da. The largest of these fragments was almost identical in molecular weight to the major peak detected by MALDI-TOF, and the peptide sequence of this fragment was found to lie within the contaminated sequence (ie. not clean) obtained for peak XV (displayed in figure 6.8). Furthermore, the removal of the peptide sequence of the 1829.97 Da fragment from the ambiguous sequence enabled the identification of a smaller fragment of myelin basic protein (1047.16 Da), also predicted by the simulated digestion. The peptide sequences of these two fragments, and the contaminated sequence (from figure 6.8) that they were both components of, are displayed in figure 6.9.

<table>
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<tr>
<th>Predicted MW (Da)</th>
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<th>7</th>
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</tr>
</thead>
<tbody>
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<td>Gly</td>
<td>Trp</td>
<td></td>
<td></td>
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<td>Asp</td>
<td>Gly</td>
<td>Gly</td>
<td>Arg</td>
</tr>
<tr>
<td>1829.97</td>
<td>?</td>
<td>?</td>
<td>Trp</td>
<td>Gly</td>
<td>Ala</td>
<td>Glu</td>
<td>Gly</td>
<td>Gln</td>
<td>Gly</td>
<td>Arg</td>
<td>Pro</td>
</tr>
<tr>
<td>1047.16</td>
<td>?</td>
<td>Thr</td>
<td>Gly</td>
<td>Ile</td>
<td>Leu</td>
<td>Asp</td>
<td>Ser</td>
<td>Ile</td>
<td>Gly</td>
<td>Arg</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.9. Primary structure of the peptide components of peak XV (figure 6.6b) obtained following in-gel tryptic digestion of the synaptosomal fraction (P2) of a Grade 2 HD brain (HC88; 17/44 repeats). Peptide fragments were identified within the primary sequence obtained for peak XV (figure 6.7) following a simulated tryptic digest of the primary structure of myelin basic protein, and analysis of mass spectrometry data (figure 6.10).
Figure 6.10. MALDI-TOF mass spectrometry of peak XV (figure 6.6b) obtained following tryptic digestion of the ~20 kDa HN1-positive band in the P2 fraction of a Grade 2 HD (HC88; 17/44 repeats; figure 6.2a). The spectrum illustrated is the average of 53 single shots of the laser in positive mode. The equipment and methods used to perform MALDI-TOF mass spectrometry are described in section 3.2.5, Chapter 3. Human insulin and somatostatin were used to calibrate the mass spectrometer. The peak at 382.1 Da represents the (2M+H) isotope of the matrix α-CHC.

Figure 6.11 illustrates the position of these two fragments within the full-length amino acid sequence of MBP.

Figure 6.11. Full-length sequence of myelin basic protein (MBP), with single letter amino acid codes, illustrating the position of the amino acid residues that were sequenced from peak XV, comprising a 1829.97 Da peptide (bold font; box) and a 1047.16 Da peptide (bold; dashed box). Genebank accession number: 1070625. Residue 1 and or 2 are shown in normal font where not sequenced. The overlap of the 1047.16 Da peptide shown here and the sequence obtained from peak III (bold font and box in figure 6.7) indicates that trypsin cleavage of MBP was probably incomplete.

An additional BLAST search was performed on each of the four fragments of MBP that we sequenced to determine which of the MBP isoforms was present in the gel. The two fragments shown in figure 6.5, sequenced from peaks III and V, were represented in all of the isoforms expressed in adult (14 kDa, 17.3 kDa, 18.5 kDa and
21.5 kDa). The 1047.16 Da fragment illustrated in figure 6.9 was also represented in all four of these, however the 1829.97 Da fragment in the same figure is not represented in the 14 kDa isoform. This suggests that the MBP isoform excised from the gel was the 17.3 kDa, 18.5 kDa or 21.5 kDa isoform. Lastly, sequencing of peak VI resulted in two consecutive proline residues before these caused the termination of sequencing. None of the other peaks that were submitted for sequencing (VII, VIII, IX, XII, XVII, XVIII, XX, XXII, XXIV, XXVI and XXVII) resulted in any sequence being obtained due either to a low level of signal or samples containing more than one peptide. MALDI-TOF mass spectrometry of peak XII detected two different masses at 492.8 Da and 663.6 Da (figure 6.12).

![Figure 6.12. MALDI-TOF mass spectrometry of peak XX (figure 6.6b) obtained following tryptic digestion of the ~20 kDa HN1-positive band in the P2 fraction of a Grade 2 HD (HC88; 17/44 repeats; figure 6.2a). The spectrum illustrated is the average of 50 single shots of the laser in positive mode. The equipment and methods used to perform MALDI-TOF mass spectrometry are described in section 3.2.5, Chapter 3. Human insulin and somatostatin were used to calibrate the mass spectrometer. The peak at 382.1 Da represents the (2M+H) isotope of the matrix α-CHC.](image-url)
As none of the predicted fragments of myelin basic protein were within the expected error range of these masses, this peak and peak XX were sent to APAF (Australian Proteomics Analysis Facility) for Q-TOF MS/MS (tandem quadrupole mass spectrometry/mass spectrometry) protein sequencing. This method has been briefly touched on in Chapter 5, and is more fully described in section 7.1, Chapter 7. Data acquired over the m/z range 200-800 was unable to detect any peptides in either sample.

6.4 Discussion

6.4.1 Two-dimensional PAGE (2D-PAGE)

Despite being a powerful technique for the resolution of different proteins within samples, 2D-PAGE was demonstrated to be an unsuitable technique for resolving poorly soluble proteins from the amyloid-enriched fractions of human brain. Previous work that resolved proteins within the soluble component of nuclear fractions by 2D-PAGE is likely to have excluded amyloid-associated proteins from these gels. Although this work could potentially lead to the identification of pathways that are turned on or off in HD, this was not the intent of the work described in this thesis, which focussed on the purification and characterisation of amyloid-associated proteins from HD brain.

6.4.2 Congo red staining of sub-cellular fractions

Most of the work in this chapter describes attempts to identify and separate N-terminal fragments of huntingtin, with a view to later purification and characterisation. Sub-cellular fractionation of tissue homogenates was introduced as a preliminary step to SDS-PAGE as a means of enriching one or more fractions in these N-terminal fragments. Congo red staining and polarised light microscopy of sub-cellular fractions revealed the presence of detectable amyloid particles only in nuclear fractions (P1) of HD tissue homogenates. This result supports the same finding described in Chapter 3, although non-nuclear fractions were not stained in those experiments.
6.4.3 One-dimensional PAGE (1D-PAGE) and Western blotting

Western blotting of polyacrylamide gels with 1C2 was expected to, but did not detect the expanded polyglutamine-containing mutant huntingtin in HD samples. It should be remembered that a negative result in this type of experiment is not as informative as a positive result – availability of epitopes and the conditions used play an important role (Harlow & Lane, 1988). Hence, a negative result does not necessarily imply that an antigen is not present. It is also conceivable that mutant huntingtin was excluded from the gel due to being in an aggregated state.

Western blotting of gels with HN1, against the N-terminus of huntingtin, revealed fragments of two different molecular weights in different sub-cellular fractions. An ~20 kDa HN1-immunoreactive protein was notably more abundant in nuclear fractions and mitochondria /synaptosome-enriched fractions of HD brain tissue relative to control. Additionally, an ~50 kDa HN1-immunoreactive protein was present in similar abundance in the cytosolic fractions of both HD and control tissue. Both of these bands were variably detected in the P3 fraction, however no difference could be distinguished between control and HD P3 fractions.

Western blotting of SDS-PAGE gels with the antibody HN2 (raised against an internal region of the protein) detected further short fragments. An ~20 kDa HN2-immunoreactive protein was present in HD and control P1, P2 and P3 fractions, however no HN2-immunoreactivity was observed in cytosolic fractions. This 20 kDa internal fragment is clearly different to the N-terminal fragment of the same size detected by HN1, as a far greater fragment would be required to contain the epitopes of both of these antibodies.

During these experiments samples were boiled prior to electrophoresis and no full-length huntingtin was observed in any fraction of either HD or control brain homogenate, with 1C2, HN1 or HN2 antibodies, despite several repeat experiments. This was surprising, as full-length huntingtin has been demonstrated previously, using the same techniques, with both the same and similar antibodies (Dyer & McMurray, 2001; Mende-Mueller et al., 2001; Schilling et al., 1995). Wood et al. (1996) do not mention the molecular weight of the protein they detected or that this was full-length huntingtin. When samples were kept unboiled prior to electrophoresis, a very faint band was detected with HN1 at ~350 kDa in the P1 fraction of HD brain and the P2 fraction of normal control brain. The apparent re-localisation of this protein, thought
to be full-length huntingtin, from the mitochondrial/synapsomal fraction to the nuclear fraction could be due to an altered conformation in those compartments leading to altered epitope availability, or could represent a genuine change in distribution. Work by Wheeler et al. (2000) demonstrates a ‘mis-localisation’ of an alternate form (amino-terminal-accessible) of mutant protein to the nucleus of medium spiny striatal neurons in a knock-in mouse model. Moreover, Kegel et al. (2001) have recently demonstrated low levels of full-length mutant huntingtin, in addition to higher levels of N-terminal and C-terminal fragments of huntingtin, localised to nuclear inclusions in cultured fibroblast cells from HD patients. The effect of boiling samples prior to electrophoresis is very interesting, and thought to be due to high temperatures assisting in the aggregation of huntingtin (Dr. L. Jones, personal communication). There was no effect due to boiling or non-boiling on the huntingtin fragments described previously.

The very low levels of full-length huntingtin that we observed could be due to a combination of proteolysis of soluble protein, and either epitope unavailability (particularly in the case of 1C2 where the antigen is the aggregating region of the protein), or exclusion from the gel of insoluble, aggregated protein. The role of proteolysis in HD has recently been investigated and this is discussed in section 6.4.5 (Dyer & McMurray, 2001; Mende-Mueller et al., 2001).

In addition to the pre-mortem proteolysis of huntingtin, some degree of proteolysis is likely to be occurring during the post-mortem delay, and further fragmentation may occur during the freezing and thawing of tissue blocks. The post-mortem delays for obtaining tissue were between 7.5 (for H123) and 19 hours (for HC88), during which time proteolytic activity is likely. Wood et al. (1996) comment on their observations that frozen brain samples and post-mortem samples generally gave a poorer signal than fresh, unfrozen tissue, and contained a series of degradation products that were not observed in the latter. Proteolysis is a common problem in the analysis of post-mortem human tissue (Walaas et al., 1989), and huntingtin has been demonstrated to be susceptible to proteolysis (Dyer & McMurray, 2001; Mende-Mueller et al., 2001).

It is interesting to note here that amyloid aggregates are highly resistant to proteolysis (Glenner, 1980). However, the methods applied in this chapter enable the separation and visualisation of soluble proteins (in contrast to the methods applied in Chapter 3). As such, aggregated amyloidogenic proteins would be excluded from the polyacrylamide gels and most likely avoid detection by antibodies. Hence, a
combination of this type of aggregation and proteolysis (both pre- and post-mortem) of soluble huntingtin, presents one possible explanation for the non-detection of full-length huntingtin described above.

6.4.4 Tryptic in-gel digestion

The ultimate goal of sub-cellular fractionation, 1D-PAGE and Western blotting, was to identify N-terminal huntingtin bands within a gel for excision and characterisation by reverse-phase HPLC and N-terminal peptide sequencing. Three different HN1-positive bands were excised from polyacrylamide gels and subjected to in-gel trypsin digestion. Peptide fragments were separated by reverse-phase HPLC, collected and submitted for N-terminal sequencing. Four internal fragments of myelin basic protein (MBP) were successfully sequenced. This protein, possibly the most common isoform (18.5 kDa), could have migrated to a similar position in the gel as the ~20 kDa HN1-positive band that was excised, and may therefore have been excised from the gel along with this HN1-positive species. BLAST searches with the peptides that were sequenced demonstrated that one of these peptides was not found within the 14 kDa isoform of MBP, suggesting that this was not the isoform excised from the gel. All four peptides were present in the 17.3, 18.5 and 21.5 isoforms. The co-migration of MBP with an HN1-immunoreactive protein is not thought to be relevant to the disease process in HD.

6.4.5 Conclusions

The distribution of huntingtin fragments described in this chapter includes nuclear fractions, mitochondria/synaptosome-enriched fractions, membrane-enriched fractions and soluble cytosolic fractions, in both control and HD-affected tissue. The relative abundance of the N-terminal huntingtin epitope (detected by HN1) in each of these fractions differed between the HD tissue (n=2 brains) and normal control tissue (n=2 brains). There was an apparent difference in the relative abundance of a small (~20 kDa) HN1-immunoreactive protein in both nuclear and mitochondria/synaptosome-enriched fractions of HD versus control brain tissue. Attempts to purify and characterise this fragment from the polyacrylamide gel resulted in peptide sequence being obtained for various fragments of myelin basic protein. Curiously, these fragments all eluted in the hydrophilic region (ie. <50% buffer B) of the
chromatograms (figure 6.5), whilst peaks eluting in the hydrophobic region (ie. >50% buffer B) of the chromatograms were unable to yield sequence data. It may be that a hydrophobic conformation is unfavourable for N-terminal sequencing of proteins or peptides. As in Chapters 3 and 5, the point in the research where progress is blocked is N-terminal sequencing, and possible reasons for this have been discussed in Chapter 3.

Finally, recent research has investigated the role of proteolysis of huntingtin in HD (Dyer & McMurray, 2001; Mende-Mueller et al., 2001). One study demonstrated that mutant huntingtin is resistant to proteolysis and that N-terminal cleavage fragments (~50-60 kDa) arise from the processing of normal huntingtin (Dyer & McMurray, 2001). N-terminal fragments of normal huntingtin were seen in both normal and HD brain, however the normally monomeric soluble fragments appeared to be sequestered to poorly soluble macromolecular aggregates in HD. These N-terminal fragments of huntingtin were not detected by the antibody 1C2 leading to the suggestion that they were derived from the normal protein and that the mutant protein is more resistant than the normal protein to proteolysis. This study supports our observation of HN1-positive, 1C2-negative N-terminal fragments of huntingtin described in this chapter. Unlike the results described in this thesis, Dyer & McMurray (2001) report full-length huntingtin (~350 kDa) detected by both the N-terminal antibody and 1C2 demonstrating the presence of mutant protein. These authors do not describe an N-terminal fragment of ~20 kDa.

A different study describes tissue-specific proteolysis of huntingtin (Mende-Mueller et al., 2001). When the pattern of proteolysis within cortex and striatum of control and HD brains was examined, three different protease-susceptible domains were identified. One domain, termed the ‘A’ domain, was postulated to reside near the N-terminus. Cleavage within this region generated four N-terminal fragments of huntingtin ranging in size from 20-50 kDa (the 20 and 50 kDa bands were prominent, whilst the 40 and 43 kDa bands were less abundant). These were detected with an equivalent antibody to HN1. N-terminal fragments of the same size have been described in this chapter. Cleavage within the second or ‘B’ domain (which is thought to lie further away from the N-terminus) resulted in 60-80 kDa fragments that are not detected by the N-terminal antibody. This result suggested that cleavage within the B domain occurs in conjunction with cleavage within the A domain, and this pattern of proteolysis was demonstrated within cortex. In contrast, cleavage within the B domain
was not observed in striatum. Proteolytic fragments from striatum suggested instead that cleavage occurred within the A domain, and a novel ‘C’ domain that was not cleaved in cortex. Of particular interest, striatum from HD brains showed increased levels of a 40-50 kDa N-terminal fragment, and a 30-50 kDa C-terminal fragment, compared with control striatum. Based on this observation of tissue-specific proteolysis, it would be interesting to extend the SDS-PAGE study described here to include striatal tissue as well.

The proteolytic fragments of huntingtin that have been reported in this chapter may be due to a mixture of non-specific proteolysis and the tissue-specific proteolysis described by Mende-Mueller et al. (2001). Despite a possible background of non-specific proteolysis however, a sub-cellular location-specific pattern of proteolysis, or localisation of proteolytic fragments, is demonstrated by the results described in this chapter.

Our observation of N-terminal fragments of 20 and 50 kDa within both HD and control cortical tissue, supports the same observation by Mende-Mueller et al. (2001). In dissecting this result even further, we have demonstrated that these two fragments also have specific sub-cellular localisation: the 50 kDa fragment was localised to the soluble cytosolic fraction, whereas the 20 kDa fragment was localised to both the nuclear pellet and mitochondria/synaptosome-enriched pellet. This smaller fragment was markedly increased in abundance in HD brain relative to control brain. A final fraction, enriched in membrane, appeared to have very low amounts of both fragments. The relevance of this is uncertain. Our result, combined with the result of Mende-Mueller et al. (2001), may be evidence for sub-cellular location-specific as well as tissue-specific proteolysis of huntingtin. Alternatively, following tissue-specific proteolysis of huntingtin, there may be a specific pattern of sub-cellular localisation for the cleavage products. There is a need for a similar dissection of striatal proteolysis, to determine if huntingtin fragments that are generated there also have a particular pattern of localisation.
Chapter 7

An Alternative Approach to Protein Purification and Characterisation

7.1 Overview

The research described in this chapter employed the use of two recent techniques for the purification and characterisation of aggregated protein from Huntington disease (HD) tissue. Firstly, laser capture microdissection (LCM) was used to extract cells containing protein aggregates directly out of tissue sections. In this way, samples could potentially be greatly enriched in the aggregated proteins that we targeted during the research described in this thesis. By this method, contamination with material derived from neurons without aggregates, non-neuronal cells, extracellular matrix and blood vessels can be decreased. Secondly, Q-TOF (tandem quadrupole-time of flight) mass spectrometry (MS) could be subsequently used for: i. Capillary LC-MS (liquid chromatography/mass spectrometry) for identification of HD ‘bio-markers’ (ie. proteins that are up- or down-regulated in HD), and ii. MS/MS for peptide sequencing of any markers identified. The research did not progress to the MS/MS stage of this strategy. Q-TOF MS was performed at APAF (Australian Proteomics Analysis Facility, Sydney). Laser capture microdissection (LCM) and Q-TOF methods (LC-MS and MS/MS) are described below.

7.1.1 Laser Capture Microdissection

LCM was conceived and first developed as a research tool at the National Institutes of Health (NIH), and through a collaborative agreement with Arcturus Engineering
(Mountain View, California), was developed into a commercial instrument (Bonner et al., 1997). LCM is described as a rapid, reliable tool to select and capture single cells from complex mixtures (Suarez-Quian et al., 1999). The capture of areas of tissue is achieved by activation of a transfer film by a solid-state near-infrared laser beam which is focussed by the operator on a selected area of tissue under the microscope (Banks et al., 1999).

![Diagram of LCM process](image)

Figure 7.1. Schematic diagram illustrating the process of LCM. The transfer film or membrane forms the bottom part of a cap that can be placed on the tissue and lifted after film activation. Caps fit within microcentrifuge tubes for extraction of DNA, RNA or protein. A. Activation of the laser leads to focal melting of the transfer film (a polymer membrane). B. Lifting of the cap selectively detaches the cells adherent to the activated film. Diagram from Fend and Raffeld (2000).

Once activated, the transfer film adheres to the selected area of tissue more strongly than the tissue adheres to the slide on which it is mounted. Thus when the transfer film is lifted, the selected cells can be lifted free of the surrounding tissue and used in subsequent analysis.
Probably due to the availability of PCR (polymerase chain reaction) and RT-PCR (reverse-transcriptase PCR), for the amplification of DNA and RNA, and the lack of a comparable technique for proteins, early applications of LCM involved subsequent analysis of nucleic acids (Fend, Emmert-Buck & Chauqui, 1999; Simone, Bonner & Gillespie, 1998). More recently, proteomic analysis of laser capture microdissected cells has been reported (Emmert-Buck et al., 2000; Ornstein et al., 2000). These studies all describe the capture by LCM of 40,000-50,000 cells and the separation of protein extracts from the cells by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The large number of cells that are required in a single sample in order to allow visualisation of proteins on 2D-PAGE gels is an unfortunate limitation of proteomic applications for LCM. In order to circumvent this problem, Arcturus Engineering have formed a collaboration with Ciphergen (Freemont, California). Ciphergen manufacture protein chips for the rapid discovery and characterisation of proteins at the femtomole level from crude biological samples.

7.1.2 Tandem Quadrupole Time-Of-Flight Mass Spectrometry
MALDI-TOF mass spectrometry has been used throughout the work described in this thesis, for the characterisation of protein and/or peptide masses in a sample. This technique is highly sensitive, but complete characterisation of proteins in a sample relies on the use of MALDI-TOF in conjunction with N-terminal sequencing methods. Tandem quadrupole time-of-flight (Q-TOF) mass spectrometry is a technique that enables protein identification by the selection of a parent ion in a quadrupole mass filter and fragmentation of this parent ion within a collision cell (representing a second quadrupole) (Loboda et al., 2000). The daughter ions then enter a reflecting TOF spectrometer, which examines the complete mass spectrum. Subsequent analysis of this tandem mass spectrum of a selected ion enables the construction of peptide sequence for that ion.

Although historically this technique was no more sensitive or powerful than N-terminal protein sequencing methods, recent developments in MS/MS technology (particularly nano-electrospray MS/MS) have made this the method of choice for protein identification when only femtomole amounts of protein are available (Shevchenko & Mann, 2000). Within the HD literature, nano-electrospray tandem mass spectrometry (whether quadrupole or not was not stated) was recently used for the identification of a 40 kDa protein (HAP40) that co-purified with huntingtin (Peters...
& Ross, 2000). Protein sequencing allowed the coding region for HAP40 to be identified as F8A; a previously putative open reading frame within intron 22 of the factor VIII gene, transcribed in the opposite direction.

A protocol for Q-TOF mass spectrometry suited to the work described in this chapter was developed in conjunction with Dr Peter Hains (APAF, Sydney). LC-MS and MS/MS can be combined (LC-MS/MS) to enable the sequencing of protein in a relatively pure sample. Because the latter of these techniques requires samples to be digested (usually by trypsin) prior to electrospray, and because our samples were expected to contain a mixture of proteins, the methods were not combined in the present research project. Trypsinisation of a mixture of proteins would be expected to result in too complex a fragmentation pattern. For this reason, the project was divided into the two parts described in section 7.2.5.

7.2 Methods and Materials

7.2.1 Preparation of tissue for LCM
As in all previous chapters, the human brain tissue was obtained from the New Zealand Neurological Foundation Human Brain Bank (School of Medicine, University of Auckland). Ethical approval for use of this tissue was obtained from the University of Auckland’s Human Subjects Ethics Committee. Tissue from three HD brains and one control brain was used for the research described in this chapter. The tissue was perfused and blocked as described in section 2.2.1, Chapter 2. The blocks were cut on a rotary microtome (Leitz) at a thickness of 8 µm and mounted on uncoated glass slides. Sections were dried for no more than 10-15 minutes at 60 °C. (NB/ Using either treated slides or drying times in excess of 15 minutes can lead to an adherence between the tissue and the slide that may be too strong and prevent LCM transfer). Mounted sections were dewaxed in xylene and then hydrated through a series of alcohols (100%, 95%, 70%, 50%) to water.

7.2.2 Staining of sections for LCM
Congo red staining of tissue sections for LCM was performed according to the method of McAnulty-Smith as described in section 2.2.2.2, Chapter 2. As in Chapter 2, a very weak cresyl violet counter-stain was used. Following dehydration in alcohols...
and clearing in xylenes, slides were left to air dry for at least 2 minutes before storage. The slides were not coverslipped.

7.2.3 LCM of tissue sections

LCM was performed using a PixCell II™ LCM system (Arcturus Engineering) at the University of Otago (Dunedin, New Zealand). This system consists of the following components: the PixCell II™, the CapSure™ Transfer Film Carrier, and the PixCell II™ Image Archiving Workstation. These components are described below:

- The PixCell II™ includes the LCM station, video monitor and controller. The LCM station integrates an Olympus IX50 inverted microscope, a low power infrared laser, a joystick-controlled stage, and custom CapSure™ handling mechanism.
- The CapSure™ Transfer Film Carrier consists of a proprietary thermoplastic polymer film. The Transfer Film Carrier is designed to fit directly onto standard microcentrifuge tubes.
- The PixCell II™ Image Archiving Workstation features custom software that records video images, and allows the annotation, storage and review of the LCM process. Unfortunately, the Image Archiving Workstation was not operational at the time of use.

LCM was performed according to the manufacturer’s instructions. A variety of laser spot-sizes was used. The best results (ie. most efficient transfer of cells) were achieved with a spot-size of 15 μm, and a laser power of 25 mW.

7.2.4 Extraction of proteins from LCM transfer film

Because there were no publications describing the extraction of poorly soluble peptides from LCM caps the following protocol was created for the work described in this thesis. Since the proteins were being extracted for LC-MS and MS/MS analysis by Q-TOF, Triton-X100 and other detergents had to be avoided. The small number of inclusion-containing cells overall (~400), and the division of these cells between 20 different LCM caps requiring pooling, introduced problems of dilution. Extraction was performed as follows:
1. 20 μl of buffer [6 M Guanidine HCl, 40 mM Tris. Cl, pH 7.4] was placed on top of inverted caps that were gently shaking at 40 °C, and left for 30 minutes.

2. The buffer on each cap was pipetted up and down several times before being transferred to one of two tubes, depending on the origin of the captured cells:
   - 1. Control + GHCl, and
   - 2. HD + GHCl

3. 20 μl of 100% (v/v) formic acid was added to the top of the inverted caps, which were still gently shaking at 40 °C. These were incubated for 5 minutes

4. The buffer on each cap was once again pipetted up and down several times before being transferred to one of two tubes, depending on the origin of the captured cells:
   - 3. Control + Formic, and
   - 4. HD + Formic

5. Tubes containing formic acid were vacuum-dried to near-dryness.

6. All tubes were packaged on dry ice and sent to APAF (Sydney, Australia) for analysis by Q-TOF mass spectrometry.

### 7.2.5 Q-TOF Mass Spectrometry

There were two separate but interconnected parts to the analysis of LCM samples by Q-TOF mass spectrometry. In the first instance, the aim was to compare the LC-MS profile of the four protein extracts described above. Once differences between HD and control samples (either guanidne-extracted or formic acid-extracted) were highlighted, these fractions were to be collected, digested with trypsin and analysed by LC-MS/MS. The methods for each part are described below. Because the equipment used was in Sydney, this part of the research was very kindly performed by Dr Peter Hains of APAF.

**Part A:**

Protein extracts were centrifuged at 14 000 x g for 20 minutes to remove particulate matter. Samples were loaded onto a Waters CapLC system equipped with a C8 capillary reverse-phase column (0.32 μm x 150 mm) that was coupled to a Micromass Q-TOF via a nanospray source. A gradient of 80% (v/v) acetonitrile, 0.1% formic acid was used to elute the sample from the column. As the ions entered the Q-TOF, the m/z range 400-1600 was scanned and a total ion chromatogram (TIC) generated.
Following manual examination of TICs, differences in observed masses were to be reported. The sensitivity of the apparatus was tested prior to sample analysis and reported to be in the range of 100-200 femtomole. Fractions that were different between control and HD extracts were to be isolated, digested with trypsin and analysed by LC-MS/MS in part B.

Because no control samples were analysed by LC-MS (see results section 7.3.2), a comparison was not possible and part B of the project (LC-MS/MS) was not required. Hence, the methods for part B are not described here.

7.3 Results

7.3.1 LCM

Examination of Congo red stained tissue sections by polarised light microscopy prior to LCM revealed the presence of a small number of pink-red inclusion bodies within or surrounding neuronal nuclei. However, birefringence could not be confirmed. The most likely explanation for this is the scattering of light at the tissue:air interface, preventing the appropriately modified light rays from passing through the analyser above the stage. When the same sections were examined by oil-immersion, birefringence of Congo red stained inclusions was observed. Because of this scattering of light from dried, uncovered tissue sections, birefringence could not be observed during LCM. The targets for transfer during LCM were therefore defined as the pink-red inclusions observed in or surrounding neuronal nuclei. These inclusions were approximately 2 \( \mu \text{m} \) in diameter, a size consistent with the amyloid-like inclusions reported in Chapter 2.

Approximately 400 Congo red-positive inclusion-containing neurons were captured from HD middle-frontal gyrus tissue sections. A further 400 neurons were captured from middle-frontal gyrus tissue sections as a control during subsequent experiments. Unfortunately, the PixCell II™ Image Archiving Workstation was not operational at the time of use. Because of this, images of tissue sections before and after LCM, or of the captured cells, are not available for this thesis.

Based on an average diameter of 2 \( \mu \text{m} \) for these inclusions, and assuming a roughly spherical shape, it is possible to calculate the total volume of Congo red-positive inclusions that were captured by LCM:
\[ V = \pi r^3 \text{(per inclusion)} \]
\[ = \pi \times 1 \times 400 \text{ (inclusions)} \]
\[ = 1.257 \times 10^{-9} \text{ cm}^3 \]

In order to calculate the amount of protein that was available for LC-MS analysis, an estimate of density is necessary. The average density of a wide range of proteins (regardless of solubility) is taken to be \( \sim 1.37 \text{ grams per cm}^3 \) (Voet & Voet, 1995). Hence, the mass of protein associated with the captured amyloid inclusions is calculated as follows:

\[ m = \text{Volume} \times \text{density} \]
\[ = 1.257 \times 10^{-9} \times 1.37 \]
\[ = 1.722 \text{ nanograms} \]

Finally, in order to calculate the amount of protein in moles in this sample, an estimate of molecular weight is necessary. The molecular weight of amylin, the protein that forms amyloid plaques in the diabetic pancreas is 3905 Da. The molecular weight of \( \beta \)-amyloid, the protein that forms amyloid plaques in Alzheimer’s disease (AD) brain is 4200 Da. The molecular weight of the uncharacterised protein (with amyloid-like properties) that we purified from amyloid-enriched extracts of Huntington disease brain, and again from polyglutamine-expressing Neuro-2A cells, is 4285 Da. Although several larger proteins have been shown to form amyloid fibrils (ie. PrP, \( \alpha \)-synuclein), in our calculations we assume a molecular weight of 4285 Da for the major amyloid-forming protein species present in HD inclusions, being approximately equal in size to the amyloid-forming proteins in AD and diabetes. Hence:

\[ n = \frac{g}{Mr} \]
\[ = \frac{1.722 \times 10^{-9}}{4285} \]
\[ = 4.02 \times 10^{-13} \text{ moles} \]
\[ = 400 \text{ femtomoles} \]

This figure is a very rough estimation and is likely to be slightly too high as amyloid has been demonstrated to contain lipid and polysaccharide components in addition to protein (Modis, 1991). As mentioned previously, the sensitivity of the Q-TOF was estimated to be as low as 100-200 femtomole. This amount of protein would then have been divided between samples extracted with GHCl and formic acid, and some protein may have been retained on the LCM transfer caps. Protein extracted from four
hundred inclusions is therefore at the extreme lower margin of what might be detected by Q-TOF mass spectrometry.

7.3.2 Q-TOF Mass Spectrometry

LC-MS analysis of the sample HD + GHCl was unable to detect any proteins or peptides within this sample. LC-MS analysis of the sample HD + Formic revealed the presence of several peptides in this sample. A total of 6 peptides and/or proteins were detected, ranging in mass from 966 daltons to 1615 daltons; 966.26 Da, 1310.02 Da, 1403.90 Da, 1504.92 Da, 1516.78 Da and 1615.76 Da. Figure 7.2 shows the MS spectra for the peptides and the equivalent monoisotopic mass (all ions are double charged).

Because two LC-MS attempts were required to detect these peptides/proteins, there was insufficient sample retained for MS/MS analysis (part B) of them. Therefore, it is uncertain what these peptides/proteins were. None are exact multiples of glutamine residues (although the 1403.9 Da peptide/protein is very close to the molecular weight of 11 glutamine residues (1409.1)). None are exact multiples of proline residues (97.1 Da). None of the peptides or proteins detected by LC-MS are equivalent in mass to GFAP, histone H3, hypothetical protein FLJ20623, myelin basic protein or known huntingtin-interactors. Additionally, none of the molecular weights of huntingtin fragments of various lengths, extending sequentially from the N-terminus, match the molecular weights that were detected. In light of the fact that a more complete characterisation of the proteins and/or peptides within the HD + formic sample could not be performed, and due to the relatively high cost of this technique, it was decided not to analyse the Control + formic sample by LC-MS.

Figure 7.2 (following page). MS spectra for the peptides observed in the sample HD + Formic. As all ions are doubly charged, the monoisotopic mass is determined by taking the mass of the lowest isotope, multiplying by 2, and subtracting 2 Da (the mass of 2H molecules).
7.4 Discussion

Two contemporary techniques were applied to achieve the aim of the research described in this thesis, which was: **to purify and characterise the aggregated protein species in HD brain.** The first of these techniques, LCM, is a preparative technique that was used to enable to first part of this aim – purification of aggregated protein by achieving molarity above background. Secondly, Q-TOF mass spectrometry is an analytical technique that it was hoped would enable the successful characterisation of purified protein. In all previous chapters, characterisation depended on MALDI-TOF mass spectrometry and N-terminal protein sequencing. Some mass values were determined for protein peaks of interest by the MALDI-TOF method. The application of N-terminal sequencing provided peptide sequence from a number of proteins: glial fibrillary acidic protein (GFAP), histone H3 protein, myelin basic protein and the hypothetical protein FLJ20623. The first of these was co-purified with amyloid components of HD brain and Chapter 4 describes investigations into the role of that protein in HD.

Myelin basic protein, co-migrated with an N-terminal huntingtin-immunoreactive species in SDS-PAGE. However, no huntingtin or polyglutamine-containing moieties were purified and sequenced. A considerable number of HPLC peaks that were submitted for N-terminal sequencing, mostly eluting under hydrophobic conditions (ie. >50% buffer B), did not yield sequence data. Some possible difficulties of applying N-terminal protein sequencing methods to polyglutamine-containing proteins are discussed in Chapter 3.

There is certainly potential for LCM and Q-TOF mass spectrometry to be used in tandem for the identification of peptide markers of disease. Given enough starting material and therefore enough sample for LC-MS and MS/MS analysis, the complete characterisation of those markers is a realistic goal. Additionally, LCM could be used in combination with protein chip technology (such as that developed by Ciphergen). With this method proteins or peptides retained on protein chip arrays are analysed by a protein chip reader. This machine uses SELDI-TOF (surface-enhanced laser-desorption/ionisation-time-of-flight) mass spectrometry, which produces a peptide ‘fingerprint’ of a sample. This type of analysis allowed changes in amyloid precursor protein (APP) processing mediated by cholesterol to be identified (Frears et al., 1999). The limitation of SELDI-TOF is that the technique is not MS/MS-capable (see
below), so that if a particular peptide fingerprint is not present within a database, the peptides cannot be fully characterised. The collaboration between Ciphergen and Arcturus is expected to enable much greater sensitivity of proteomic analysis on LCM-obtained cells than 2D-PAGE (Paweletz et al., 1999). At the time of the research described in this thesis there were no protein chip readers available in Australasia, and so this technology was not applied to the research project. More recently, however, a handful of these systems have been installed in Australian universities and institutes, and there are plans to use these for the analysis of human HD tissue in the future.

LCM enabled rapid and accurate microdissection of inclusion-containing neurons out of the surrounding tissue. It is unfortunate that due to the non-operation of the Image Archiving Workstation it was not possible to show the effectiveness and accuracy of LCM for the reader’s information. The technique was easy to use, and the collection of 800 neurons took approximately a day and a half. The more traditional biochemical methods for purification, such as those described in Chapter 3, can take up to a week to purify proteins for mass spectrometry or sequencing. Although the amount of protein obtained using traditional methods is far greater, much of this is unwanted ‘background’ protein and the point of using LCM was to achieve molarity above background for the proteins of interest. Contemporary characterisation techniques such as Q-TOF mass spectrometry are sufficiently sensitive to enable analysis of femtomole amounts of protein.

Q-TOF mass spectrometry was chosen due to the low level of protein that was expected to be extracted from 400 cells. This number is 100-fold lower than the number of cells captured by LCM in studies that use two-dimensional polyacrylamide gel electrophoresis. It is unlikely that any proteins extracted from these cells would have been detectable by UV had they been injected on to a reverse-phase HPLC column. Therefore, capillary-LC directly coupled to a Q-TOF mass spectrometer was selected as the most suitable technique for separation and analysis of proteins in these extracts. This was performed at APAF as described, and calculations in section 7.3 demonstrate that the amount of protein likely to present in the samples that were sent to APAF is at the borderline of what the Q-TOF machine could detect. The protein extraction method from LCM caps was designed with LC-MS in mind, however due to the relatively small number of target cells there was no opportunity to refine this extraction protocol. No proteins or peptides were detected in the sample HD + GHCl,
however several peptides ranging in mass from 966.26 to 1615.76 daltons were observed in the sample HD + Formic. It was unfortunate that two attempts were required to obtain a mass spectrum for this sample, and as such insufficient sample remained for MS/MS analysis, and therefore characterisation of these peptides. Hence, the project was halted. Because of this, neither control sample was analysed by LC-MS. The masses that were detected are not consistent with the molecular weights of any of the known huntingtin-interacting proteins or proteins that localise to neuronal inclusions (see table 8.1, Chapter 8).

If sufficient sample is available, Q-TOF MS/MS may be able to overcome the potential technical limitations of N-terminal sequencing (discussed in Chapter 3) such as N-terminal blockage, that may be the reason sequence data was not obtained for certain HPLC peaks. The work described in this chapter represents an initial investigation into the viability of new technologies for purifying and characterising proteins extracted directly from tissue sections. The results demonstrate that even at the limits of sensitivity (due to the very low amount of protein expected to be present in 400 inclusions), proteins and or peptides are detected by Q-TOF mass spectrometry. If half of the HD + formic acid sample had not been applied to LC-MS analysis when the Q-TOF was operating sub-optimally, it is possible that MS/MS analysis of the remainder would have enabled characterisation of the peaks that were observed by LC-MS. These results warrant a continuation of the project described in this chapter, perhaps with a greater number of Congo red-positive inclusions being obtained. Additionally, Arcturus have recently developed kits for extracting protein from LCM caps that may be more successful than the method described in this chapter and therefore allow more protein to be submitted for Q-TOF analysis. Due to financial constraints, the project was not expanded for the purpose of this thesis.
**Chapter 8**

**Concluding Discussion**

8.1 Overview

Huntington disease (HD) is an insidious disease that despite a relatively low prevalence is tragic for patients and their families. Although the genetic cause of HD was discovered almost ten years ago, there remains no treatment available for sufferers of this disease. The work that has been described in this thesis was performed to elucidate the biochemical basis of HD, in particular to purify and characterise the aggregating protein species that are seen in post-mortem HD brain. This chapter is intended as a summary of the results of the research performed for this thesis and to draw conclusions based on those results. Possible future avenues of research, which may complement or follow on from the research, are also discussed.

We have described the presence of amyloid-like inclusion bodies in Huntington disease brains. Polyglutamine has been hypothesised to form amyloid structures (Perutz, 1996; Perutz *et al.*, 1994), and amyloid structures formed from expanded polyglutamine-containing proteins have been demonstrated *in vitro* (Scherzinger *et al.*, 1997). Our finding, using polarised light and confocal microscopy, is the first report of amyloid *in situ* in HD brain, resulting from a reduction of counter-stain intensity to very low (McGowan *et al.*, 2000; Appendix 2).

In an effort to purify and characterise the protein component(s) of these amyloid-like inclusions, protein extracts from HD brain were enriched in amyloid based on the resistance to proteolysis and relative insolubility of amyloid. Control brain extracts were treated by the same enrichment process and proteins were purified and characterised based on a difference between HD and control brain extracts visualised by HPLC. Three different proteins, GFAP, histone H3, and hypothetical protein FLJ20623 were co-purified with the amyloid component of HD brain based on their increased abundance relative to control brains. The role of GFAP in HD was subsequently investigated as described in Chapter 4, whilst the potential role of
CHAPTER EIGHT – CONCLUDING DISCUSSION

Histone H3 in HD is discussed in Chapter 3. In addition to these proteins, an ~4285 dalton protein was co-purified with the amyloid component of HD brain. This protein was absent in control brain and displayed unusual properties (discussed in section 8.2.2). An amino-acid sequence was not determined for this protein, possibly due to limitations of N-terminal protein sequencing as discussed in Chapter 3.

Much of the work described in this thesis involves method development, in particular, to increase the relative abundance of target amyloid-associated proteins, to improve the solubility of these, or to improve the likelihood of obtaining sequence data from them by enzymic or chemical cleavage into peptides. Additionally, the methods used were tested on polyglutamine-expressing cultured cells that form poorly soluble protein aggregates. Most interestingly, an ~4285 dalton protein was purified from the cultured cells, with similar properties to the protein of the same approximate molecular weight described above. Because the transgene in the cultured cells is different to the HD gene except for the polyglutamine stretch, this protein is probably unrelated to huntingtin. However, because it is expressed in both Neuro-2A cells and human brain, and either upregulated or altered in conformation (leading to purification from the insoluble fraction) in HD, this protein might be important in the disease process.

In all, a very broad range of biochemical and histochemical techniques were applied during the research described in this thesis. These include histology, immunohistochemistry, confocal and polarised-light microscopy, protein extraction and sub-cellular fractionation, HPLC using a wide variety of conditions, MALDI-TOF mass spectrometry, N-terminal sequencing, 1D- and 2D-PAGE, Western blotting, LCM (laser capture microdissection), and Q-TOF tandem mass spectrometry.

The aims of the research project were ambitious, particularly considering the small size and scarcity of amyloid-like inclusions we observed, but potentially very rewarding. Precise knowledge of the aggregating fragment of huntingtin would reveal the relevant cleavage site(s) within huntingtin and enable therapeutic strategies aimed at preventing cleavage at those sites to be developed. Furthermore, it was hoped that other, presently unidentified, protein components of the HD amyloid or associated with it, if they exist, would be identified during the research. It was hypothesised that this information may lead to new insights into the neuropathogenic mechanism...
operating in HD. (c.f. the discovery of amylin in amyloid plaques from the diabetic pancreas).

The major contribution of the research project to the knowledge of HD, is the first description in the literature of in situ amyloid-like inclusions (McGowan et al., 2000) bringing HD, and by association the other polyglutamine diseases, into the expanding family of late-onset neurodegenerative diseases associated with amyloid. Additionally, the work described in Chapter 6, taken in conjunction with the work of Mende-Mueller et al. (2001), suggested a possible pattern of sub-cellular localisation of different sized proteolytic fragments.

With the advent of more sensitive characterisation techniques such as Q-TOF tandem mass spectrometry the aims of the thesis could conceivably be achieved in the near future. Although this technique was applied on three occasions in the late stages of the work described in this thesis, the method was not fully developed during this project due to the overseas location of the equipment.

8.2 Review of Experimental Results

8.2.1 Identification of amyloid-like inclusions in HD brain

The possibility that expanded polyglutamine stretches could form amyloid structures was first proposed several years ago (Perutz et al., 1994). Evidence for the involvement of amyloid in HD is provided by studies using cell culture models (Scherzinger et al., 1997), and protein extracts from HD brain (Huang et al., 1998). We describe the first finding of in situ amyloid-like inclusions in HD brain. The relationship between the amyloid-like inclusions we detected and the immunohistochemically-detected inclusions that are reported in the literature is uncertain. The presence within amyloid-like inclusions of either polyglutamine, N-terminal fragments of huntingtin, or ubiquitin was not confirmed due to the incompatibility of the amyloid-staining protocols and immunohistochemistry protocols. The frequency of amyloid-like inclusions was considerably lower (5-20 per 150 mm² section; n=11) than the reported frequencies of immunohistochemically-detected inclusions. There are at least two possible interpretations of these results. Firstly, the Congo red-stained, birefringent inclusions we observed could represent a discrete set of inclusions unrelated to those reported in the literature. No data is
currently available on the protein constituents of these inclusions. The only contradictory ‘evidence’ to this possibility is the similar size of the inclusions we describe and the immunohistochemically-detected inclusions reported in the literature, although this could be coincidental. A second possibility is that the amyloid-like inclusions we describe are a subset of the immunohistochemically-detected inclusions reported in the literature. In this case, amyloid-like inclusions would be likely to contain one or more of the proteins localised to neuronal inclusions (described in section 1.8, Chapter 1). It may be that the abundance of other protein components in many of these inclusions, prevents the regular binding of Congo red, and birefringence observed by polarised light microscopy. Only inclusions that are abundant in the particular protein(s) responsible for amyloid staining characteristics would allow these characteristics to be observed.

The definition of amyloid is generally accepted to include: (i) green birefringence under polarised light when stained with Congo red, and (ii) a fibrillar morphology observed by electron microscopy (EM). In the present study, we observed Congo red staining of inclusions, which were birefringent under polarised light. DiFiglia et al. (1997) report “parallel and randomly oriented fibrils” observed in HD brain by electron microscopy. Huang et al. (1998) demonstrate Congo red staining with green birefringence for aggregates purified from HD brain. Collectively, these three independent studies provide good evidence for the presence of amyloid in HD brain, although the protein constituents have yet to be determined, as has its role.

The relevance of a lack of amyloid-like staining in R6/2 transgenic mice has been discussed in Chapter 2. Due to the higher than endogenous expression level for the transgene and the extreme length of the glutamine repeat (144-190), the dynamics of aggregation in the mice are different to those of the human disorder. In this respect, the R6/2 mouse model more closely resembles juvenile-onset HD than the more common adult-onset disease. Whether or not amyloid-like inclusions are present in the brains of juvenile-onset HD patients is uncertain. In the research described in this thesis, we did not examine tissue from any juvenile-onset cases. One study reports Congo red staining and polarised light microscopy of a juvenile-onset case (Karpuj et al., 1999). In that study, no amyloid staining properties were observed and the authors conclude that immunohistochemically-detected inclusions were non-amyloidogenic. They also report using a “brief hematoxylin counterstain” and it is possible that this may have masked detectable amyloid. Based on the results of this study and our own,
it is possible that in both juvenile HD cases and in the R6/2 mice, protein aggregation occurs at too fast a rate for ordered structures to be formed, possibly due to the incorporation of other proteins. Hence, the rate of aggregation may be an important determinant in the presence or absence of detectable amyloid, as regular structures are required for Congo red binding and birefringence to be observed.

8.2.2 Purification and partial characterisation of amyloid associated protein(s)

A large proportion of the research effort was spent performing the work described in Chapter 3. Ultimately, three proteins that co-purified with the amyloid component of HD brain tissue were characterised, and another potentially amyloid-associated protein was partially characterised (mass spectrometry data only). Following the initial purification and characterisation of a novel fragment of GFAP using the method of Cooper et al. (1987), a number of refinements were made to this method. The most successful modification was the separation of nuclei from the remainder of the cellular material, at the beginning of the purification process. Introduction of this step, and the use of a less 'sticky' reverse-phase column (C5 Jupiter; better suited to the separation of hydrophobic proteins), enabled the purification of an internal fragment of histone H3. The relevance of this protein has been discussed in Chapter 3. In addition to histone H3 another protein was identified in the amyloid-enriched extract of HD brain that was absent in control brain. This protein eluted at either 32%B or 62%B, and had a mass spectrum with molecular weights of 4285 Da (± 5 Da) and 8572 Da (± 4 Da, 1 outlier). N-terminal sequencing failed and therefore complete characterisation for these proteins was not achieved. Despite this, there were a number of protein property clues that imply a possible amyloidogenic nature for these proteins. Firstly, the same mass spectrum was observed in reverse-phase peaks from completely different hydrophobicities (32%B & 62%). Secondly, the peaks would often not elute during the chromatography run on which the sample was injected but during the subsequent blank run (GHCl). It is important to note that successive injections of the same GHCl solution did not produce these peaks; only blank runs that immediately followed sample runs produced these peaks, with a decrease in peak height in subsequent blank runs to baseline by the third or fourth consecutive blank run. Thirdly, the same mass spectrum was observed in reverse-
phase peaks originating from three different gel filtration fractions (IV, V and VI) with completely different expected mass ranges.

Taken together, these observations suggest that the protein or proteins we detect are capable of undergoing a major conformational change between hydrophilic and hydrophobic states, and of forming oligomers and multimers enabling their elution from gel filtration at a variety of expected molecular weights. Whether the two masses that were detected by mass spectrometry represent differently charged ions of the same protein or two different proteins is uncertain. However, it is likely considering the proximity of the larger mass to double the smaller mass, that they represent the same protein. Which of these masses represents the monomeric state of that protein is the next issue to be resolved. In most cases, the approximately 4285 Da peak was the higher of the two indicating that this is likely to represent the monomeric state. Where mass spectrometry peaks represent differently charged ions of a particular protein, the monoisotopic mass is easily calculated. If the larger mass we detected was 2M+H of the smaller mass, then the relationship between the two would be $x = (y-1)/2$, where $x$ is the smaller mass, and $y$ the larger (1 Da being the mass of a hydrogen atom).

Conversely, if the smaller mass we detected was M+2H of the larger mass, then the relationship between the two would be $y = 2x - 2$ for the same parameters. Note that generation of multiply charged ions is a phenomenon of mass spectrometry, and does not usually represent the presence of dimers, or other n-mers of a particular protein. In the current study however, none of the mass pairs that were detected within the various reverse-phase peaks were consistent with either of these formulae. It is therefore likely that both monomer and some dimer were present in those samples. The approximately 8570 Da mass could not represent the monomer, because the smaller peak would then have to be consistent with the formula for M+2H, which is not the case. No consistent relationship between x and y values could be determined for all peaks (VIII-XI). Therefore, it is likely that the smaller mass we detected is due to a mixture of the monomeric mass and the M+2H peak of the dimer. Conversely, the larger mass that we detected would be due to a mixture of dimeric mass and the 2M+H peak of the monomer. These differently charged ions would have been selected together with the genuine monomer or dimer mass due to their close proximity, thereby skewing the relationship between the two masses. It is highly unlikely that these two masses represent different proteins. None of the other
modifications that were made to the purification protocol improved the separation of protein samples, solubility of proteins, or sequencing success any further. The same purification methods were applied to cultured Neuro-2A cells expressing different lengths of polyglutamine that were shown to form aggregates. The reason for doing this was not to characterise the aggregating proteins in these cells - aggregation is visualised by fluorescence from GFP, which is translated downstream of the polyglutamine stretches. Rather, the reason for purification of aggregates from cultured cells was to determine the efficacy of the purification and protein characterisation methods that were used in Chapter 3, particularly when dealing with polyglutamine-containing proteins. The use of gel filtration and reverse-phase HPLC techniques enabled the purification of two protein peaks from cell homogenates expressing 30 or 100 glutamine residues. These peaks were absent in cell homogenates expressing 0 glutamines (ie. vector only). Partial characterisation of one of these peaks was achieved by mass spectrometry, however as before N-terminal sequencing was not successful. It is curious that the masses detected in this peak are very similar to those described above. The smaller mass (4289.5 Da) is within the range of the smaller masses described, and the larger mass (8588.6 Da) is only slightly outside the range for those described. Again the relationship between the smaller and larger masses cannot be explained by differently charged ions of the same protein, and in this case, the larger is approximately 10 Da larger than double the smaller mass. This difference could be due to a calibration problem caused by the low level of detection of these peaks.

Whether this partially characterised protein from expanded polyglutamine-expressing cells is the same protein we purified and partially characterised from HD brain tissue is uncertain. Two possibilities were suggested in Chapter 5. One possibility is that the purified proteins consist mostly of a stretch of glutamine residues, being derived in the human tissue from proteolysis of huntingtin. This hypothesis is unlikely for a number of reasons. Firstly, the masses we observed are not divisible by 128.1, the molecular weight of glutamine. The smaller mass falls between 33 and 34 glutamines, and the larger mass is very close to 67 glutamines however none of the human tissue we used had a CAG repeat that long. Secondly, the difference might have been due to some flanking sequence, however there is no homology between the linker peptide used to insert polyglutamine and the polyglutamine-flanking sequences in huntingtin. Thirdly, Huang et al. (1998) suggest on the basis of immunohistochemistry using a
range of different huntingtin antibodies, that the N-terminal fragment of huntingtin that aggregates in the nucleus is between 179 and 595 residues from the N-terminus. This peptide would have a molecular mass approximately between 20-66 kDa, far greater than the small peptide we purified.

The second possibility suggested in Chapter 5 is the presence in amyloid/aggregates of an as yet uncharacterised protein expressed in both Neuro-2A cells and HD middle-frontal gyrus. This protein might be upregulated or switched on where expanded-polyglutamine containing proteins are expressed, or it may interact with these proteins becoming recruited into poorly soluble aggregates. An investigation of the molecular masses of the proteins known to either interact with huntingtin, or co-localise to inclusions with huntingtin, yielded no match for the mass we detected. Table 8.1 lists the molecular weights of all of these proteins.

Table 8.1. Molecular weights for all of the known huntingtin-interacting proteins, and proteins known to co-localise with huntingtin to intracellular aggregates or inclusions. Proteins are ranked by decreasing molecular weight. Most molecular weights are estimates based on the number of amino acids. Mdm-2 is expressed as different isoforms with a wide range of molecular weights. HYPA, HYPB and HYPF are WW domain proteins that were found to interact with huntingtin. TFIID is the subunit of TATA-binding protein (TBP) that has been localised to inclusions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBP</td>
<td>268 620</td>
</tr>
<tr>
<td>N-CoR</td>
<td>268 400</td>
</tr>
<tr>
<td>CA150</td>
<td>120 780</td>
</tr>
<tr>
<td>HIP1</td>
<td>109 000</td>
</tr>
<tr>
<td>HSP70</td>
<td>70 000</td>
</tr>
<tr>
<td>Cystathionine beta-synthetase</td>
<td>60 610</td>
</tr>
<tr>
<td>Mdm-2</td>
<td>11 220-55 232(^1)</td>
</tr>
<tr>
<td>HYPA(^2)</td>
<td>49 720</td>
</tr>
<tr>
<td>p53</td>
<td>43 230</td>
</tr>
<tr>
<td>SH3GL3</td>
<td>38 170</td>
</tr>
<tr>
<td>TFIID(^3)</td>
<td>37 698</td>
</tr>
<tr>
<td>CREB</td>
<td>37 510</td>
</tr>
<tr>
<td>GAPDH</td>
<td>37 000</td>
</tr>
<tr>
<td>HAP1</td>
<td>36 816</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>25 746</td>
</tr>
<tr>
<td>E2-25K</td>
<td>22 000</td>
</tr>
<tr>
<td>HYPF(^2)</td>
<td>15 840</td>
</tr>
<tr>
<td>HYPB(^2)</td>
<td>13 970</td>
</tr>
</tbody>
</table>

The approximately 4285 dalton protein we observed could conceivably be a protein for which a relationship to huntingtin or to HD has not yet been established. Alternatively, the protein we purified from human brain and Neuro-2A cells and partially characterised, might represent a peptide fragment of one of the proteins listed
above, or a smaller fragment of N-terminal huntingtin than the one described by Huang et al. (1998). 'In-silico' digestion or cleavage of huntingtin with trypsin, clostripain or formic acid produced numerous fragments, however none were of the sizes we detected using mass spectrometry.

8.2.3 SDS-PAGE and Western Blotting

SDS-PAGE of amyloidogenic proteins is typically very difficult (G. Cooper, personal communication). Even using high concentrations of the chaotropic substance urea and the detergent SDS, these proteins are not well solubilised. Electrophoresis of these proteins therefore usually results in a considerable amount of smearing and poor separation. Often, these proteins will remain in the wells and not migrate into the gel. Polyglutamine-containing proteins also display unusual electrophoretic properties, migrating through polyacrylamide gels at a different rate to other proteins of the same size. Two-dimensional SDS-PAGE was investigated as a technique for the separation of amyloid-associated proteins from HD brain, but this method achieved poor separation and resolution of these proteins. An interesting additional project would be the 2D-PAGE of soluble protein extracts from control and HD brain to identify changes in protein expression in the disease state. HPLC using strongly denaturing solvents (such as 6 M guanidine hydrochloride) was more successful (see above) and used instead of 2D-PAGE.

As an aside from efforts aimed at purifying amyloid-associated proteins, we looked for soluble N-terminal fragments of huntingtin in sub-cellular fractions of HD and control tissue. It was thought that some N-terminal fragments might be present in a soluble state as an intermediate between cleavage from full-length huntingtin and aggregation into inclusions. Hence, SDS-PAGE of soluble proteins offered an alternative means to purify at least one of the aggregating proteins in HD.

Western blotting of gels revealed some consistent differences in the sub-cellular localisation of N-terminal fragments of huntingtin between control and HD. In particular, an approximately 20 kDa band was observed to be markedly increased in the nuclear fraction and mitochondrial/synaptosomal fraction of HD brain homogenates relative to control brain homogenates. This short fragment of N-terminal huntingtin might be the molecular species that aggregates within neuronal nuclei to form intranuclear inclusions. N-terminal sequencing of tryptic peptides from this
fragment did not result in huntingtin sequence, however four tryptic peptides were shown to be different fragments of myelin basic protein. This protein is thought to have co-migrated (due to being a similar molecular weight) with the N-terminal huntingtin fragment that was detected by Western blotting. Interestingly, myelin basic protein has been reported to be increased in Alzheimer's disease cortex (Selkoe et al., 1981). Furthermore, both myelin basic protein and GFAP have been demonstrated to serve as substrates of brain transglutaminase, a calcium-dependent enzyme that is also implicated in HD (see section 1.10.4, Chapter 1; Selkoe et al., 1982a). Hence, a role for these proteins in HD can not be entirely ruled out.

This sequencing of myelin basic protein fragments indicates that N-terminal sequencing methods are useful when applied to proteins that are compatible with them. Several other tryptic peptides from the same digestion mixture did not provide sequence data and these may be from the huntingtin fragment. The presence within this fragment of (i) a long stretch of glutamine residues, and (ii) a moderate stretch of proline residues (usually between 7-12), is most likely the reason for its apparent incompatibility with N-terminal sequencing methods. Even short stretches of proline cause the sequencing reaction to stop (I. Anthony, personal communication), and N-terminal glutamine residues are subject to cyclization under acidic conditions to pyroglutamyl (Allen, 1986) leading to blockage of Edman degradation. Although not very satisfying, it is possible that the amyloid-associated proteins from HD brain discussed above (section 8.2.2) were N-terminal fragments of huntingtin, or other proteins with similar motifs, due to the problems that we encountered with N-terminal sequencing.

The identification of different N-terminal fragments of huntingtin in control and HD brain indicates disease-specific proteolysis. It also appears that a degree of protein degradation had occurred in all cases (HD and control) during post-mortem delay and processing of tissue. This is evidenced by the extremely weak detection of full-length huntingtin on Western blots, and only in unboiled samples, despite the detection of small fragments of huntingtin (both N-terminal and internal), and despite several repeats. Lastly, 20 kDa is a rough estimate for the mass of the N-terminal fragment of huntingtin we report given the irregular migration of polyglutamine-containing proteins in polyacrylamide gels. It is conceivable that the ~4285 Da protein we purified from amyloid extracts could migrate at an apparent molecular weight of 20 kDa, if it contained a large proportion of glutamine residues. However, 20 kDa is
within the range predicted by Huang et al. (1998) and the fragment we detected is both enriched in HD brain (relative to control) and localised to the nucleus. Polarised-light microscopy of Congo red stained nuclear fractions from HD brain revealed amyloid characteristics that may, or may not have been caused by the N-terminal fragment of huntingtin we detected.

8.2.4 LCM and Q-TOF Mass Spectrometry

These two contemporary techniques were applied to achieve the aims outlined in Chapter 1. Firstly, laser capture microdissection of HD sections enabled fast and accurate microdissection of inclusion-containing neurons from the surrounding tissue. No images of this procedure could be obtained due to the Image Archiving Workstation being non-operational at the time of use, however the sensitivity of the laser enabled very precise microdissection. A method was developed to extract poorly soluble proteins from ~400 inclusions on LCM caps for Q-TOF tandem mass spectrometry. The amount of protein associated with this number of inclusions was estimated to be at the lower level of sensitivity for Q-TOF mass spectrometry. LC-MS analysis was performed by Dr Peter Hains of APAF, Sydney. Two different samples were analysed: (i) inclusion-containing neurons extracted with guanidine-HCl, and (ii) inclusion-containing neurons extracted with formic acid. No proteins or peptides were detected in the former sample, however several proteins and/or peptides were detected in the latter sample on a second analysis. These ranged in mass between 966-1615 daltons. Because the LC-MS analysis this analysis had to be performed twice, there was not sufficient sample remaining for MS/MS tandem mass spectrometry of these peptides. Hence, these peptides were not fully characterised and their identity is unknown. No match for the molecular weights any of these proteins or peptides could be found with multiples of glutamine residues, multiples of proline residues, huntingtin fragments extending from the N-terminus, or known huntingtin-interactors. Furthermore, the addition of the molecular weights of various permutations of these proteins/peptides did not enable a sum of ~4285 daltons to be obtained, suggesting that the proteins/peptides were not derived from the protein of that molecular weight described previously as being enriched in HD brain. Used together, these two techniques offer a powerful means of identifying and characterising proteins at very low levels, extracted from very specific cell types.
Neuro-2A cells expressing polyQ form aggregates

Amyloid-like inclusions identified in situ, in HD brain

Nuclear separation and amyloid enrichment

Sub-cellular fractionation – amyloid detected in nuclear fractions

HPLC

SDS-PAGE

~4285 dalton protein or peptide with amyloidogenic-like behaviour

~20 kDa (apparent molecular weight) N-terminal fragment of huntingtin

Incompatible with N-terminal sequencing techniques

In-gel tryptic digestion generating peptides

Figure 8.2. Schematic illustration of a subset of the results described in the thesis research. Dotted arrow represents possible identity of peptides due to the abnormal migration of polyglutamine containing proteins in polyacrylamide gels. LCM results and investigations of IGFAP are not included in this schematic.
8.3 Future directions

It seems clear from the results described in the thesis that the aims of the research outlined in Chapter 1 will be attained with the availability of more sensitive and powerful sequencing techniques such as tandem mass spectrometry. Furthermore, the results of Chapter 6 taken together with the results of Mende-Mueller et al. (2001) and Dyer & McMurray (2001) discussed in that chapter, warrant the investigation of the physiological proteolysis of huntingtin. A dissection of pre-mortem and post-mortem proteolysis would be difficult to achieve due to extensive proteolysis in post-mortem human brain, however this could illuminate potential alterations in huntingtin cleavage mediated by the expanded polyglutamine tract. Fragments of huntingtin detected by Western analysis were previously assumed to be degradation products (Metzler et al., 2000; Wood et al., 1996). Although many of these fragments are likely to represent post-mortem proteolysis, consistent detection of particular fragments of huntingtin enriched in HD brain, and with a particular sub-cellular localisation, might provide evidence of physiologically relevant proteolysis of huntingtin (Mende-Mueller et al. (2001); this study). Such a model could explain the discrepancies in the literature describing huntingtin localisation, based on the use of antibodies raised against different regions of the protein. Western analysis of mouse and rat brain tissue provides no evidence of endogenous proteolysis of the huntingtin homologues in these species.

Another hypothesis that might account for the presence of truncated N-terminal fragments in HD brain is the occurrence of a process termed ‘molecular misreading’. This process describes a novel type of mutation in susceptible dinucleotide repeats in messenger RNA, leading to truncated proteins with abnormal C-terminii. Molecular misreading of both APP (β-amyloid precursor protein) and ubiquitin have been demonstrated in Alzheimer’s disease and Downs syndrome brain tissue (van Leeuwen, Burbach & Hol, 1998a; van Leeuwen et al., 1998b). Antibodies were raised against the predicted aberrant C-terminii based on a proposed dinucleotide deletion of GAGAG motifs to GAG and subsequent frameshift. The so-called ‘+1’ proteins were detected in dystrophic neurites forming neuritic plaques, neuropil threads and neurofibrillary tangles of AD patients and tangles in the brains of non-demented elderly controls. The same mutant proteins were also detected in apparently healthy looking neurites of Down syndrome patients.
Investigation of the cDNA sequence of the *HD* gene revealed a number of GAGAG motifs, one of which would produce a truncated fragment of huntingtin in the range predicted by Huang *et al.* (1998) if a dinucleotide deletion occurred. This motif is in exon 12 of the *HD* gene, and a ‘GA’ deletion is predicted to produce a 493 residue protein with an aberrant C-terminus from residue 448 inclusive. The predicted molecular weight of this peptide is 54.389 kDa, similar to the cytosolic N-terminal fragment of huntingtin we observed in both HD and control brain as reported in Chapter 6. The isoelectric point (8.33) and extinction coefficient (19 600) were also predicted. Immunohistochemistry of HD brain tissue with an antibody raised against the aberrant C-terminus would enable the existence of this predicted +1 protein to be determined.

### 8.4 Conclusions

Much of the HD research found in the literature comes from cell-culture or animal models of the disease. Although these models provide very useful information about the behaviour of huntingtin, the conditions under which aggregation occurs, and the biochemical pathways (particularly those that are involved in cell death) that are invoked, it is extremely important not to lose sight of the abnormal context of huntingtin in most of these cases. A mouse that expresses a fragment of human huntingtin introduces into its cellular milieu a molecule that could interact with other molecules in unpredictable ways that need not mimic interactions occurring in the human disorder. A mouse expressing endogenous huntingtin with an expanded polyglutamine tract may offer more clues to the disease process but the relatively short lifespan of mice suggests that this might be a good model for juvenile-onset HD only. The availability of human brain tissue from HD patients and normal controls is a highly precious resource that enables us to investigate potential mechanisms of the disease directly. There are however a number of confounding factors including post-mortem processes, the occurrence of death long after the onset of symptoms, medications that patients may have been treated with, and factors associated with the cause of death.

Using post-mortem HD brain tissue, we describe the first observation of in situ amyloid-like inclusions. Amyloid-enrichment led to the purification and
characterisation of fragments of GFAP, histone H3 and hypothetical protein FLJ20623, as well as the purification and partial characterisation (mass spectrometry data only) of a protein or peptide of approximately 4285 Da molecular weight. A protein or peptide of the same approximate molecular weight was also purified from polyglutamine-expressing cells, yet absent in control cells. This molecular weight does not match the estimated molecular weight of a range of huntingtin-interacting or huntingtin co-localising proteins. Nor does this mass match the expected molecular weight of any huntingtin fragments generated by trypsin, clostripain or formic acid. A 20 kDa (apparent molecular weight estimated from a gel) N-terminal fragment was found to be enriched in HD nuclear fractions, which also display amyloid-like staining characteristics. It is possible that this fragment is related to the ~4285 Da protein that we purified from amyloid-enriched HD brain, with the 20 kDa fragment representing multimers of the 4285 Da monomer. Alternatively, aberrant migration in a polyacrylamide gel of the ~4285 Da protein, possibly due to the presence of an expanded polyglutamine domain, could result in an apparent molecular weight of 20 kDa. This relationship could not be confirmed due to an ostensible incompatibility of N-terminal sequencing methods with some of the peptides or proteins we purified. This incompatibility might be expected for proteins and peptides containing stretches of glutamine and/or proline residues for reasons that have been discussed in Chapter 3. Ultimately, the research described in this thesis demonstrates amyloid in situ in the human HD brain, and raises the possibility that a monomer of ~4285 Da, which may have identity with an N-terminal portion of huntingtin, or may represent a novel protein, is a major protein component of these structures. This finding places HD and by association the other polyglutamine disorders into the category of amyloid diseases, and suggests that strategies to prevent amyloid accumulation, a focus of Alzheimer’s Disease research, may be of much wider application to glutamine repeat disorders such as HD or vice versa.
LITERATURE CITED


LITERATURE CITED


LITERATURE CITED


Appendix 1

Mass spectrum and High Performance Liquid Chromatography data supplied by Auspep Pty. Ltd. (Parkville, Australia) following synthesis of custom peptide.
CERTIFICATE OF ANALYSIS

Product: Custom Synthesis Peptide

Code: CS           Batch No: K20800
Molecular Weight: 3072
Peptide Weight: 10mg           Purity: >70%

Comments: Product molecular weight confirmed by mass spectral analysis.
Cysteine free sulfhydryl detected by Ellmans test.

Certified: Quality Control Manager

High Performance Liquid Chromatography

Column: MERCK, Superspher®, 250-4, LiChroCART, 100 RP-18
Solvent A: 0.1% Trifluoroacetic Acid (TFA)
Solvent B: 90% Acetonitrile/Water + 0.09% TFA
Gradient: 0%B to 70%B, 35 minutes, Linear
Wavelength: 220nm
Flow Rate: 1.0ml/min

HPLC CHROMATOGRAM

'pCSK20800'

Auspep Pty. Ltd. A.C.N. 006 673 347
P.O. Box 806
Parkville 3052

Telephone +613 9328 1211
Fax +613 9326 8810
Email: auspep@auspep.com.au
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Publication arising from this thesis:

Letter to Neuroscience

AMYLOID-LIKE INCLUSIONS IN HUNTINGTON’S DISEASE


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Key words: Alzheimer’s disease, Congo Red, neurodegeneration, polyglutamine, prion.

Huntington’s disease is a progressive, autosomal dominantly inherited, neurodegenerative disease that is characterized by involuntary movements (chorea), cognitive decline and psychiatric manifestations.7 This is one of a number of late-onset neurodegenerative disorders caused by expanded glutamine repeats, with a similar likely biochemical basis.9 Immunohistochemical studies on Huntington’s disease tissue, using antibodies raised to the N-terminal region of huntingtin (adjacent to the repeat) and ubiquitin, have recently identified neuronal inclusions within densely stained neuronal nuclei, peri-nuclear and within dystrophic neuritic processes.12 However, the functional significance of inclusions is unknown. It has been suggested that the disease-causing mechanism in Huntington’s disease (and the other polyglutamine disorders) is the ability of polyglutamine to undergo a conformational change that can lead to the formation of very stable anti-parallel β-sheets; more specifically, amyloid structures.13 We examined, using Congo Red staining and both polarizing and confocal microscopy, post mortem human brain tissue from five Huntington’s disease cases, two Alzheimer’s disease cases and two normal controls.Brains from five transgenic mice (R6/2)12 expressing exon 1 of the human huntingtin gene with expanded polyglutamine, and five littermate controls, were also examined by the same techniques. We have shown that some inclusions in Huntington’s disease brain tissue possess an amyloid-like structure, suggesting parallels with other amyloid-associated diseases such as Alzheimer’s and prion diseases. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

There has been a developing debate over the functional significance of inclusions, with some evidence suggesting that they may be secondary to the disease process and may arise due to a proteolytic protective mechanism.10,14 The hypothesis that amyloid structures are formed in Huntington’s disease (HD) is supported by evidence that polyglutamine forms amyloid-like protein aggregates in vitro, which stain with Congo Red (a histological stain for amyloid) and exhibit green birefringence under polarized light.15 The two factors which are important in Congo Red staining and birefringence are the linearity of the dye molecule (a recurring active group at 10.3Å intervals) and the β-sheet configuration of substrate, which should also create a reflectance signal detectable by confocal microscopy.1 Further support for amyloid-like protein aggregation occurring in HD is provided by evidence of Congo Red staining with green birefringence, of purified aggregates from HD brain captured on cellulose acetate filters.6 Immunohistochemical investigation of these in vivo aggregates revealed the presence of an amino terminal segment of huntingtin, as well as the polyglutamine-containing TATA-binding protein.

In the present study, Congo Red-stained birefringent inclusions, were observed in cortical and striatal neurons in all five HD cases, but were absent in two normal brains and the two cases of Alzheimer’s disease (AD; data not shown). Inclusions were observed both within neuronal nuclei (Fig. A, B) and peri-nuclear (Fig. IC, D). Regions that contained these inclusions were middle-frontal gyrus, middle-temporal gyrus, caudate nucleus, amygdaloid nucleus and globus pallidus. Cerebellum and hippocampus appeared to be spared. The CAG-repeat size range in the five HD cases ranged from 42 to 52. There was no correlation between repeat size and the number of Congo Red-stained birefringent inclusions. The AD cases showed typical extracellular plaque morphology and no intracellular birefringent inclusions. When the HD sections were examined by confocal microscopy, using transmitted light (Fig. 1E) and reflection modes (Fig. 1F), the reflectance signal was seen to co-localize with the birefringent neuronal inclusions. These results provide high-resolution evidence that the inclusion bodies contain amyloid-like protein aggregates. The inclusions observed averaged 1.5–2 μm in diameter, a size consistent with the immunohistochemically detected inclusions reported by other investigators, however they were very infrequent with a range of 5–20 per 150 mm² section. This variability between sections could be explained by masking of the Congo Red stain by the counterstain (see Experimental Procedures). Immunohistochemical staining of sections with a ubiquitin-detecting antibody (Fig. 1H) revealed a much higher frequency of inclusions. However, staining of adjacent sections with an

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Abbreviations: AD, Alzheimer’s disease; DAB, diaminobenzidine tetrahydrochloride; HD, Huntington’s disease; PBS, phosphate-buffered saline.
Fig. 1. Congo Red staining and immunohistochemistry of inclusion bodies in HD brain. (A) A neuronal intranuclear inclusion in the basal ganglia of a Grade 3 HD case stained with Congo Red when viewed in bright-field and polarized light (B). Bright-field (C) and polarized light (D) views of a Congo Red-stained perinuclear inclusion in a middle frontal gyrus neuron of a different Grade 3 HD case. (E) Transmitted light image of a Congo Red-stained inclusion corresponding to the reflectance signal detected by confocal microscopy (F). (G) N-terminal huntingtin-positive and ubiquitin-positive (H) neuronal intranuclear inclusions in middle frontal gyrus of a Grade (3) HD case. Scale bars = 5 μm.
antibody raised against the N-terminal 17 amino acids of huntingtin (Fig. 1G), detected only 1–2% of this number, falling within the range of Congo Red-stained, birefringent inclusions.

We were unable to successfully Congo Red-stain tissue previously processed for immunohistochemistry or vice versa, to confirm or otherwise the presence of huntingtin in these inclusions. Also, because of the small size of the Congo Red-positive inclusions we were unable to find in serial sections, a huntingtin-positive, Congo Red-stained, biecstic inclusion. The observation that membrane-trapped aggregates purified from HD brain stain with Congo Red, display birefringence, and react with antibodies to N-terminal epitopes of huntingtin,6 supports the theory that there is at least some overlap between amyloid-like inclusions and the immunohistochemically detected inclusions. The presence of TATA-binding protein and ubiquitin in the immunohistochemically detected inclusions could prevent the linear binding of the Congo Red dye molecule, thereby impeding staining and birefringence in the majority of inclusions. This heterogeneous aggregation could explain the relatively low abundance of amyloid-like inclusions.

No Congo Red staining with birefringence was observed in the transgenic mouse brains or littermate controls, despite clear and abundant immunohistochemically detected inclusions.7 The absence of Congo Red staining indicates that detectable amyloid did not form in these mice. This could be due to the incorporation of other proteins, an effect of localization, protein concentration, or possibly smaller undetectable β-sheet inclusions in the mice. In vitro studies have shown the aggregation of polyglutamine-containing proteins to be length dependent; longer repeat lengths lead to faster and more complete protein aggregation.11 The repeat length in the transgenic mouse (141–157) examined is in the range of the very severe juvenile-onset disease in humans. Protein aggregation in these mice is, therefore, likely to occur much faster than in the normally slowly progressive human disorder, potentially leading to the incorporation of other proteins into the aggregates and preventing linear binding of Congo Red. Alternatively, the short lifespan of these mice (killed at five to eight weeks of age) may not be sufficient to lead to the formation of the amyloid-like ordered aggregates observed in human disease tissue.

The term “amyloid” refers to a disease-associated mammalian polymer exhibiting fibrillar structures and green birefringence after Congo Red staining.5 In the present study, we have observed only the latter of these two defining features, and therefore describe amyloid-like inclusions. Ultrastructural analysis of neuronal inclusions in human and transgenic mouse tissue, has shown a mixture of granules and filamentous structures in both, with parallel and randomly oriented fibrils observed only in humans.5,23 The independent observations in HD tissue of fibrils in neuronal inclusions and Congo Red staining with birefringence of inclusions that we have described, support the hypothesis of amyloid formation occurring in HD. This finding makes it more likely that HD and the other polyglutamine disorders fit into the category of amyloid diseases together with AD and prion disease. Whilst the role of aggregates remains contested in all of these diseases including HD, our suggestion that the inclusions in HD have an amyloid-like structure raises the possibility that strategies to prevent amyloid accumulation being developed for one neurodegenerative disease (ie, AD) may be of much wider application to glutamine repeat disorders such as HD or vice versa.

**EXPERIMENTAL PROCEDURES**

The human tissue was obtained from the New Zealand Neurological Foundation Brain Bank, and the diagnosis of HD was confirmed unequivocally by genetic analysis of the CAG-repeat length. Tissue for Congo Red staining was perfused and blocks were embedded in paraffin. The blocks were cut on a rotary microtome (Leitz) at a thickness of 8 µm, and mounted on Apes-coated glass slides (2% amine- propyltrimethoxysilane in acetone). Mounted sections were de-waxed in alcohols and xylene and taken to water. Sections were then incubated in an alkaline salt solution (0.01% NaOH in NaCl-saturated 80% alcohol) for 20 min and stained in a 3% Congo Red solution (3% Congo Red; 0.01% NaOH; NaCl-saturated 80% alcohol) for a further 20 min. Following Congo Red staining, sections were very weakly counterstained in Cresyl Violet and then washed in water, dehydrated in alcohol, cleared in xylene and coverslipped using Histomount mounting media (Hughs and Hughs, UK). Only the very weakest counterstain allowed detection of Congo Red-stained inclusions, probably contributing to the variability between sections, as the counterstain appeared to mask Congo Red staining. Slides were examined on a Leitz Dialux 20 EB polarizing microscope or a Leica laser scanning confocal microscope.

Mouse brains from line R6/2 were perfused with 4% paraformaldehyde, fixed overnight and transferred to phosphate-buffered saline (PBS) with 0.02% azide. Sections were cut at 8 µm on a frozen microtome and stained as described above. Tissue for immunohistochemistry was perfused and blocks were cryoprotected. Sections of 50 µm were cut on a freezing slide microtome and collected in PBS with 0.1% sodium-azide. In order to compare and contrast the cellular localization of ubiquitin and huntingtin, immunocytochemistry for each protein was performed on immediately adjacent sections. Ubiquitin was localized with a rabbit polyclonal ubiquitin antibody (DAKO Corporation). For detection of the huntingtin protein, a rabbit polyclonal antibody was used, raised against amino acids 1–17 of huntingtin protein (kind gift of Dr. A.L. Jones). Sections were incubated in 0.2% Triton in PBS (PBS-Triton) at 4°C overnight, transferred to 1% H2O2 in 50% methanol for 20 min, washed in PBS-Triton (3 × 10 min), and then incubated for three days at 4°C in primary antibodies diluted in 1% goat serum in PBS with 0.2% Triton and 0.4% methanol. Primary antibodies were used at a dilution of 1:2000. The sections were washed with PBS-Triton (3 × 10 min) and then incubated with donkey anti-rabbit immunoglobulin bietinated secondary antibody (1:1000, Amersham Life Sciences) overnight at room temperature. The sections were washed, incubated in StreptAvidin–biotinated horseradish peroxidase complex (1:1000, Amersham) for 3 h, washed again in PBS-Triton and the antigen was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H2O2 to produce a brown reaction product. The sections were mounted on chrome album coated slides, air dried, and lightly counterstained with Cresyl Violet. Finally, the sections were dehydrated and coverslipped.

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