MICROGLIAL DYSFUNCTION INDUCED BY MUTANT HUNTINGTIN

by

Colúm Connolly

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Abstract

Huntington’s disease (HD) is a devastating, late-onset neurodegenerative disorder that causes profound behavioral abnormalities, language impairment, and alterations in personality in affected patients. HD is an autosomal dominantly inherited disease, caused by a CAG trinucleotide repeat expansion in the huntingtin gene. HD effects up to 1 in 10,000 in populations of European ancestry, but with at least a 10 fold reduced prevalence rate in individuals in Asian or African descent. The critical mechanisms by which the expansion in the huntingtin gene leads to selective neurodegeneration in HD are poorly understood. The purpose of this thesis was to better understand the microglial dysfunction caused by mutant huntingtin and the potential role this microglial dysfunction may play in the pathogenesis of HD. Huntingtin, the protein (HTT) expressed from the huntingtin gene, is ubiquitously expressed in many tissues, with the highest expression levels in brain and testis. Over the last 20 years there have been multiple scientific breakthroughs allowing the development of an array of model systems to investigate HD pathogenesis. Immune dysfunction has recently been implicated in a number of neurodegenerative diseases, including HD. In conclusion, the mechanism of neurodegeneration is not well understood in HD, inflammation could play a pivotal role in the progression of the disease. Inflammation is altered in immune cells containing mutant HTT (mHTT), and although I was unable to provide conclusive evidence that mHTT-induced microglial dysfunction and related neuroinflammation are required for neurodegeneration in an HD mouse model, my work highlights the importance of critically evaluating proposed new disease mechanisms as many will not be directly involved in HD neurodegeneration. My
research provides concrete evidence that immune dysfunction occurs in monocyte cells expressing mHTT, however, this cell intrinsic dysfunction does not play a major role in the HD phenotype of the BACHD mouse model.
Preface

All of the work presented henceforth was conducted at the Center for Molecular Medicine and Therapeutics, part of the Child and Family Research Institute at the University of British Columbia, Children’s and Women’s Hospital campus. All projects and associated methods were approved by the University of British Columbia’s Animal Care Committee (Certificate # A09-0673).

Chapter 1: Introduction is an updated and reworked version of published material based on the publication of my book chapter. Connolly C, Hill A, Leavitt BR. Microglia in Huntington disease. Microglia; Biology, Functions and Roles in Disease.

Chapter 2: Replicates of Figure 2.1 as well as some text describing the methodology for the generation of both bone marrow monocytes and peritoneal monocytes have been published in Trager U, Andre R, Magnusson-Lind A, Miller JR, Connolly C, Weiss A, Grueninger S, Silajdzic E, Smith DL, Leavitt BR, Bates GP, Bjorkqvist M, Tabrizi SJ. Characterization of immune cell function in fragment and full-length Huntington’s disease mouse models. 2014 Neurobiol Dis 2014 73: 388-398. The other figures from this chapter (Figure 2.2-2.7) along with figures 3.6 and 3.8 has been compiled in a manuscript that has been submitted and is currently under review. Colúm Connolly, Anna Magnusson-Lind, Ge Lu, Pamela K Wagner, Amber L Southwell, Michael R Hayden, Maria Björkqvist, and Blair R. Leavitt Enhanced Immune Response to MMP3 stimulation in Microglia Expressing MHTT. mRNA generated for figure 2.3 was also used in for investigation of FoxP1 levels in the

I led all aspects of experimental design for this chapter and conducted all experiments with assistance from P. Wagner. I was responsible for all major areas of data collection and analysis.

Chapter 4: Figures 4.4 - 4.10 as well as some of the text outlining the methodology will be incorporated into a paper. I was responsible for all aspects of experimental design, collection and subsequent analysis for Figures 4.1- 4.4. For Figures 4.5-4.10 myself, A Hill and BR Leavitt had significant input into conceptual design of the experiments. The BACHD mouse trial was conducted by J Yang, subsequent analysis was of the trial was conducted as previously described by Fransciosi et al., 2012. J Blanco performed microscopy and cell counts. The BACCre trial paper is currently in preparation, Connolly C, Blanco J, Hill A, Yang J, Lu G, Leavitt BR. Microglial deletion of mHTT in the BACHD mouse model of HD.
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List of Abbreviations

3-hydroxykynurenine (3-HK)
cannabinoid receptor 2 knockout (CB2KO)
central nervous system (CNS)
control standard endotoxin (CSE)
enzyme linked immunosorbent assay (ELISA)
förster resonance energy transfer (FRET)
glial fibrillary acidic protein (GFAP)
huntington’s disease (HD)
huntingtin (HTT)
k keratinocyte-derived cytokine (KC)
kynurenic acid (KYNA)
kynurenine pathway (KP)
kynurenine 3-monooxygenase (KMO)
lipopolysaccharide (LPS)
N-methyl-D-aspartate (NMDA)
matrix metalloproteinases (MMPs)
medium spiny neurons (MSNs)
meso scale discovery (MSD)
positron emission tomography (PET)
quinolinic acid (QUIN)
tissue inhibitors of metalloproteinases (TIMPs)
toll like receptor 4 (TLR4)
wild-type (WT)
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1. Introduction

1.1 Thesis Introduction

Huntington's disease is a neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin (HTT) gene resulting in expression of an uninterrupted polyglutamine stretch within the N-terminus of its protein product HTT. The HTT protein is expressed in a wide range of cells, but the role of (wild-type) WT HTT is not yet fully understood. HD neuropathology is characterized by selective neurodegeneration of medium-sized spiny neurons (MSNs) of the caudate and putamen, collectively known as the striatum. Neuroinflammation is a typical feature of many neurodegenerative diseases, including HD and is associated with characteristic pathological changes within the affected areas of the brain. The neurodegeneration in HD is accompanied by inflammatory changes in the brain that are thought to be an aberrant immune response in the presence of mHTT. However, immune response in HD is a relatively unexplored area in the field, and the immune response of the central nervous system (CNS) immune response even less so. Therefore, the aim of my thesis was to explore the potential role of inflammation on the progression of HD.
1.2 Huntington’s Disease

1.2.1 History

The first widely-accepted description of Huntington’s disease was provided by George Huntington in 1872 in a report entitled “On Chorea” which appeared in the Medical and Surgical Reporter of Philadelphia (Huntington, 1872). This concise four-page treatise outlined the distinctive choreiform movement disorder, the hereditary nature of the chorea, and the frequent association of the chorea with psychiatric disease. In 1993 ‘The Huntington Disease Collaborative Research Group’ determined the genetic cause of the disease as a CAG tri-nucleotide repeat expansion in a gene initially called IT15 (interesting transcript 15). It was later known as the Huntington or HTT gene (Gusella et al., 1983; Huntington’s Disease Collaborative Group, 1993). Despite the development of a sufficient clinical description of the disorder 140 years ago, an understanding of the genetic basis, and incredible progress in identifying potential therapeutic targets in HD, there has to date been no translation of these discoveries into effective therapies to slow or reverse the relentless progression of disability and loss of function in HD.

1.2.2 Clinical Features

The first symptoms of HD can appear at any age, but commonly occur in mid-life, with a mean age of clinical disease onset of approximately 40 years old (Andrew,
The disease is characterized by the insidious development of motor, cognitive, and psychiatric disturbances with an inexorable progression towards complete disability and death. HD is found in all populations around the world, with an estimated prevalence of up to 1 in 10,000 in populations of European ancestry and a 10-fold reduced prevalence rate in individuals of Asian or African descent (Harper et al., 1991). The formal clinical diagnosis of HD is based on the presence of specific motor symptoms (an otherwise unexplained extra-pyramidal movement disorder) in the setting of a known family history of HD. Typically, the clinical diagnosis is confirmed through direct genetic testing for the disease allele. Prior to the clinical diagnosis (the pre-manifest phase) many patients already exhibit subtle changes in personality, cognition, and motor control. In many cases the individuals are often unaware of these changes (Paulsen et al., 2008; Tabrizi et al., 2009). Large longitudinal studies of pre-manifest and early manifest HD subjects, such as PREDICT-HD and TRACK-HD, have identified neurobiological and clinical alterations that are quantifiable and that occur long before obvious clinical diagnosis and motor symptoms are detected (Paulsen et al., 2008; Tabrizi et al., 2009).

The movement disorder in HD consists of both abnormal involuntary movements such as chorea and dystonia, as well as abnormalities of voluntary movement such as abnormal eye movements, bradykinesia (slowed voluntary movements), rigidity, dysphagia, dysarthria and gait disturbance (Hayden et al., 1981). Chorea is slowly progressive and is generally observed during the early stages of HD, peaks in the mid-stages, and then in later stages becomes replaced by a clinical picture dominated
by bradykinesia, dystonia and rigidity as the disease progresses (Leavitt, 2000). In comparison, voluntary motor dysfunction (such as motor impersistence, the inability to maintain a constant voluntary muscle contraction) arises and progresses independently of chorea and correlates with disease progression and disability (Feigin, 1995). Quantifiable abnormalities in fine motor skills such as tongue force and finger tapping rhythm and rate may be useful measures in early HD (Pausen et al., 2008; Tabrizi et al., 2009) whereas changes in gait, balance and posture are often observed later (Busse et al., 2009; Grimbergen et al., 2008; Rao et al., 2009).

Cognitive dysfunction can vary greatly in different individuals, affecting executive functions such as organizing skills and problem solving, but also visuo-spatial abilities, emotional processing and smell identification (Sprengelmeyer et al., 1996). Cognitive impairment in HD often progresses at a similar rate to the motor disturbances and is generally classified as a progressive “subcortical dementia”. Loss of cognitive speed, flexibility, and concentration are common early deficits, with memory loss, aphasia and agnosia being less prominent. These changes can appear early in pre-manifest subjects before motor symptoms are noticeable and once they occur they tend to worsen as the disease progresses (Pausen et al., 2008; Tabrizi et al, 2009; Montoya et al., 2006). Depression, apathy, and irritable or impulsive behavior are common neuropsychiatric manifestations of HD, and obsessive-compulsive symptoms or psychosis also occasionally develop during the course of the illness. The expression of neuropsychiatric symptoms in HD varies widely in prevalence, age of onset, and rate of progression (Anderson et al., 2001).
Common systemic features of HD include testicular degeneration (Van Raamsdonk et al., 2007), sleep disorders (Morton et al., 2005), and weight loss. The weight loss appears to be independent of dysphagia and occurs despite the maintenance of a normal caloric intake (Sanberg et al., 1981). Cachexia is an almost invariant feature of HD, and suggests that an altered metabolic state may be associated with progression of the disease (Pratley et al., 2000). Juvenile-onset HD, a rapidly progressive form of the disease sometimes termed the Westphal variant of HD, occurs in approximately 5% of patients these generally present with a predominantly akinetic-rigid clinical picture with prominent spasticity, bradykinesia, dystonia, and rapid intellectual decline, often complicated by myoclonus and seizures beginning prior to age 20 (Nance and Myers 2001).

1.2.3 Neuropathology

The most prominent pathologic feature in HD is the selective neurodegeneration of MSNs of the caudate and putamen, collectively known as the striatum (Vonsattal et al., 1985). MSNs receive excitatory (glutamatergic) inputs from the neocortex and thalamus and make efferent inhibitory projections (GABAergic) to the globus pallidus and substantia nigra (Albin et al., 1989). The neurodegeneration observed in HD is highly specific as aspiny interneurons, the other major neuronal cell type in the striatum, are relatively resistant to this cell death (Cabresi et al., 1998). Other brain regions also display selective neurodegeneration but not to the extent
seen in the striatum; these include the pyramidal projection neurons in layers V and VI of the cerebral cortex, and the CA1 region of the hippocampus. As the disease progresses neurodegeneration also occurs in the globus pallidus, subthalamic nucleus, substantia nigra, cerebellum and thalamus (Hedeen, 2006; Richards, 1997).

Changes at the subcellular and molecular levels are critical features of pathology in the neurons of HD. In terms of neuronal morphology, the neurons of effected individuals contain nuclear and cytoplasmic intracellular inclusions (DiFiglia et al., 1997.). These inclusions are also a common pathologic feature of other polyglutamine diseases. They are insoluble, ubiquitinated protein aggregates formed from several cellular proteins including full-length and truncated HTT (Sieradzan et al., 1999). Despite the fact that these inclusions are a pathologic feature of HD and other polyglutamine diseases, their role in the disease process remains unknown. The neuropathological hallmark of HD is the degeneration of the caudate nuclei (Graveland et al., 1985). As the disease progresses, there is dramatic neuronal loss in the caudate along with increasing numbers of both reactive astrocytes and microglia in the grey matter of the caudate, this is in contrast to the reported pathology of early stage HD brains in which minimal gliosis was observed (Pavese et al., 2006; Tai et al., 2007). In the neostriatum, mHTT is found in the cell bodies and synaptic processes of surviving neurons and glial cells. Interestingly, despite the selective neurodegeneration seen in HD, the expression of HTT is ubiquitous and not limited to the brain or to brain regions selectively affected (Sathasivam et al., 1999).
1.2.4 Genetics

The genetic defect that underlies and causes HD is the expansion of a CAG trinucleotide repeat in the first exon of the \textit{HTT} gene. The expansion results in the production of HTT with an expanded polyglutamine tract. Alleles containing greater than 35 CAG repeats are usually associated with the clinical phenotype of HD, with an earlier age of onset occurring with higher repeat sizes (Andrew \textit{et al.}, 1993). The length of the CAG repeat is unstable during intergenerational transmission of the gene from parent to child. The unstable nature of the CAG repeat in the \textit{HTT} gene provides the molecular basis for the clinical phenomenon of anticipation. Anticipation is defined as increasing severity of disease or decreasing age of disease onset in successive generations. Larger CAG repeat sizes exhibit greater instability than shorter repeat lengths, and paternal transmission of expanded CAG repeat alleles is more likely to result in expansion rather than contraction of the CAG repeat length (Wheller \textit{et al.}, 1999.). The majority of early-onset or juvenile HD cases are due to paternal transmission of a large expanded allele (Telenius \textit{et al.}, 1993). Individuals with intermediate alleles, defined as 27-35 CAG repeats, do not have any risk of developing HD, but the CAG repeats can sometimes expand into the pathogenic range during intergenerational transmission, causing HD in the offspring (Semaka \textit{et al.}, 2006). These intergenerational expansions from intermediate alleles often are identified as HD without a family history, this occurs more commonly than previously
thought potentially being responsible for up to 10% of all HD cases (Falush et al., 2001).

1.2.4.1 Mouse Models of HD

Since the identification of the disease-causing gene in 1993, a number of genetically modified animal models of HD have been generated (Table 1.1). The first transgenic mouse models, R6/2 lines, were established nearly 20 years ago (Mangiarini et al., 1996). The R6/2 mice represent the best-characterized and the most widely used model to study pathogenesis of HD and therapeutic interventions. The R6 mouse models are “fragment models” expressing the N-terminal first exon (of 67 total exons) of HTT driven by the human HTT promoter. The R6/2 mouse model develops symptoms rapidly and has a very short lifespan of 13-16 weeks.

N171-82Q mice are another mouse model of HD, in this case expressing a N-terminal fragment of HTT that incorporates both exon 1 and exon 2 of the HTT gene (Schilling. et al., 1999). The N-terminal fragment is expressed from the mouse prion promoter resulting in expression that is restricted to neurons (i.e. with no expression from other cells in the CNS) The mice contain two copies of the WT mouse gene and one copy of the mutant gene. The phenotype is similar but less severe than the R6/2 mice.

Mouse knock-in models are also used in the HD field (Wheeler et al., 2002). In this case, the endogenous CAG repeat of the mouse homolog of HTT is expanded
and the expression of mHTT is thus controlled by the endogenous mouse promoter, e.g. Hdh111. Polyglutamine expansions of varying lengths have been modeled in this context but these mouse models are used less frequently as the phenotype are comparably mild and the mice live as long as WT mice, representing a poor approximation of the human disease.

There are two widely used full-length mouse models of Huntington’s disease, the YAC128 (Yeast Artificial Chromosome 128) mouse and the BACHD (Bacteria Artificial Chromosome Huntington’s disease) mouse (Slow et al., 2003; Gray et al., 2008). They both display neurodegeneration specific to areas affected in human patients and undergo progressive degeneration of the striatum and cortex. The BACHD model was designed to incorporate LOXP sites for future research experimentation and this method of HTT reduction has been used to study neuronal reduction of mHTT in the striatum, the cortex or both (Wang et al., 2014).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Repeat length</th>
<th>Allele Type</th>
<th>Standard nomenclature</th>
<th>Gene characteristics</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6/2</td>
<td>116</td>
<td>Tg Fragment</td>
<td>Tg[Hd exon1]62 Gpb/1J</td>
<td>HTT promoter, exon 1 of human huntingtin</td>
<td>Most commonly used fragment mouse model of HD, widespread neurodegeneration</td>
</tr>
<tr>
<td>N171-82 Q</td>
<td>82</td>
<td>Tg Fragment</td>
<td>Tg[HD82Gln]81D bo/J</td>
<td>Prnp promoter, 171AA of human huntingtin</td>
<td>Widespread expression under the prion promoter, not the huntingtin promoter</td>
</tr>
<tr>
<td>Hdh111</td>
<td>116</td>
<td>Knock in</td>
<td>Httm5Mem/111J</td>
<td>Endogenous murine Htt gene, chimeric human/mouse exon 1</td>
<td>Mild phenotype</td>
</tr>
<tr>
<td>YAC128</td>
<td>100, 126 mixed CAAAG repeat (7% CAA)</td>
<td>Tg full length</td>
<td>Tg[YAC128]S3Ha y/J</td>
<td>Full length human huntingtin</td>
<td>Random insertion site with 4 copies inserted</td>
</tr>
<tr>
<td>BACHD</td>
<td>97 mixed CAA-CAG repeat (50% CAA)</td>
<td>Tg full length</td>
<td>Tg[HTT*97Q]LX wy/J</td>
<td>Full length (exon 1 fixed); human huntingtin</td>
<td>Random insertion site, estimated to have 5 copies – differences to YAC128 include SNPs and CAA-CAG repeat</td>
</tr>
</tbody>
</table>
Table 1.1. Overview of mouse models of HD. Differences in repeat length were also created for most mouse models, these are the most commonly used mouse models in the HD literature.

1.2.4.2 HTT Function

Even though the discovery of the causative mutation in HTT gene was reported in 1993, much about the WT and mHTT proteins remains unknown. HTT is abundantly expressed in the brain and testes with moderate expression observed in other organs such as liver, heart and lungs (Walker et al., 2007). Even though the exact function of HTT remains unclear, HTT has been suggested to play a role in a number of cellular processes including cytoskeletal anchoring and transport of mitochondria, vesicle trafficking to mediate endocytosis, and has been implicated in embryogenesis and development (Orr et al., 2008; Reiner et al., 2003). The deletion of this gene leads to embryonic lethality in homozygous Hdh null mice (HTT knockout mice) (Nasir et al., 1995). HTT is important during the post-embryonic stage including craniofacial development, forebrain formation especially the cortical and striatal areas, and also in neuronal survival (Reiner et al., 2001). The active participation of HTT in brain development and maintenance illustrates the critical importance of HTT to the CNS.

WT HTT is important for embryogenesis, as mouse homozygous knockouts are embryonic lethal, but can be rescued if HTT is expressed in extra-embryonic tissues (Nasir et al., 1995). HTT is also involved in neurogenesis. If HTT levels are reduced...
after gastrulation, there is a reduction in the number of neurons formed as well as malformations in the cortex and striatum. HTT also plays a role in adult neuronal survival as conditional HTT deletion in adult mouse forebrain causes late-onset neurodegeneration (Dragatsis et al., 2000). In addition, increasing WT HTT appears to protect adult neurons from apoptosis in response to toxic stimuli in a dose dependent manner indicating HTT may have a neuroprotective role in the adult CNS (Leavitt et al., 2006).

1.2.4.3 Putative Mechanisms of mHTT Toxicity

In HD patients, the expanded CAG repeats in the HTT gene leads to a polyglutamine expansion in mHTT protein (mHTT). This repeat expansion is primarily thought to confer a novel gain of function to the HTT protein, although loss of WT HTT function may also be involved in pathogenesis (Cattaneo et al., 2001) Expression of this toxic polyglutamine-containing mHTT induces age-dependent neurodegeneration and neuronal dysfunction that lead to the eventual manifestation of clinical symptoms. Although the mechanism through which mHTT gains toxicity still remains under debate, mHTT can self-aggregate and forms inclusion bodies containing fibrillar mHTT fragments within neurons in HD brain (DiFiglia et al., 1997; Scherzinger et al., 1997). The identification of mHTT aggregates in the R6/2 mouse model led to the hypothesis that pathogenesis in HD was directly the direct result of these neuronal intranuclear inclusions (Davies et al., 1997). However, the pathogenic role of these aggregates in
HD is now debated (Slow et al., 2005). Recent data suggest that neuronal dysfunction and death is independent of formation of mHTT inclusions (Arrasante et al., 2005). Nevertheless, HTT aggregates have been suggested to disrupt neuronal transcription, axonal transport, and mitochondrial function (Richards et al., 1997). Mitochondrial dysfunction with associated aberrant energy metabolism in striatal neurons has been widely implicated in HD (reviewed in Ray et al., 2011). Transcription of brain derived neurotrophic factor (BDNF), an important pro-survival factor for MSNs, is decreased by mHTT expression, suggesting that disruption of trophic support may play a role in the selective degeneration of MSNs in HD (Josep et al., 2004). The fact that BDNF levels increase as WT HTT levels increase is further evidence of BDNF-dependent neuroprotective role for WT HTT.

Among the many potentially toxic effects of mHTT observed in a variety of neuronal and other cell types altered ubiquitin-proteasome system function, mitochondrial dysfunction, impaired axonal transport, and transcriptional dysregulation may also contribute to disease pathogenesis. (Reviewed in Ramaswamy et al., 2007). Proteolytic cleavage of expanded HTT into pathogenic mHTT fragments appears to be critical for neurodegeneration in HD, with both caspase and calpain-mediated cleavage of the full-length mHTT protein playing a role (Wellington et al., 2002; Gafni & Ellerby 2002). Mutation of the caspase-6 cleavage site prevented the development of neuropathology in YAC128 mice (Graham et al., 2006) suggesting that generation of an N-terminal expanded HTT fragment may be an initiating event in HD. A number of the cellular changes observed in neurons expressing mHTT converge upon
increased sensitivity to NMDA receptor-mediated excitotoxicity and altered intracellular calcium homeostasis leading to the initiation of cell death pathways (reviewed in Milnerwood et al., 2010).

The HD field has focused on the effect of mHTT in neurons as the majority of potential mechanisms of mHTT toxicity proposed to play a role in the pathogenic process in HD have been studied in neurons. In contrast, the role of these pathways in glial cells, or other cell types such as immune cells (see next chapter) have received comparably little attention in HD pathogenesis to date.

1.3 Inflammation and Huntington’s disease

The three major cell types in the brain are neurons, microglia and astrocytes. Microglia are monocyte lineage cells, and the represent the major immune cell type in the brain where they constantly survey the microenvironment (Lawson et al., 1990). Dysfunctional signaling between the three major cell types in the brain can have disastrous consequences (Zhan et al., 2014; Cerebai et al., 2012). Under normal physiological conditions, microglial remain in a quiescent state with extremely sensitive ‘spidery’ processes that extend outward from the cell body to monitor the microenvironment around them, and lacking expression of MHC class I and II proteins (Nimmerjahn et al., 2005). Microglia activation is the primary cellular event in acute neuroinflammation and is essential for wound healing in the CNS (Streit & Kincaid-Colton 1995). Upon activation, microglial cells adopt the role of antigen presenting
cells and therefore can activate T cells (Streit et al., 2004). Although inflammation is not typically viewed as an initiating factor in neurodegenerative disease, there is emerging evidence from a number of animal models that sustained inflammation responses involving microglia and astrocytes contribute to disease progression (Schwab et al., 2009). Activated microglia change their morphology, express increased MHC class I and class II antigens, and secret proteases and pro-inflammatory cytokines (Bonifati et al., 2007). These cytokine and protease signals may stimulate a number of signaling cascades in both neurons and astrocytes, which in turn influences neuronal cell death. Activated microglia are also centrally involved in the sprouting of axons from dopaminergic neurons in the damaged striatum (Batchelor et al., 1999).

1.3.1 Brain inflammation in HD

Until recently, the brain was thought to be immunologically privileged, and unable to generate humoral and cellular immune responses (Owens, T. & Babcock, A. 2002). The blood brain barrier (BBB) was believed to assist in the separation of the systemic immune system from the CNS (Muldoon et al., 2012). This is true for the healthy CNS, but not in the case in the setting of an inflammatory or neurodegenerative disorder. As the immunology field in HD research is relatively new and small, there has been limited amount of work on the BBB. In the YAC128 mouse model, neurovascular changes and cerebral blood vessels were assessed, and the BBB showed a
thickened basal lamina and a narrowed of the lumen (Fransciosi et al., 2012). Similar alterations were described in Alzheimer’s Disease, Parkinson’s Disease and normal aging (Brown & Thore 2011). Additionally, leakage of the BBB, increased blood vessel density and a reduced of vessel diameter was demonstrated in the stratum of R6/2 mice, which in turn correlated with reduced expression of tight junction associated proteins (Drouin-Ouellet et al., 2015). Similar vascular alterations were observed in HD patients (Drouin-Ouellet et al., 2015). Expression of mutant huntingtin in astrocytes and neurons is sufficient, leading to increases in brain vessel density (Hsiao et al., 2015).

Direct pathological injury within the brain can initiate an immune response. In neurodegenerative diseases, an immune response to abnormally folded proteins and protein aggregates may trigger active neuroinflammation that in turn increases neuronal degeneration (Floden et al., 2005). Consistent with the presence of neuroinflammation in other neurodegenerative diseases, mHTT aggregates are observed co-incident with neostriatal atrophy (Becher et al., 1998). A striking pathologic feature in HD brains is massive neurodegeneration in the neostratium, i.e. the putamen and caudate. However, expression of mHTT has not yet been demonstrated to directly induce an inflammatory response from microglia or peripheral macrophage. In other neurodegenerative diseases, such as Alzheimer’s Disease, the pathogenic protein and amyloid beta aggregates are deposited extracellularly in the brain and the antigen presenting cells of the local immune system, microglial cells recognize these aggregates (Wood, 2003.). It is possible that mHTT causes neuronal
death and dying neurons can secondarily activate microglia and the CNS innate immune system. Pre-manifest HD, where the carriers of the gene do not yet exhibit the classical signs and symptoms of the typical HD patient display evidence for the presence of activated microglia in the striatum (Politis et al., 2008). This may be as a result of mHTT toxicity and early neuronal dysfunction including elevated pathogenic extrasynaptic NMDA receptor signaling, reduced synaptic connectivity and loss of brain-derived neurotrophic factor (BDNF) (Milnerwood et al., 2011). A direct cell-autonomous role of mHTT in microglial dysfunction is currently being investigated by several groups, but microglial involvement in HD has not been well-characterized.

Ninety percent of the cells in the brain are glial cells. Their main function is to provide neurons with neurotrophic factors, as well as structural and functional support (Temburni & Jacob 2001). These cells are responsible for maintaining the normal physiology within the CNS and the normal brain functions in an immune privileged environment. A series of events and cascades due to infections, trauma, toxins and stroke can lead to similar forms of pathological neuroinflammation and neurodegeneration that resemble those seen in most neurodegenerative diseases, including HD (Streit et al., 2004; Bonifat et al., 2007).

Microglial cells are resident macrophages of the CNS that are quiescent to maintain a healthy brain. When microglia are acutely stimulated by infections or direct trauma they can take on a phagocytic role and secrete various cytokines and chemokines (Wood, 2003). However, this type of inflammation is usually a short-lived phenomenon and considered an acute neuroinflammatory response. Despite the
resulting oxidative and nitrosative stress, the acute neuroinflammatory response is seldom harmful to long term neuronal survival. In fact, it minimizes further damage to brain cells and facilitates repair of damaged tissue (Ziebell & Morganti-Kossmann 2010.). On the other hand, chronic neuroinflammation appears to be associated with exacerbation of neuronal damage (reviewed by Campbell, A. 2004). Chronic neuroinflammation not only includes the extended activation and proliferation of microglia, for example by amyloid beta in AD, but also leads to increased secretion of pro-inflammatory cytokines and increased superoxide and nitric oxide production (Barger & Harmon 1997). This prolonged inflammation affects the permeability of the BBB that in turn supports the infiltration of macrophages and myeloid progenitor cells into the brain parenchyma and further intensifies the already ongoing neuroinflammation (Ryu & McLarnon 2009). Thus, the detrimental effects of chronic inflammation may exacerbate the direct toxic neurodegeneration caused by mHTT in HD.

Under normal conditions in the adult brain, microglial cells exist in a quiescent state with extremely sensitive spidery processes monitoring the microenvironment around them and do not express MHC class I and II proteins (Aloisi 2001). Microglia activation leads to upregulation of cell surface antigens and secretion of pro-inflammatory cytokines. Upregulation of cytokines like IL6, IL-12 and TNFα leads to a communication with neighbouring microglial cells, astrocytes, T-cells, neurons and myeloid progenitor cells (Kim et al., 2005). Secretion of proinflammatory cytokines can lead to a wide variety of neurodegenerative processes like free radical production,
NMDA-mediated excitotoxicity and caspase activation (Allan 2000). In the event of trauma, axotomy, ischemia, and neurodegeneration, the immune system within the CNS becomes activated (Banati et al., 1993). In HD, the precise mechanism by which mHTT leads to region-specific neuronal death is still unclear. A number of studies have suggested a role for immune components that might boost or initiate gliosis and neurodegeneration in HD (Rus et al., 2006; Janssen et al., 2005).

Astrocytes are another source of inflammation in the brain; they are support cells for neurons and potentially play an important role in HD. Although the focus of my thesis is on microglial cells, it is important to note the role astrocytes may play in neuronal protection throughout HD. Expressing an N-terminal fragment of HTT from the Glial Fibrillary Acidic Protein (GFAP) promoter caused astrocyte-specific expression and phenotypes similar to those presented in HD (Bradford et al., 2009). Astrocytes are more commonly thought to have a large role in regulation and support of neuronal networks in addition a potential capacity to present antigen and activate T cells has been described, but remains controversial (Chastain et al., 2011).

1.3.2 Complement System and HD

The complement system is the most important and powerful humoral component of the innate immune system (Dunkelberger & Song 2010). The vital functions of the complement system include host defense against pathogenic microorganisms, removal of immune complexes and apoptotic cells, and facilitating adaptive immune
responses (Dunkelberger & Song 2010.). It also mediates the production of anaphylatoxins (C3a, C4a and C5a) that trigger degranulation, cell lysis and phagocytosis via inducing chemotaxis and cell activation (Huber-Lang et al., 2006; Zhou et al., 2006.). The complement system may also be a key factor in several neurodegenerative disease, as it is a key player in immune surveillance in the CNS and is activated upon stimulation by toxic peptides such as mHTT (McGeer et al., 1989).

The complement system gets activated via three pathways depending on target ligands and the recognition complement component (reviewed in Sarma & Ward 2011). The common purpose of all the three pathways is to activate the central component of the complement system. Altered complement system activation is considered important causative factors in a number of inflammatory, neurodegenerative and cerebro-vascular diseases (Rus et al., 2006.). Abnormal expression of a several complement components such as C1q C chain, C1r, C4, C3, as well as the complement regulators, C1 inhibitor, clusterin, MCP, DAF, CD59 in HD brain samples with severe atrophy has suggested the recruitment of the complement system during HD progression (Singhrao et al., 1999). Proteomic profiling of plasma and CSF from HD patients detected up-regulation of acute-phase protein α2-macroglobulin (α2M) and clusterin, as well as other pro-inflammatory cytokines (especially IL6) (Dairymple et al., 2007).

Clusterin is a multifunctional glycoprotein that is involved in diverse mechanisms of cytoprotection, membrane recycling and regulation of membrane attack complex
(Hochgrebe et al., 1999). Up-regulation of clusterin has been implicated in a variety of physiological and pathological states including apoptosis and response to injury. Clusterin has been previously associated with other neurodegenerative disorders such as Alzheimer’s disease (AD) where its plasma level correlates with the degree of neurodegeneration (Thambiesetty et al., 2010). Interestingly, clusterin has also found to be expressed in both the peripheral plasma and in the CSF thus suggesting widespread immune activation. Up-regulation of α2M was observed peripheral plasma and its release is stimulated by pro-inflammatory IL6 (Kim & Choi 2011). These findings are consistent with observations in AD where in α2M is up-regulated in reactive astrocytes and is also observed to bind to Aβ which is the pathogenic peptide responsible for the formation of senile plaques in AD (Desikan et al., 2014.). Thus, a simultaneous up-regulation of immune proteins and cytokines is evident in both the central and peripheral nervous system in HD.

1.3.3 Neuroinflammation in HD

The primary report identifying microglial activation in HD used morphologic changes in HD brains by immunocytochemical staining of HD brain compared to control with thymosin B-4, an antigen that is increased in reactive microglia(Sapp et al., 2003). Reactive microglia were evident in all grades of HD pathology, and the number of immunostained microglia increased with increasing grade (Sapp et al., 2003). Microglial morphology was altered in the striatum and in the cortex.
Positron emission tomography (PET) is a nuclear imaging technique that produces an image or picture of functional processes in the body. Three-dimensional images of tracer concentration within the body are constructed by computer analysis. PET scans are commonly used in HD to image the striatum using raclopride (RAC), which labels dopamine D2 receptors. PK11195 (PK) a ligand that binds selectively to peripheral benzodiazepine binding sites, is used to label microglia. Although there are two benzodiazepine binding sites expressed in the brain, activated microglia are the only cells that express the peripheral benzodiazepine binding site on the outer membrane of the mitochondria.

Notably, PET imaging identified increased striatal PK binding in HD patients (Pavese et al., 2006). The amount of PK binding was significantly correlated with disease severity. Disease severity was assessed by the striatal reduction in RAC binding, the Unified Huntington’s Disease Rating Scale (UHDRS) score, and the patients’ CAG repeat index. These findings provide strong in vivo evidence that the level of microglial activation correlates with clinical severity in HD. Microglial activation measured with PK PET and linked changes in striatal neuronal dysfunction measured with RAC PET, suggesting an application for PK PET as a marker of subclinical disease progression in HD pre-symptomatic HD patients (Tai et al., 2007). These pre-symptomatic individuals had lower striatal RAC binding than the controls, but significantly higher striatal and cortical PK binding. Individual levels of increased striatal PK binding correlated with lower striatal RAC binding and a higher probability of developing HD in 5 years. The inverse association between striatal PK and RAC
binding using PET imaging was consistent for the presymptomatic period to moderate stages of HD. Five year probability of HD onset was predicted using the association of widespread in vivo microglial activation associated with striatal neuronal dysfunction using MRI PK and RAC PET in CAG repeat-matched groups of pre-manifest and symptomatic HD gene carriers (Politis et al., 2009). Again the results of the 32 subjects (8 premanifest HD brains, 8 symptomatic HD brains, and 16 control normal brains) were correlated to UHDRS scores. These data suggests that PET imaging of pathologically activated microglia in striatum and other associated areas is a valid predictor of clinical onset. Altered inflammatory profile, increases in IL1B and TNF-a in striatum of HD post mortem brain is consistent with a pattern of microglial activation (Silverstroni et al., 2009).

A number of studies in chemical rat models of HD have also suggested a role of inflammation in HD. Pyruvate was described as protecting subtypes of neurons in a chemical model of HD, quinolinic acid (QUIN) injected rat striata (Ryu et al., 2003). Further work using these chemical models of HD have assessed the effects of inflammatory treatments and prevention of oxidative stress in HD. Both minocycline a broad-spectrum tetracycline antibiotic) and pyruvate treatment enhance the inhibition of inflammation, neuronal loss and oxidative damage in quinolinic acid injected rat striata (Ryu et al., 2006). Another study demonstrated a reduction in the microglial activation by the addition of the peripheral benzodiazepine receptor ligand using the same model (Ryu et al., 2005). A possible link between vascular
remodelling and microglial activation through thalidomide administration has also been suggested (Ryu et al., 2009).

Whether inflammation in the CNS is in response to neuronal death caused by the toxic mHTT is debatable. MHTT is likely to be a key factor in promoting inflammation in HD, either directly or indirectly. However, the question that now arises is whether this inflammation is brought about by mHTT induced neuronal injury, or by inflammation due to a direct effect of mHTT on activated microglia.

1.3.4 Peripheral vs. Central Inflammation in HD

Outline a topic, build flow – organization. Human peripheral monocytes of HD patients display abnormally high levels of the pro-inflammatory cytokine IL6 in response to lipopolysaccharide (LPS) stimulation compared to controls (Bjorkqvist et al., 2008). Peripheral macrophages from the YAC128 mice and microglia from R6/2 mice also showed a similar phenotype. Additionally microarray profiling of various brain regions of the CNS of HD patients revealed expression of GFAP, complement protein and other inflammation-related genes, indicative of microgliosis. coding genes (Hodges et al., 2006). These increases are even more pronounced in the caudate and putamen where HD patients the brain pathology is most severe (Bjorkqvist et al., 2008). However, it remains unclear if the microgliosis seen in HD brains is pro- or anti-inflammatory.
Neurovascular alterations including decreased cerebral blood flow in the caudate have been reported from imaging studies in both symptomatic and presymptomatic HD patients (Harris et al., 1996). Pathologically, cerebral blood vessel lumen narrowing and increased angiogenesis in post-mortem HD brain tissue has also been identified (Harris et al., 1999). In the presence of mHTT, microglia dysfunction leading to altered signalling between microglia, neurons, astrocytes and the neurovasculature exacerbating the pathogenesis of HD. Another postulated cause for neuroinflammation in HD is a leaky BBB caused by sustained peripheral inflammation. This potential abnormality remains largely unexplored in HD, but has a serious effect on the prognosis of MS patients (Vis et al., 1998). Prolonged inflammation affects the BBB that in turn BBB malfunction supports the infiltration of macrophages and myeloid progenitor cells into the brain parenchyma, further intensifying the inflammatory processes (Stolp, H. B. et al., 2005).

1.3.5 Potential Roles of Microglial Dysfunction in HD
1.3.5.1 Caspase Involvement in Microglia Activation

Neuroinflammation has been closely linked to caspase activation in microglia, a new role for caspase signaling in primary microglia has recently been demonstrated (Burguillos et al., 2011). Specifically, caspase 8 and caspase 3/7 were found to control microglia activation and brain inflammation, with caspase 8 acting as an initiator and caspase 3/7 as activator proteases in this process. Inhibition of the caspase 3/7
pathway resulted in reduced microglia activation and inhibition of downstream IKK/NFKB signalling. Importantly, microglia exposed to LPS (a pro-inflammatory signal) were not cytotoxic to adjacent neurons when caspase 3/7 activation was inhibited either chemically or by siRNA knock-down.

1.3.5.2 Caspases and HD

Caspases play key roles in HD pathogenesis by cleaving and modulating HTT. Evidence for proteolytic cleavage of HTT by caspase-3 in apoptotic cells was initially demonstrated in 1996 (Goldberg et al., 1996). N-terminal fragments of mHTT are found in the brains of HD patients and in mice transgenic for full-length mHTT (Difiglia et al., 1997). Caspase-mediated cleavage of mHTT occurs in the HD brain as an early step in the during HD pathogenesis (Wellington et al., 2002). In addition, the discovery that N-terminal fragments of mHTT are more toxic than full-length mHTT in many systems, which links HTT cleavage with neuronal injury (Li et al., 2000). This model of HD pathogenesis was first proposed by Hayden and colleagues and is known as the “toxic fragment hypothesis” (Wellington et al., 1997). Site-directed mutagenesis of caspase cleavage sites in HTT were altered to generate caspase-resistant mHTT, which demonstrated significantly reduced toxicity in cultured neuronal cells and in mouse models of HD (Graham et al., 2006). Caspase activation has also been suggested to play a critical role in other neurodegenerative disorders including other polyglutamine disorders, Alzheimer’s disease and A.L.S. (Ehrnhoerfer et al., 2009).
Caspase resistant HTT would suggest that in addition to generating toxic fragments of N-terminal polyglutamine containing HTT fragments, depletion of endogenous WT HTT by caspase-mediated cleavage would result in a loss of the anti-apoptotic function of HTT, rendering neurons more vulnerable to cellular stress and neurodegeneration. Caspase-resistant mHTT in these studies was expressed in all cell types of the brain, including microglia, but to date there have been no data published on the effects of mHTT on microglia from these mouse models.

Factors such as caspase-mediated proteolytic cleavage that decrease the endogenous levels of WT HTT may predispose microglia to a neurodegenerative role contributing to the selective striatal cell death seen in HD (Wellington et al., 2002). Modulating HTT levels either by selectively decreasing mHTT or by increasing HTT levels within brain cells is neuroprotective (Leavitt et al., 2006). This may represent a novel therapeutic approach for neurodegenerative diseases. The link between caspases, microglia and HD is a relatively new one. Caspases have been central in HD research, but again this research had previously taken a neurocentric role. In the near future, research into caspase activation in microglia as potential contributors to disease will provide a clearer picture of HD pathogenesis.

1.3.5.3 The Kynurenine Pathway

Metabolites of the kynurenine pathway (KP) have been suggested to play a major role in excitotoxicity (Lim et al., 2010). Excitotoxicity is characterized by
excessive stimulation of glutamate receptors, increased intracellular calcium and mitochondrial dysfunction (Pipovarova N. B & Andrews B. S. 2010). The KP pathway is the major route of tryptophan metabolism in mammals and leads to the generation of either A). QUIN, a selective N-methyl-D-aspartate (NMDA) receptor agonist found in mammalian brain or B). Kynurenic acid (KYNA), which is formed in a side pathway of the KP, is also thought to modulate excitotoxicity and neurodegeneration by antagonizing QUIN (Chen, Y. & Guillemin G. J. 2009). Genetic reduction in KYNA formation enhances vulnerability to an excitotoxic insult whereas modest increases in brain KYNA reduce extracellular glutamate levels in brain by inhibiting presynaptic a7 nicotinic receptors (Campesan et al., 2011).

1.3.5.4 Alterations in the KP in HD

Many neuropathological features and chemical impairments in HD can be duplicated in experimental animals by an intrastriatal injection of QUIN (Beal et al., 1996). These findings led to the hypothesis that QUIN contributes to the pathophysiology of HD. The neostriatal and neocortical levels of the KP metabolites 3-hydroxykynurenine (3-HK), a free radical generator that mediates neuronal cell death and QUIN are significantly elevated in early pathological-grade HD brains, whereas KYNA levels are decreased (Guidetti et al., 2004). Moreover, cerebral 3-HK and QUIN concentrations are also increased in mouse models of HD (Sathyasaikumar et al., 2010). In the serum of HD patients, tryptophan levels are reduced, and the
Kynurenine:tryptophan ratio is elevated, coinciding with increased production of proinflammatory cytokines and chemokines (Stoy et al., 2005). Changes in KP metabolite levels are found in the CNS and in the periphery in HD, and it is widely hypothesized that these events are early contributors to the pathophysiology of disease (Heyes et al., 1992).

1.3.5.5 Targeting the KP in HD

Microglia are capable of secreting QUIN through the induction of this pathway, however it has been reported that macrophages from the periphery are capable of secreting over 20 times more QUIN than microglia when stimulated ex vivo Raison et al., 2010). Enrichment of gene expression involved in key rate-limiting steps of the KP have been demonstrated in HD, also indicating a perturbed pathway leads to increased QUIN leading to excitotoxicity in HD (Campesan et al., 2011.). Recently a Kynurenine 3-monoxygenase (KMO) inhibitor was found to alter QUIN and KYNA levels in the R6/2 mice and in AD mouse models (Zwilling et al., 2011). KMO inhibition mediates KP metabolism toward enhanced KYNA production and may therefore reduce neuronal vulnerability. KMO inhibitor treatment of the R6/2 mice improved the phenotype of the mice and prevented neurodegeneration, specifically restoration of synaptophysin levels, and restoration of calcium regulated neuronal activity levels, that are decreased in R6/2 at 12 weeks. Interestingly, high doses of the KMO inhibitor resulted in a decrease in IBA1 immunostaining (a microglial marker) in
R6/2 to WT levels. This paper highlights peripheral cell types altering neurons indirectly and also reinforces the hypothesis that dysregulation of the KP is centrally involved in HD pathogenesis (see review paper by Schwartz et al., 2004).

1.3.5.6 Microglia Signaling Pathways in HD

The specific activation state of microglia in HD is not yet well characterized. Generally in the literature, LPS or (Lipopolysacharide) a cell wall component of gram-negative bacteria is used for pro-inflammatory activation and IL4 and or IL13 stimulation are used for anti-inflammatory conditioning. These models of microglia/macrophage activation are not ideal for the study of microglia in HD because the proximal cause of inflammation in HD is not known. Nevertheless, these approaches could help understand how microglia are altered in the presence of HD. MHTT is localized to the nuclei of glial cells where it can act on gene transcription, widespread transcriptional dysregulation occurs in fragment models of HD. MHTT, but not WT HTT can activate the IKK complex, leading to the phosphorylation induced degradation of IKBs which results in increased NF-kB translocation to the nucleus in mouse striatal cells (Khoshman et al., 2004). This provides a candidate mechanism by which mHTT could directly alter the activity of immune cells in HD patients leading to abnormal immune responses. Immunological protection by microglia may not be as important in HD as the functional neuroprotection provided by these cells through
various mechanisms including secretion of growth factors, cytokines and neurotrophins, glutamate uptake, phagocytosis of debris and iron acquisition.

1.3.5.7 Iron Regulation in the Brain

Iron plays an essential role in normal neurobiological functioning, such as in neurotransmitter synthesis and in myelin production. Iron levels in brain tissue are found to be elevated in numerous neurological disorders, including MS (Vine et al., 1997; Craelius et al., 1982). Pathogenesis of neurodegenerative disorders may be influenced by iron through the promotion of oxidative stress, subsequently leading to direct tissue damage. Moreover, increased deposition of non-heme iron, predominantly in the basal ganglia, is also related to the normal aging process (Daugherty, A. & Razi, N. 2013). Iron deposition may derive from myelin/oligodendrocyte debris, destroyed macrophages, or it can be the product of hemorrhages from damaged brain vessels. Oxidative mitochondrial injury through Fenton reaction and release of phospholipid-rich cellular membrane elements, with the generation of toxic free radicals, may also be another important source of iron overload in neurologic disease.

Iron is a powerful chemotactic stimulus that attracts macrophages and contributes to or causes initial activation of T-cell autoimmunity in patients with MS (Zamboni et al., 2009). On the other hand, an alternative hypothesis could be that decreased blood flow in brain parenchyma of MS patients could result from vessel
congestion or occlusion due to inflammatory cells, fibrin deposits, or other factors. In this case, iron deposits could develop as a consequence of inflammatory reactions rather than causing them. Iron accumulation has been related to microglia activation using rat ischemic models in the 1990’s, accumulation of ferritin immunopositive microglia paralleled iron accumulation in brain. Following this finding BV2 microglia cell lines have demonstrated that iron can alter gene expression prior to LPS stimulation through microarray analysis (Lund et al., 2006).

1.3.5.8 Iron in Huntington’s Disease

Alterations in iron homeostasis and neurodegenerative diseases have been closely linked; iron chelators and antioxidants have therefore been suggested as potential therapies for neurodegenerative diseases. Iron levels in the brain are relatively high, especially in the striatum (Martin et al., 1998). It is not however, well understood if iron in the brain predominantly comes from neurons or glia. Oxidative free radical processes catalyzed by iron lead to an increase in lactate and a decrease in pH can lead to a release of iron from ferritin (potentially leading to oxidative stress). Moreover, brain iron also accumulates with increasing age (Bartzokis, G. et al., 1994), further suggesting that it could be involved in age related. Increased iron levels are a common risk factor for neurodegenerative disease; increased levels are found in Alzheimer’s disease, Parkinson’s disease and Huntington’s disease. MRI studies on 11 HD patients compared to 27 controls and determined the increased iron in HD
patients is greater than that of AD and PD patients (Bartzokis et al., 1999). Studies conducted in the R6/2 mice showed increased striatal iron levels starting as early as 2-4 weeks of age i.e. preceeding what!? (Simmons et al., 2007). Iron levels also increased in hippocampus and cortex but only from 5-7 weeks on. These changes were also described as being predominantly localized to microglia. These results indicate an early pathologic disturbance in iron metabolism in microglia in a model of HD. Microgla stimulated with LPS in the presence of iron have an altered gene expression profile (Saleppico et al., 1996).

1.3.5.9 Cannabinoids in HD

Cannabinoid signalling is another important regulator of microglial function is cannabinoid signalling (Merighi et al., 2012). Components of the endocannabinoid system are found throughout the body. Currently there are two identified cannabinoid receptors (CB1 and CB2) and two major endogenous cannabinoids (Anandamide and 2-Arachadonylglycerol) are known. CB1 is primarily located within the CNS and is expressed on neurons and astrocytes. CB1 receptors are most highly expressed in the caudate and putamen, regions of the brain primarily affected in HD (Mailleux et al., 1992). Activation of CB1 reduces the amount of glutamate released from stimulated cells and is also been linked to pro-survival signaling molecules such as BDNF and AKT (Ozaita et al., 2007; Molina-Holgado et al., 2007; Khaspekov et al., 2004). CB2 is principally located on immune cells of the body including microglia, the immune cell of
the brain (Stela et al., 2010). Activation of CB2 in microglia causes decreased secretion of pro-inflammatory cytokines from microglia when stimulated (Correa et al., 2011).

Excitotoxicity and neuroinflammation are both implicated in HD pathogenesis (Blazquez et al., 2011; Palazuelos et al., 2009) making the endocannabinoid system an attractive target for therapy. CB1 has been identified as the first receptor lost in HD (Glass et al., 2000). This, along with the ability of CB1 activation to inhibit the synaptic release of glutamate (Gerde and Lovinger 2001) makes it a key target of interest. Recent experiments demonstrate that genetic deletion of CB1 compounds the HD phenotype in both genetic and chemical models of HD (Meivis et al., 2011; Blazques et al., 2011) further implicating cannabinoid signaling in HD pathogenesis. Increased levels of CB2 mRNA have been observed in genetic mouse models of HD and human post-mortem tissue (Palazuelos et al., 2009). It is not clear at this point if the increase is due to an upregulation of the receptor itself or an increase in microglial number. An increase in lesion volume after injection of QUIN in CB2 knockout animals suggests a protective role of microglia following excitotoxic injury further linking this system full circle to HD pathogenesis (Palazuelos et al., 2009).
Table 1.2 Pathways with roles both in HD and inflammation.
1.4. Conclusions

HD is a progressive age-dependent neurodegenerative disease in which significant immune activation has been described in both the CNS and periphery. A number of studies have identified immunological biomarkers that appear to correlate with HD severity. Microglial function is altered in HD, perhaps directly by mHTT expression (i.e. caspase activation induced by mHTT) or through alternative secondary effects such as in response to mHTT-induced neuronal injury (See Table 1.2). Microglia normally maintain homeostasis in the brain through tight control of neurotrophic factors. Microglia activation occurs in HD, however, it remains unknown if this activation plays a role in prevention of neuronal death, or if these are actions of the mutant protein leading to neuroinflammation in a relatively healthy brain (reviewed by Soulet & Cicchetti 2011). Small microenvironmental changes in the brain can affect microglia in a profound way, leading to activation of astrocytes and neuronal death. Peripheral inflammation can have an impact on both neurodegeneration and the degree of inflammation seen in the brain. Studies that improve our understanding of the role of microglia in the CNS and the regulation of neurodegeneration and neuroinflammation could pave the way to an improved prognosis for patients affected by HD.

Considerable overlap in both microglia function and HD mechanisms of pathogenesis implicate microglia as having a central role in this disease. Research in the HD field has generally focused on the effect of mHTT on the MSNs. These neuron-centric approaches ignore glial cells that play a critical role in brain
homeostasis. In the future, targeting microglial activation, either by increasing pro-
inflammation or decreasing anti-inflammation, may prove to be a beneficial approach
to therapeutic interventions for HD. Normal brain function requires important cell-to-
cell communication; microglia together with astrocytes play a key role in brain cell 
signaling and maintenance of homeostasis. Further research on the role of microglia 
in HD is clearly required, and this area of research provides hope for the development 
of eventual treatments for this devastating neurodegenerative disease.

1.5 Objectives

The objectives of this thesis were to 1) establish a model to assess mHTT 
induced-inflammation in HD 2) to study inflammation levels in this model following 
treatments with targeted therapeutics, and 3) to manipulate the inflammation caused 
by mHTT in vivo by genetic means and to assess alterations on HD progression.
Chapter 2: Increased activation of the immune system in the presence of mutant huntingtin.

2.1 Introduction

HD is a neurodegenerative disorder caused by a polyglutamine expansion in the HTT protein encoded by a CAG trinucleotide repeat expansion in the HTT gene. Previously, the brain was thought to be immunologically privileged, and unable to generate humoral or cellular immune responses, but abnormal immune cell activation as recently suggested to play a pathogenic role in a number of neurodegenerative diseases, including HD (Bjorkqvist et al., 2008). Microglia are macrophage-like cells of the myeloid lineage and the major immune cells in the brain. These cells constantly survey the microenvironment and produce factors that can influence the surrounding astrocytes and neurons. Activated microglia change morphology, express increased MHC class I and class II antigens, and secrete proteases and pro-inflammatory cytokines (Bonifati et al., 2007).

The YAC transgenic mouse model for HD (Table 1; Slow et al., 2003) faithfully recapitulates key features of the human disorder. These mice are the primary transgenic mouse model used in my laboratory as they develop progressive age-related motor phenotypes and show selective degeneration of neurons within the striatum and cortex, similar to the phenotypes of human HD. The progressive motor
phenotype has been demonstrated in the YAC128 transgenic mouse model using a standardized series of behavioural tests (Van Raamsdonk 2007b).

Cytokines are inflammatory proteins that are secreted by cells of the monocyte lineage in response to many different stimuli (Turnbull et al., 1999). Up-regulation of cytokines like IL6, IL-12 and TNFα leads to altered communication with neighbouring microglia cells, astrocytes, neurons and myeloid progenitor cells in vivo (Kim et al., 2005). Pro-inflammatory cytokine production can lead to a wide variety of processes involved in neurodegenerative pathology, such as free radical production, NMDA-mediated excitotoxicity, and caspase activation. Notably, microglial activation has recently been implicated in HD pathogenesis (Moller et al., 2010). Increased levels of inflammatory cytokines IL-1β and TNFα have been observed in the striatum of HD patients, while microglia activation was separately associated with the progression of the disease (Silverstroni et al., 2009). PET studies identified increased striatal microglia numbers in HD patients, and have correlated striatal neuronal dysfunction with increased microglial signal in HD (Tai et al., 2007). There are a number of inflammatory linked molecules that may mediate these changes in HD, including cannabinoids and MMPs.

Matrix metalloproteinases (MMPs) and their regulators tissue inhibitors of metalloproteinases (TIMPs) play important roles in inflammation, wound healing, and metastatic cancer (Page-McCaw et al., 2007, Von Lampe et al., 2000). Glia including microglia? produce MMPs and free radicals in response to pro-inflammatory stimuli (Tarnuzzer et al., 1996, Qiuatresooz et al., 2003) and MMP3 knockout mice display reduced neurodegeneration and microglial activation in response to MPTP, a
chemical model for Parkinson’s Disease (Kim et al., 2007). MMP3 has recently been recognized as a secreted neuronal signal that induces inflammatory cytokine release from microglia (Kim et al., 2005). MMPs have also been identified as potential modifiers of HTT proteolysis and toxicity in HD (Miller 2010). MMP activity is increased in the striatum of HD mice (Sathasivam et al., 1999).

HTT is expressed ubiquitously, peripheral monocytes from both HD patients and HD mouse models have an altered cytokine secretion phenotype (Sathasivam et al., 1999; Bjorkqvist et al., 2008). Other phenotypes have also been demonstrated, including cell migration and cannabinoid receptor 2 signalling (Kwan et al., 2012; Bouchard et al., 2012). Furthermore recent, work has looked specifically at myeloid cells and mHTT and the dysfunction in migration of these cells (Crotti et al., 2014). Other in vitro work found altered NF-κB signalling in microglia from HD mice (Hsiao et al., 2013) and human cells (Trager et al., 2014). In my thesis I further investigated the nature of CNS inflammation in HD by studying the effects of increased WT HTT, and decreased amounts of mHTT. I also examined the response of primary microglia from HD mice to an endogenous brain stimuli MMP3 compared to LPS stimulation.

Once symptoms have manifested, microglia activation correlates with disease severity. In the CNS, microarray profiling of several brain regions from HD patients and controls provides evidence for increased microgliosis and expression of inflammation-related genes, including GFAP and complement protein (Bjorkqvist et al., 2008). Increases of inflammatory signals are also more pronounced in the caudate and putamen, where the brain pathology is most severe in HD patients (Dalrymple et al., 2007). Neurodegeneration prior to the onset of symptoms in HD could accelerate
an inflammatory problem causing feedback loops that lead to further neurodegeneration (reviewed by Glass et al., 2010). Along with collaborators we have demonstrated support for this hypothesis, monocytes from HD patients secrete abnormally high levels of the pro-inflammatory cytokine IL6 in response to LPS stimulation (Bjorkqvist et al., 2008). Peripheral macrophage from the YAC128 and microglia from R6/2 mouse model also showed a similar phenotype. MHTT is localized to the nuclei of glial cells where it can act on gene transcription (Shin et al., 2005). MHTT, with the exception of WT HTT, can activate the IKK complex. This major kinase that leads to the phosphorylation induced degradation of IKBs, leading to increase NF-kB translocation in mouse striatal cells. This provides a candidate mechanism by which mHTT could alter the activity of immune cells in HD patients leading to abnormal immune responses.

Microglia activation is the main cellular event in acute neuroinflammation and essential in wound healing in the CNS. Although inflammation is not typically viewed as an initiating factor of neurodegenerative disease, there is emerging evidence in animal models that sustained inflammation responses involving microglia and astrocytes contribute to disease progression (Nazem et al., 2015). However, it is possible that the level of immunological protection by microglia may not be as important as the level of neuroprotection that is provided by these cells by various mechanisms including growth factors, cytokines and neurotrophins, glutamate uptake, phagocytosis of debris and iron acquisition (reviewed by Michell-Robinson et al., 2015). The concept of microglia residing in a spectrum of states not defined solely as
pro- or anti-inflammatory cells many intermediates is a relatively new concept. This idea has been widely accepted in the macrophage field, but only recently been adapted to microglia in the literature.

Microgliosis occurs prior to clinical symptoms being present in HD (Tai et al., 2007). Based on both mouse models and human data, peripheral monocyte cells have altered immune secretions in the presence of mHTT (Bjorkqvist et al., 2008). The objective of this chapter is to understand if microglial functions are altered in the presence of mHTT.

2.2 Methods

YAC128 mice of mixed gender and their non-transgenic littermates maintained on the FVB/N (Charles River, Wilmington, MA) strain were used for these experiments (Slow et al., 2003). All experiments were carried out in accordance with protocols approved by the UBC Animal Care Committee.

2.2.1 Isolation of Bone Marrow Monocytes

Mice were sacrificed by neck dislocation or by rising concentration of CO₂. Femur and tibia were dissected at the hip joint and any remaining muscle tissue was carefully removed. The bones were placed in a petri dish filled with cold RPMI-1640 media and cut at the joints. Bone marrow was flushed out by rinsing the shaft with media using a 5 ml syringe and 26 gauge needle. Lumps of cells were disaggregated
by pipetting up and down several times before the cells were passed through a 70 µm nylon cell strainer. After washing with RPMI-1640 media (centrifugation at 300 × g for 5 min) cells were counted using a Neubauer counting chamber. The cell suspension was labelled with 10 µl anti-mouse CD11b magnetic beads and 90 µl MACS buffer per 1 × 10^7 cells, and sorted as described above. When seeded in culture the isolated CD11b positive cell population resembled an early monocyte population, which could then be differentiated into bone marrow-derived macrophages. For the differentiation, sorted bone marrow cells were cultured in R10 media (RPMI-1640 supplemented with 10% FBS, 2 mM l-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin supplemented with 20 ng/ml recombinant murine M-CSF). After 3 days cells were provided with fresh media and growth factor. The cells resembled a macrophage phenotype from day 6.

2.2.2 Isolation of Peritoneal Macrophages

In order to prevent bleeding into the abdominal cavity, mice were killed using a rising concentration of CO₂. Fur covering the peritoneum was carefully removed and 5 ml of ice-cold, serum free RPMI1640 medium was injected into the abdominal cavity using a 26 gauge needle. After massaging the mouse abdomen for 1–2 min, cells were recovered through a small incision using a sterile plastic Pasteur pipette and put on ice as quickly as possible. The centrifuged (5 min at 300 ×g) cell suspension was incubated with 1 ml red blood cell lysis for 5 min at room temperature. Afterwards, the
cells were washed with PBS (centrifuged at 300 × g for 5 min). The resultant cell suspension contained macrophages as well as neutrophil, so cultures were enriched for macrophages by removing non-adherent neutrophils by media changing and washing the cells after 2 hours in a culture. Cytokine profiling of YAC128 mice used peritoneal macrophages isolated from WT and YAC128 mice and were seeded in 24-well plates at a density of 500,000 cells per well. Bone marrow-derived macrophages were used for stimulation experiments after differentiation in 6-well plates at $3 \times 10^6$ cells per well. All cultures were primed with 12.5 ng/ml murine IFNγ and stimulated with 100 ng/ml CSE. After 24 h, supernatants were collected for analysis of IL6 levels by ELISA according to manufacturer's instructions (e-Bioscience). Cells were lysed in RIPA buffer (25 mM Tris–HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) for protein measurement by BCA assay (Thermo Scientific).

2.2.3 Isolation of Primary Microglia Cells

Whole brains were obtained from post-natal day 1 to 3 WT, YAC18 and YAC128 mouse pups on the FVB/N strain background as previously described (Saura et al., 2003). Briefly, the meninges were removed carefully, and the remaining brain tissue was placed into growth medium (DMEM, 10 % FBS, 1 % L-glutamine, 1 % penicillin/streptomycin) and homogenized using a 5 ml pipette. Cells of each brain were pelleted, re-suspend in growth medium, transferred into a T150 flask, and cultured at
37°C with 5% CO₂. Growth medium was replaced after 24 hours and then every 7 days. After 18 to 21 days in culture, loosely attached microglia were harvested and seeded at 1.4x10⁵ cells/ml with pre-incubated conditioned media into 96-well PRIMARIA™ tissue culture plates or onto glass coverslips.

2.2.4 Isolation of Primary Medium Spiny Neurons

Medium spiny neuron (MSN) cultures were prepared from embryonic E 15.5 WT and YAC128 embryos as described previously (Zeron et al., 2002). Briefly, striata were dissected and tissue digested using a 0.05% trypsin solution in HBSS for 15 minutes at 37°C. The striata were then triturated 5 times through a 5mL pipette and an additional 5-7 times through a 200µL pipette tip. Cells were pelleted by centrifugation for 5 minutes at 1000 rpm, the supernatant was removed, and the pellet was re-suspended in 5mL HBSS. This process was repeated twice before re-suspension in warm neurobasal media supplemented with B27 (Gibco), 2mM L-glutamine and 1% penicillin/streptomycin. MSNs were plated onto poly-D lysine-coated 24-well plates at a density of 1.5 x 10⁵ cells/well. Cells were maintained at 37°C in a humidified atmosphere 5% CO₂.

2.2.5 Control Standard Endotoxin (CSE) Stimulation

After the initial isolation, primary microglia were seeded at a density of 1.4x10⁵
cells/ ml onto 96-well gelatin-coated plates or 96-well PRIMARIA™ tissue culture plates respectively. 24 hours later the culture media was replaced with growth medium containing 1% FBS either alone or containing Interferon-γ (INF-γ; final concentration 10 ng/ ml) with or without CSE, highly purified LPS) (a widely used standard for endotoxin testing, purified from Escherichia coli O113:H10, final concentration 100ng/ml (Associates of Cape Cod). Supernatants were collected at specified times and stored at -20°C. Cells were lysed and total protein levels were determined using the micro BCA kit (Thermoscientific).

2.2.6 Immunocytochemistry (ICC)

Cells were cultured on glass-coverslips. After 24 Hours post isolation of pure primary microglia the culture media was aspirated, cells were washed once with PBS, and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT). Cells were washed and permeabilized three times for 5 minutes at RT with PBS-T (T = Triton X-100; 0.3 % in PBS), followed by blocking with 10% normal goat serum in PBS-T for 30 minutes at RT. Primary antibodies rabbit polyclonal anti-IBA1 (1:1000; Wako Chemicals USA) and rabbit polyclonal anti-GFAP (1:1000; Abcam Eugene, USA) diluted in PBS-T and 2% NGS were incubated overnight at 4°C. Cells were washed three times for 5 minutes with PBS and treated with fluorescent secondary antibodies diluted in PBS. All following steps were performed at RT with minimum light exposure. Cells were incubated in secondary for 1 hour, washed twice for 3 minutes with PBS, and counter-stained with 200nM 4',6-diamidino-2-phenylindole (DAPI)
solution for 5 minutes. After a final 5 min PBS wash, the coverslips were air-dried, cleared in xylenes, and mounted onto glass slides using DePeX MOUNTING MEDIUM (Electron Microscopy Sciences).

2.2.7 Cytokine Quantitation

Cytokine concentrations were quantified in supernatants from independent wells for each condition using either the mouse IL6 (eBioscience, ready set go IL6 ELISA) or Meso Scale Discovery (MSD®, Gaithersburg, MD, USA) electrochemiluminescence assays as per the manufacturer’s protocol. Briefly, ELISA: Nunc 96 well plates were coated with coating buffer and capture antibody 24 hours prior to use. Plates were washed 5 times and blocked in assay diluent for 1 hour at room temperature. Again plates washed 3 times and samples and standards were added to the plates in duplicate and incubated overnight at 4°C. The following day plates were washed and detection antibody was added to all plates and incubated at room temperature for 1 hour. Plates were washed and incubated with Avidin-HRP for 30 minutes at room temperature. Finally, plates were washed 7 times and substrate solution was added to each well for 15-minute incubation at which point substrate stop solution was added. Plates were read at 450nm and 570nm. 570nm values were subtracted from 450 values (background subtraction). Samples were diluted 6X to attain values within the readable range. All washes were conducted with 1X PBS containing 0.05% Tween 20. MSD; Supernatant samples were thawed and centrifuged to remove insoluble material prior to use in the MSD mouse custom 9-plex kit assay. Levels of the following
cytokines were analyzed: IL-1β, IL-10, IL-12, IL-4, IL-2, IL-5, keratinocyte-derived cytokine (KC), TNFα, and IFN-γ. The calibrator standards or supernatant samples were dispensed (25µl/well) into duplicate wells of the MSD plate. The plate was sealed with an adhesive plate seal and incubated for 2 hours with vigorous shaking (1000 rpm) at RT. The detection antibody solution was added to the wells (25µl/well), and the plate was incubated for 2 hours with vigorous shaking (1000 rpm) at RT. The plate was washed three times with 1X PBS containing 0.05% Tween 20, and 150µl of reading buffer was added to each well. The plate was read on an MSD plate reader (Sector Imager 6000) and the concentration of cytokine (pg/ml) was determined and compared to total cell protein.

2.2.8 Real-time Quantitative-PCR (RT-qPCR)

To obtain pure cultures of microglia, loosely attached microglia were gently detached from astrocyte microglia culture in the culture medium at 18 days ex vivo. Following centrifugation, cells were counted and plated in pre-conditioned media (from the co-culture of astrocytes and microglia) in 6 wells plates (620,000 cells per well in 3mls media). 24 Hours following isolation, cells were washed with PBS and media was replaced with 1% FBS media. 24 hours later, cells were either stimulated with CSE or media alone was added to the well. Following 9 hours stimulation, media was aspirated and cells were again washed with PBS. Cells were lysed in Invitrogen RNA mini kit lysis and stored at -80°C until RNA preparation using Invitrogen RNA mini kit. cDNA Vilo synthesis was used to synthesize cDNA from RNA according to the
manufacturer's protocol. Briefly, 180 ng of total RNA was used as input for the cDNA synthesis. Following this, cDNA was diluted 3X in H₂O for a total input of 3ng into each well of the PCR reaction. Fast Sybr (ABI) was used for all primary microglia PCR. qPCR was conducted on Step-One ABI machine for the following targets IL6, IL-10, TNFα, KC, IL-12, IL-1β (Table 2.1).

2.2.10 MMP3 Stimulation

Microglia were isolated from both WT and YAC128 as described above. Media was changed to 1% FBS on the pure microglia cultures 24 hours post isolation. A further 24 hours after this, microglia were stimulated with 400µM MMP3. Microglia supernatant was collected 24 Hours post-stimulation, and the cell lysate used for total protein quantification.

2.2.11 Human MMP studies

2.2.11.1 Participants and ethical approval

CSF donors were recruited through the University of British Columbia HD Medical Clinic. Gene-positive subjects were staged presymptomatic, early/mid or late stage and demographic details were described previously (Dalrymple et al., 2007). Subjects were also assessed on the Unified Huntington's Disease Rating Scale (UHDRS). CSF was collected as described previously (Huntington Study Group, 1996). The clinical
characteristics of subjects were as follows; 10 per disease stage; presymptomatic: 5 female: 5 male, mean age: 39 (with a distribution of 23-68); early/mid stage HD: 5 female, 5 male, mean age: 53 (38-72); late-stage HD: 5 female, 5 male, mean age: 54 (29-67); and 10 age-matched control subjects: 6 female, 4 male, mean age: 47 (25-67). Collection of CSF was performed in accordance with the declaration of Helsinki and approved by University of British Columbia (UBC) Clinical Research Ethics Board. All subjects gave informed written consent.

2.2.11.2 MMP and TIMP-1 analysis in CSF

The CSF samples were diluted 1:50 (for the TIMP-1 assay) or used undiluted (for the MMP assay) and assayed in duplicates using ultra-sensitive MSD. The MMP ELISA was used with the following modifications of the manufacturer’s protocol: a 30 µl sample volume was used, a 10-point standard curve ranging up to 2500 ng/ml was used, the sample and calibrator were incubated on the MSD plate over night at 4°C (instead of 2 hours at RT), and a 3 hours instead of 2 hours detection antibody incubation was used. Results were analyzed on a SECTOR™ 6000 instrument (MSD). The human TIMP-1 ELISA (MSD) kit was used according to manufacturer’s protocol. The operator was unaware of the disease state of each sample during processing, and statistical analysis was performed independently.
2.2.12 Quantification of HTT levels

For quantification of mHTT protein by Förster resonance energy transfer (FRET), 10 μg of total protein from microglial cell lysates was mixed with 0.2ng/ml terbium (TB) (FRET donor)-labeled BKP1 anti-HTT antibody (Wellington, 2002) and 2 ng/ml of D2 (FRET acceptor)-labeled MW1 anti-expanded CAG antibody (Ko, 2001), in a white 384-well plate. After excitation at 340 nm, FRET was measured as the ratio of 655 nm (D2)/615 nm (TB) emission. For the quantification of mHTT protein by Western blotting, 40 μg of total protein were denatured in LDS sample buffer (Invitrogen) with 100 mM dithiothreitol and heated to 70°C for 10 min. Samples were resolved on 3-8% low-bis acrylamide gels. Proteins were transferred to 0.45 mm nitrocellulose. Membranes were blocked with 5% milk in PBS, and then blotted for HTT (MAB2166, Millipore) and calnexin (Sigma C4731) loading control. Proteins were detected with IR dye 800CW goat anti-mouse (Rockland 610-131-007) and AlexaFluor 680 goat anti-rabbit (Molecular Probes A21076)-labeled secondary antibodies and the Li-Cor Odyssey Infrared Imaging system.
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Table 2.1 Primer sequences for qPCR analysis.
2.3 Results

2.3.1 IL6 Secretion Changes in Peripheral Monocytes Expressing MHTT

I first wished to understand whether and how peripheral immune cells were impacted in the presence of mHTT. Peritoneal macrophage isolated from WT and YAC128 mice have an increased IL6 secretion compared to WT, with no genotypic difference detected in bone marrow of these cells (Figure 2.1) (Trager et al., 2014).
Altered cytokine production in macrophages from YAC128 mice.

Peritoneal macrophages obtained via peritoneal lavage were stimulated with CSE for 24 hours before IL6 levels were measured using ELISA. A significant increase in cytokine production by YAC128 peritoneal macrophages was found when compared with WT when stimulated (interaction p=0.0025, treatment p<0.0001, genotype p=0.0043). Macrophages were also differentiated from bone marrow monocytes of three-month-old YAC128 and WT mice using M-CSF before stimulation with CSE for 24 hours. Measuring IL6 levels using ELISA showed no difference in cytokine production from YAC128 bone marrow-derived cells compared with WT controls (interaction p=0.2313, treatment p<0.0001, genotype p=0.2342). Graphs show mean concentrations corrected to protein content ±SEM. Unpaired two-tailed Student's t-tests.
2.3.2 Microglia expressing mHTT have an Altered IL6 Response when Stimulated

Microglia cultures were isolated and characterized as over 99% pure, based on immunocytochemical analysis using an antibody raised against ionized calcium binding adaptor molecule 1 (Iba1), a marker frequently used to assess microglia purity (Ito et al., 1998). Cultures of Iba1 positive microglia cells (Figure 2.2A.i) contained less than 1% glial fibrillary acid protein (GFAP) positive astrocyte cells (Figure 2.2A.ii). I quantified both mouse and human HTT levels in WT and YAC128 microglia cells as well as in neuronal cells (Figure 2.2A.iv). To investigate whether the presence of mHTT would cause microglial dysfunction, I evaluated microglia response to LPS stimulation. Microglia secrete the pro-inflammatory cytokine IL6 when stimulated with CSE (Control Standard Endotoxin), highly purified LPS, by activating toll like receptor 4 (TLR4). Microglia from YAC18 and YAC128 mice overexpress human WT or mHTT protein, respectively. No significant changes in IL6 secretion were observed from microglia derived from YAC18 mice, expressing non-pathogenic human HTT protein, or their WT littermates (Figure 2.2B). In contrast, YAC128 microglia expressing mHTT displayed significantly increased IL6 cytokine secretion upon CSE-stimulation when compared to littermate WT cultures (Figure 2.2C).
Figure 2.2 Microglia from the YAC128 mouse model of HD release increased IL6 cytokine in response to a pro-inflammatory stimulus.

Cultures of microglia isolated from co-cultures with astrocytes were characterized as over 99% pure Iba1 positive (A.i) microglia cells. Less than 1% GFAP (A.ii) positive astrocyte cells were present. Iba1 antibody was selective to primary microglia (A.iii) No Primary Control (NPC), absence of primary antibody, but with the presence of the secondary antibody, giving background signalling. I quantified the amount of both mouse and human HTT present in WT and YAC128 microglia cells compared to neuronal HTT using western blotting (A.iv). No significant changes in IL6 were determined from cultures derived from YAC18 or WT littermates by ELISA (B). Microglia from the YAC128 mouse model of HD have increased stimulation-induced IL6 cytokine secretion compared to littermate WT cultures (C). (treatment: F=23.08, p<0.0001; genotype: F=56.17, p<0.0001; interaction: F=4.960, p=0.162, two-way ANOVA). Stimulation experiments were repeated twice and conducted with an N of 6 per condition.
Unstimulated controls were ommitted as IL6 was not detectable in these samples. Graphed data with an N of 6 per condition represented as mean with standard error of the mean.

2.3.3 No effect of MHTT on Cytokine mRNA when Stimulated

Cytokines are produced and secreted in response to a wide variety of stimuli. I next investigated if the elevated secretion from YAC128 microglia resulted from increased cytokine presence at the mRNA levels or elevated secretion from individual microglia. mRNA was isolated from microglia at 9 hours post-stimulation. Cytokine mRNA levels were significantly increased upon stimulation in WT, YAC18 and YAC128 microglia. However, no significant difference between these three genotypes was identified in IL-12, IL-10, TNFα, IL-1β, IL6, or KC (mouse IL-8) cytokine transcripts (Figure. 2.3A-F), indicating that increased cytokine release of YAC128 microglia does not result from increased cytokine mRNA transcription.
Figure 2.3 Pro-inflammatory stimulation of microglia leads to an increase in cytokine transcription regardless of genotype.

Microglia from WT, YAC18 and YAC128 cultures were isolated and stimulated with CSE and INF-γ. Following stimulation, total RNA was purified and cDNA was amplified for qPCR. Target genes were normalized to the three housekeeping genes Rpl13A, Rplo, and Paklip1 to produce a normalization factor using GeNorm (Vandesompele 2002). This experiment was repeated twice, and was conducted with a minimum N of 6 per condition. Graphed data is represented as mean with standard error of the mean. Two-way ANOVA was used to determine significance of both WT and YAC18 and WT and YAC128 cultures independently, although there was a highly significant increase in the changes in condition, there was no genotypic differences between groups.
2.3.4 Widespread Changes in Cytokine Secretion from YAC128 Microglia following CSE Stimulation.

Primary microglia from YAC128 mice display increased IL6 cytokine secretion when stimulated with CSE (which Figure?), with no differential transcript levels compared to WT or YAC18 microglia. Next, I determined if this effect was limited to IL6 cytokine, investigating the effect of stimulation on a wider range of cytokines. IL-12, IL-10 IL-1β, IL-2, IL-5 and KC were all significantly different between WT and YAC128 in at least one assay using post hoc tests (Figure 2.4 A, B, D, E, G, H). No post hoc differences were identified in TNFα or IL-4 in any condition (Figure 2.4 C, F; see Table 2.2 for statistical analysis). Under control conditions, cytokine levels were undetectable in all assays (data not shown). A twelve-hour time course was also conducted (Figure 2.5), which highlighted alterations in cytokine secretions over time.
Figure 2.4 Cytokine profiling of YAC128 cultures demonstrates significant changes in release of various cytokines in response to CSE.

Supernatants from WT and YAC128 stimulated microglial cultures were simultaneously analyzed for multiple cytokines using the Meso-Scale Discovery assay. (A) Following different treatments, microglial cultures differed significantly from WT and YAC128 cultures in IL-12 secretion.
(B) Following different treatments, microglial cultures were significantly different in IL-10 secretion upon different treatments between WT and YAC128. (C) Following different treatments, microglial cultures were not significantly different in TNFα secretion upon different treatments but were significantly different between WT and YAC128. (D) Following different treatments, microglial cultures were significantly different in IL1β secretion upon different treatments between WT and YAC128. (E) Following different treatments, microglial cultures were significantly different in IL-2 secretion upon different treatments between WT and YAC128. (F) Following different treatments, microglial cultures were significantly different in IL-4 secretion upon different treatments with no genotypic difference identified between WT and YAC128. (G) Following different treatments, microglial cultures were significantly different in IL-5 secretion upon different treatments between WT and YAC128. (H) Following different treatments, microglial cultures were significantly different in KC secretion upon different treatments between WT and YAC128. N= 6 per condition. Graphed data is represented as mean with standard error of the mean. Graphed data is represented as mean with standard error of the mean (see Supplementary Figure 1 for detailed statistical analysis using two-way ANOVA).
Figure 2.5 Cytokine profiling of YAC128 cultures demonstrates significant changes in release of various cytokines in response to stimulus over 24-hour time-course.

Consistent with data from 9 hours post stimulation, large alterations in a number of cytokines. Alterations are exacerbated by the presence of IFNγ. WT (blue) vs YAC128 (red) outlines stimulation in the presence of IFNγ and CSE. WT (black) vs YAC (green) outlines alterations in the absence of IFNγ but the presence of CSE. Graphed data N=5 per condition and data is presented as mean with standard error of the mean.
<table>
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<td>IL-2</td>
<td>**</td>
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<td>F=1.508</td>
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<td>IL-4</td>
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<td>F=1.829</td>
<td>F=0.08144</td>
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<td>IL-5</td>
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<td>KC</td>
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<td>F=2.777</td>
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Table 2.2 Statistics for figure 2.4 outlining alterations in cytokine secretions from microglia at 9 hours post stimulation.
2.3.5 Region-specific Changes in Secretion Profile in YAC128 Microglia

I demonstrated altered cytokine secretion in response to a pro-inflammatory stimulus in the presence of mHTT with no change in cytokine transcription. To investigate the relevance of this phenotype in HD and to identify regional differences in cytokine release that were not correlated with differences in HTT expression levels, I isolated microglia from several different brain regions and performed immunological stimulations. Microglia isolated from striatum, cortex, and cerebellum from WT and YAC128 showed differential responses to CSE, further substantiating my primary finding of increased IL6 secretion from YAC128 microglia (Figure 2.6A-C). However, hippocampal microglia did not show genotypic differences in IL6 responsiveness (Figure 2.6D). Furthermore, no regional differences in murine HTT level, or human mHTT level in the case of YAC128 mice, were identified (Figure 2.6E).
Figure 2.6. Region-specific microglia activation differences in YAC128 primary microglia cultures despite similar mHTT levels.

Region specific microglial cultures extracted from WT and YAC128 treated with l for 1 hour prior to CSE show an increase in IL6 secretion as measured by ELISA. (A) Striatal microglial cultures from WT and YAC128 animals were significantly different (p=0.0134, non-paired t-test). (B) Cortical microglial cultures from WT and YAC128 animals were significantly different (p=0.0113, non-paired t-test). (C) Cerebellar microglial cultures from WT and YAC128 animals were significantly different (p=0.081, non-paired t-test). (D) Hippocampal microglial cultures from WT and YAC128 animals were not significantly different (p=0.379, non-paired t-test).* p<0.05. (E) Western blotting of pure microglia culture protein lysates illustrating changes in both murine and human HTT levels between brain regions. Stimulation experiments were repeated twice and conducted with an N of 6 per condition. Graphed data is represented as mean with standard error of the mean.
2.3.6 MMP3, an Endogenous Stimulus, can Evoke an Increase in YAC128 Microglial Cytokine Secretion

Using CSE stimulation I have identified alterations in cytokine release following stimulation in microglia isolated from YAC128 mice. I then investigated whether cytokine release by other disease-related pathways and mechanisms is altered in YAC128 microglia using an endogenous brain-specific microglial stimulus, MMP3. I demonstrated that MMP3 stimulation can illicit an immune response from microglia containing mHTT. MMP3 stimulation of primary microglia causes a similar IL6 response as CSE and a similar fold increase in IL6 cytokine release in YAC128 compared to WT microglia (Figure 2.7). Increases in MMPs were also identified in late stage HD CSF samples, as both MMP3 and MMP9 were significantly increased compared to control samples (Figure 2.7). This indicates a potential role for MMPs in the activation of the enhanced cytokine secretion phenotype in HD.
Figure 2.7 MMPs regulate cytokine release from microglia and play a role in HD.

Similar to CSE stimulation, YAC128 microglia respond to MMP3 treatment with increased IL6 release compared to WT littermate controls. Bonferonni Post-hoc test CSE (p<0.001), MMP3 (p<0.05). (Treatment: F=1.8, p<0.3586; Genotype: F=55.68, p<0.0001; Interaction: F=1.8, p=0.3586, two-way ANOVA). MMP3 and 9 are significantly increased in CSF from HD patients compared to controls. No significant changes in TIMP1 or MMP1 were detected. (One-way Anova, MMP3, Treatment F=3.15, p<0.0412; MMP-9 Treatment F=4.19, p=0.0147).
2.4 Discussion

Here, I have demonstrated that both cell intrinsic and extrinsic mechanisms lead to microglia dysfunction in HD, ultimately leading to disease-related dysfunction of CNS inflammatory cells. Several studies have provided evidence for abnormal microglial morphology and active neuroinflammation in the HD brain (Vonsattel et al., 1985). I have recently defined similar microglial and microvascular changes in YAC128 mice (Franciosi et al., 2012). Unbiased proteomic profiling of plasma and CSF from HD patients has detected up-regulation of inflammatory cytokines and other innate immune proteins such as the acute-phase protein α2-macroglobulin and clusterin (Dalrymple et al., 2007). In a follow-up study, I, along with collaborators demonstrated that up-regulation of a number of pro-inflammatory cytokines such as IL6, IL-8 and TNFα in the plasma and CSF of HD patients correlates with disease stage (Bjorkqvist et al., 2008). My laboratory has seen evidence for increased gliosis using magnetic resonance spectroscopy (Sturrock et al., 2010), and PET studies on pre-symptomatic and manifest HD patients have illustrated selective microgliosis in brain regions affected by HD (Tai et al., 2007). Interestingly, the degree of microglial activation on PET scans correlates with HD severity in symptomatic patients (Politis et al., 2008).

I hypothesized that the presence of mHTT has a functional effect on microglia. The main finding is that perturbations in microglial function are regulated by mHTT levels leading to an increase in secretion but not transcription of multiple cytokines. MHTT
expression significantly increased IL6 release from microglial cells following stimulation with CSE (Figure 2.2). In addition, microglial secretion of various other cytokines (Figure 2.4) was also elevated from microglia expressing mHTT, but transcription of these cytokines was not altered (Figure 2.3). In addition, altered NFκB signalling has been identified as dysregulated in HD microglia associated with increased cytokine release (Hsaio et al., 2013). This non increase in transcript was unexpected, previous research had indicated that monocytes from R6/2 mice and from HD patients containing have an increase in IL6 transcript levels, these occur at late stage of disease progression in human samples (Trager et al., 2014). Bone marrow transplantation studies and KMO inhibitor administration studies in mouse models of HD have both suggested that the targeting the peripheral immune system may modify disease progression (Zwilling et al., 2011).

The alterations in my primary microglial culture model may have an increased effect on cytokine regulation at later stages of disease as microglia become more active over time (Lou et al., 2010). I further investigated how an increase in extracellular protein levels occurred without concomitant increase in transcript. At a specific time point (9 hours) I observed a small but significant decrease of intracellular IL6 levels in YAC128 microglia, indicating that the elevated IL6 levels occurred due to increased secretion rather than increased transcription. Accumulation of cytokines in the media with increased secretion in the absence of increased cytokine transcription levels would be predicted to decrease intracellular cytokine levels during the stimulation. Also, p65 driven luciferase activity was not different between WT and YAC128 microglia cultures when stimulated, again suggesting a lack of transcriptional
dysregulation in YAC128 microglia and supporting dysregulated vesicle-mediated secretion.

In HD, the striatum and cortex are primarily affected, with some secondary effects in hippocampus and even lesser effects in the cerebellum. I found regional differences in the activation of mHTT-expressing microglia from striatum, cortex, and cerebellum, but not in microglia originating from the hippocampus (Figure 2.6). No correlation between regional differences and mHTT levels was observed. Interestingly, given that hippocampal microglia express similar levels of mHTT, these results indicate that mHTT may have cell-autonomous effects that can indirectly alter the responsiveness of primary microglia cells.

The original description of microglial activation in the HD brain was based on morphological changes identified by immunocytochemical staining with thymosin B-4, an antigen increased in reactive microglia (Sapp et al., 2004). Reactive microglia were evident in all grades of HD pathology, and the number of immunostained microglia increased with increasing grade. Microglial morphology was also altered in the striatum and cortex. My data suggests that under basal conditions microglia containing mHTT are primed to secrete increased amounts of cytokines in response to both exogenous stimuli LPS and more importantly an endogenous brain stimuli MMP3.

PET scans using raclopride (RAC), which labels dopamine D2 receptors, are commonly used in HD to image the striatum, while PK11195 (PK), a ligand that binds selectively to peripheral benzodiazepine binding sites, is used to label microglia. Tai et al., 2007, measured microglial activation with PK PET and linked changes to striatal
neuronal dysfunction measured with RAC PET, proposing a role for PK PET as a marker for subclinical disease progression in pre-symptomatic HD gene carriers. These pre-symptomatic gene carriers had lower striatal RAC binding than the controls but significantly higher striatal and cortical PK binding. My data supports these pre-symptomatic microglial changes, as the primary microglia are isolated five months before any neurological defects are observed in the YA128 model. Altered inflammatory profile from HD post mortem brain tissue. IL-1β and TNFα were increased in striatum, consistent with a pattern of microglial activation (Silverstroni et al., 2009).

I have demonstrated a phenotype that is synergistic with microglial activation, demonstrating increased cytokine release in the presence of human mHTT. Specifically, mHTT has an intracellular effect on the release of cytokines in response to pro-inflammatory stimuli. This effect may be related to perturbations in vesicle trafficking in the presence of mHTT. Recently, I described altered microglia morphology at 12 months of age in the YAC128 mouse model, but no dramatic increases in numbers or size of microglia (Franciosi et al., 2012).

YAC128 mice model early HD, and my data would suggest that a subtle inflammatory process is occurring in YAC128 striatum. Microglia in these mice display an activated morphology (Franciosi et al., 2012) and secrete increased levels of inflammatory cytokines, but large numbers of microglia are not recruited to the striatum at this stage of the disease. This increase in microglial activation at 12 months could play a role in the neuronal loss (10%) typically seen at this time point. However, a potential role of this microglial activation in repairing the diseased striatum
cannot be ruled out without further in vivo studies. In my primary microglia model of inflammation, YAC128 microglia secreted increased IL6 in response to CSE, and responded to MMP3 in a similar fashion. Interestingly, when I analyzed CSF from HD patients, both MMP3 and MMP9 protein levels were significantly increased in HD patients. These expression changes also correlate with changes in microglial morphology observed in mice and may be directly related (Fransciosi et al., 2012). Microglia morphological changes and perturbations in neurovasculature have been correlated with both presymptomatic HD and early stages of HD progression (Lin et al., 2013).

My data implicates MMPs as potential modulators of neuroinflammation in HD. MMP3 stimulation of microglia has a similar effect on cytokine release as CSE stimulation, this increase secretion of YAC128 microglia. This demonstrates that mHTT-dependent alterations in microglial function are not Toll-like Receptor 4 dependent. These data also demonstrate that the hyper-responsiveness of YAC128 microglia occurs in response to an endogenous stimulus that is elevated in the HD brain. mHTT is likely a key factor in promoting inflammation in HD, either directly or indirectly. However, the question that now arises is whether mHTT induces neuronal injury or directly mediates inflammation by intrinsic effects on microglia, or both. Interestingly MMP activity is increased in the striata of both YAC128 and R6/2 mice (Miller et al., 2010). Increases in MMPs in CSF from HD patients correlate with HD disease severity (Figure 2.8). It is important to note that there may be compensatory mechanisms hiding the alterations seen in HD until a later stage of the disease. In the data presented here, the R-square value was less than 0.4 indicating the correlation,
suggesting that less than 40% of the variance is shared between "MMP levels" and "HD progression"; although this is concerning, The role of MMPs in HD requires further investigation and need to be understood in the context of mHTT. Insights into microglial-mediated neuroinflammation and the interaction between HTT and the immune system, may identify new drug targets in HD and will hopefully lead to novel therapeutic approaches for this devastating neurodegenerative disease.
3 Utilizing the HD microglial model system to test therapeutic agents

3.1 Introduction

Generally, therapeutic approaches in HD do not selectively target neuronal cells or neuronal processes, although these cells are usually analyzed for their response to therapeutics because, in HD, brain dysfunction is thought to be due to mHTT expression in neuronal cells. As I had previously identified an enhanced immune response in microglia expressing mHTT, I wanted to test whether therapeutically targeting mHTT would modulate this effect. From the perspective of utilizing my system as a model of illustrating immune dysfunction in HD, it is important to show both that the immune response can be modulated and that the effect of mHTT is a direct effect on microglia. Furthermore, modulating immune response ex vivo could inform future experiments aimed at tweaking the immune system of HD mouse models in vivo. To better understand the enhanced immune response in the presence of mHTT in microglia, I treated these primary cells with different therapeutics both aimed at decreasing the mHTT HTT load in these cells, and at enhancing endogenous anti-inflammatory signals.
3.1.1 Immune modulation by laquinimod

Laquinimod, which is thought to modulate the immune system and have neuroprotective actions, was previously assessed for actions against MS (Haggiag *et al.*, 2013). Here, I utilized laquinimod to modulate the immune phenotype in primary microglia isolated from WT and YAC128 mice (primary culture).

3.1.2 Activation of the NRF2 Pathway by MIND4-17

Activation of the NRF2/KEAP1/ARE pathway is a promising therapeutic strategy to counteract neuroinflammation-induced neurodegeneration (Thimmulappa *et al.*, 2007). Previous work described Nrf2 activation in BV2 cells and its role in redox homeostasis, BV2 cell line are a model for microglia (Innamorato *et al.*, 2008). In my experiments, I used the compound MIND4-17, which is based on a novel structural scaffold and activates NRF2 by targeting KEAP1, thus causing nuclear translocation of NRF2 and enhanced expression of NRF2 responsive genes.

3.1.3 AA-5HT

Microglia represent a candidate target for endocannabinoid signaling due to the immunomodulatory actions of endocannabinoids, and due to the presence of cannabinoid receptors and cannabinoid biosynthesis and degradation enzymes in
microglia (Ueda et al., 2011). The cannabinoid anandamide, synthesized by microglia, is increased in diseased brain lesions and protects neurons from inflammatory damage (Van der Stelt et al., 2001). Anandamide also inhibits the expression of inflammatory IL-12p70/IL-23 subunits, and is a potent inducer of IFNγ and stimulator of T helper cells (Ribeiro et al., 2010). Furthermore, anandamide enhances IL-10, an anti-inflammatory cytokine through a cannabinoid receptor 2 (CB2)-mediated pathway in diseased microglia. Pharmacological stimulation of CB2 receptors by anandamide in diseased microglia may lead to alteration cytokine expression contributing to the accumulation of anti-inflammatory microglia at lesion sites (Correa et al., 2011). Endocannabinoids are local messengers connecting the immune system to the brain and represent a new mechanism of neuroimmune communication during CNS pathologies.

3.1.4 Modulation of HTT Levels via Anti-Sense Oligonucleotides (ASO)

ASOs can be used to silence genes, and as the specific mechanism (if there is one) of mHTT dysfunction is unknown, ASOs hold great potential as therapeutics for HD. Over the last six years, the concept of HTT lowering has been widely discussed in the HD field; this past year, an ASO targeting both WT HTT and mHTT in human patients was announced (Gold, 2015). Modulating the levels of HTT and showing a functional effect is important for the progress of this therapeutic approach;
additionally, delineating direct and indirect (downstream) changes due to HTT reduction will be important.

3.2 Methods

3.2.1 MIND4-17

Pure primary microgila cultures from both WT and YAC128 were treated with (0.5- 20µg/ul) concentrations of MIND4-17 or DMSO for 24 hours prior to stimulation with IFN-γ and CSE. Media was collected for cytokine detection and cells were collected and lysed for quantification of total protein analysis.

3.2.2 Laquinimod Treatment

Pure primary microglia cultures from both WT and YAC128 were treated with 5µM laquinimod or PBS for 24 hours prior to stimulation with IFN-γ and CSE. Media was collected for cytokine detection and cells were collected and lysed for quantification of total protein analysis.

3.2.3 AA-5-HT Treatment

Arachidonoyl serotonin (AA-5-HT) is a fatty acid amide hydrolysis (FAAH) antagonist, FAAH itself leads to the degradation of Anandamide. Pure primary microglia cultures from both WT and YAC128 were treated with 150µM AA-5-HT
(Sigma) or PBS for 24 hours prior to stimulation with IFN-γ and CSE. Media was collected for cytokine detection and cells were collected and lysed for quantification of total protein analysis.

3.2.4 Anti-Sense Oligonucleotide Treatment

Both human HTT specific and control off-target antisense oligonucleotides (ASOs) were provided by ISIS pharmaceuticals, previously this HTT specific ASO has been used in ex vivo and in vivo studies to reduce levels of the mHTT protein (Carroll, 2011). 24 hours post isolation from co-culture with astrocytes, pure primary microglia cultures were treated with 1µM ASO diluted in culture medium. 5 days later, microglia cells were stimulated for 24 hours with IFN-γ and CSE. Cells were then collected and lysed for quantification of HTT knockdown or supernatant was collected and cells lysed for both cytokine and total protein analysis.

3.3 Results

3.3.1 Laquinimod has Minimal Effect on the Microglial Immune Response to CSE

Laquinimod is thought to modulate the immune system and have neuroprotective actions, I assessed laquinimod treatment in my microglial cell model. My anecdotal evidence having used laquinimod repeatedly over the span of 2 years, there were some indications that laquinimod was being effective in decreasing the enhanced immune phenotype I previously described in YAC128 primary microglia. However, upon repetition of experiments the effect of laquinimod on cytokine release
from microglia was not reproducible. Figure 3.1 is a representative graph of my assessment of laquinimod. I identified laquinimod as effecting the transcription of IL6 cytokine (Figure 3.2), I tested whether levels of p65 translocation were also altered by laquinimod (Figure 3.3). As the effect of laquinimod on cytokine release was not significant in my microglial model of enhanced immune response, I also assessed Intracellular IL6 levels to determine if the extracellular levels of IL6 were representative, finding no overall changes in IL6 levels intracellularly (Figure 3.4). I also assessed if limiting the amount of CSE would improve the therapeutic window and enable me to demonstrate an effect of laquinimod within this system (Figure 3.5) in a more robust way, however I was unable produce a more reproducible laquinimod effect.
Figure 3.1 Dose response of laquinimod pre-treated microglia.

Alterations in YAC128 microglia treated with 5uM laquinimod were identified in IL6 secretion levels, N=4 per condition, this experiment was repeated over 8 times, small trends were observed but no significant or consistent drug effect was demonstrated (Interaction $F=34.33 \ p=0.0041$, Treatment $F=3.776 \ p=0.4438$, Genotype $F=19.6 \ p=0.0085$, TWO WAY-ANOVA).
Figure 3.2 Transcript levels of cytokine IL6 assessment of 5uM laquinimod treated WT and YAC128 microglia.

A small trend of a difference was noted in laquinimod treated microglia N=4 per condition. (Two-way ANOVA Treatment p=0.0059, Genotype p=0.8692, Interaction p=0.8369)
Figure 3.3 p65 levels of WT and YAC128 microglia were also assessed post CSE treatment.

No significances were observed across conditions N=3 per condition. (Two-way ANOVA Treatment p= 0.0203, Genotype p=0.6529, Interaction p=0.8864)
Figure 3.4 Intracellular levels of IL6 were assessed in both WT and YAC128 microglial protein samples.

CSE leads to increased intracellular levels of IL6 as basal condition was not detectable. Laquinimod lead to a small decrease in the level of intracellular IL6. Brefeldin A was used as a control, there was no IL6 secretion detected in these samples and an increase in intracellular IL6 was detected.

(Two-way ANOVA Treatment p=<0.0001, Genotype p= 0.0103, Interaction p=0.2431)
Figure 3.5 CSE dose modification did not affect the therapeutic benefit of laquinimod on WT or YAC128 microglia.

Limiting CSE stimulation dose overall leads to a decrease in IL6 release from primary microglia, the addition of laquinimod did not impact the levels of IL6 secreted by these cells.
3.3.2 MIND4-17 reduces the Enhanced Immune Response in YAC128 Microglia

To test whether the NRF2/Keap1/ARE pathway would be a potential therapeutic I used MIND4-17 as an NFR2 activator and used IL6 secretion as a readout for secretion changes from microglia cultures. NRF2/KEAP1/ARE pathway is a promising therapeutic. I found that MIND4-17 reduced the amount of IL6 secreted from WT and YAC128 primary microglia in a dose-dependent manner (Figure 3.6). Interestingly, at low doses, MIND4-17 inhibited IL6 release in YAC128 cells only, compensating for the apparent pro-inflammatory hyper-secretion response of HD microglial cells.
Figure 3.6 MIND4-17 treatment has a significant effect on IL6 levels from both WT and YAC128 primary microglia.

Above is a combination graph of two independent experiments outlining the effects of a MIND on microglial activation upon stimulation with CSE. CTRL is the absence of MIND but stimulated with CSE. (Two-Way ANOVA Treatment $p<0.0001$ $F=76.53$, Genotype $p=0.1180$ $F=4831$, Interaction $p=0.0179$ $F=3.489$)
3.3.3 Cannabinoid Modulation Alters Cytokine Release in YAC128 Primary Microglia

To test the hypothesis that cannabinoids can be used to modulate neuroinflammation through direct targeting of microglial cells, I next examined the effects of cannabinoids in my ex vivo model of inflammation in HD. Manipulation of the cannabinoid pathway can have immunomodulatory effects on microglial cytokine release and I tested this effect on microglia in the presence of mHTT. Pre-treatment of microglia with AA-5-HT, a fatty acid amide hydrolase (FAAH) antagonist, leads to build up of the endocannabinoid anandamide. YAC128 microglia treated with PBS illustrated the usual increased IL6 secretion phenotype (Figure 3.7A). However, when microglia were exposed to AA-5-HT, IL6 secretion was significantly reduced in both WT and YAC128 microglia (Figure 3.7B). Interestingly, no significant differences were identified between WT PBS and AA-5-HT treated YAC128 cultures, demonstrating the effectiveness of cannabinoids in normalizing the YAC128 inflammatory phenotype (Figure 3.7).
Figure 3.7 Increasing endogeneous cannabinoid levels decreases IL6 secretion by YAC128 microglia.

Microglia cultures were treated with either PBS or AA-5-HT 30 hours prior to stimulation (A). AA-5-HT led to a decrease in secretion from both WT and YAC128 cultures (Bonferroni post-hoc test). AA-5-HT treatment reduced the increased IL6 secretion in YAC128 primary microglia to that of WT levels (B) (Treatment: F=52.48, p<0.0243; Genotype: F=92.03, p<0.001; Interaction: F=6.182, p<0.001, two-way ANOVA,). Stimulation experiments were repeated twice with an N of six per condition. Unstimulated controls were omitted from graphs as IL6 below detection limits using this assay. Figure 3.7 are representatives of two independent experiments. Graphed data is represented as mean with standard error of the mean.
3.3.4 Modulating mHTT Levels in YAC128 Microglia Ameliorates Secretion Phenotype

As AA-5-HT was effective in modulating the inflammatory phenotype of both WT and YAC128 microglia, I next wished to investigate whether this immune phenotype was caused by the presence of mHTT in microglia (direct effect) or, alternatively, a result of microglia being exposed to an HD-like environment (indirect effect). I utilized ASOs to selectively knock down mHTT in YAC128 microglia, achieving a reduction of approximately 45% as judged by both Western blotting (Figure 3.8 A) and FRET (Figure 3.8 B) analyses. Importantly, I observed a reversal of the elevated cytokine release in YAC128 microglia, essentially leading to normalization of the YAC128 microglia phenotype (Figure 3.8 C) with 55% reduction in overall HTT levels.
Figure 3.8 Altering mHTT levels can alter the inflammatory phenotype of YAC128 primary microglia.

WT or YAC128 microglia were either untreated (UT), treated with an off-target oligonucleotide (OT), or treated with a human HTT-specific anti-sense oligonucleotide (HTT) for 6 days and then stimulated with CSE and INF-γ. Western blot (Figure 3.8A) and FRET (Figure 3.8B) were performed on cell lysates to confirm HTT knockdown. (Figure 3.8C) ASO treatment of microglial cultures lowered the overactive IL6 response in YAC128 compared to identically treated WT microglia (treatment: F=4.25, p<0.0237; genotype: F=19.88, p=0.0001; interaction: F=14.78, p<0.0001, two-way ANOVA). YAC128 microglia secrete similar amounts of IL6 as WT controls when treated with a human HTT ASO indicating a mHTT protein dependent IL6 secretion in response to a pro-inflammatory
stimulus. Graphed data is represented as mean with standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.4 Discussion

In this chapter I demonstrated that pharmacological manipulation can modulate cytokine release in an HD model of enhanced immune response in microglia. Laquinimod had a minimal effect on microglial cytokine secretion and this was investigated extensively. I conducted 9 independent experiments with laquinimod and the effect on cytokine release was significant in only two of these experiments. Optimizing the experimental conditions of this model system, I tried to identify a set of conditions in which the initially-observed trends would become more robust. However, limiting the dose of CSE or altering the stimulation protocol did not lead to a more robust therapeutic effect of laquinimod in the model I used. I also assessed the alteration in mRNA, intracellular protein and p65 activation indicative of transcriptional changes. Very subtle alterations were observed in response to laquinimod treatment. However, I did not find a reproducible change in extracellular secretion of cytokines, my primary endpoint for these studies. Laquinimod may play a modulatory role in other types of microglial signalling in HD, but my data is most consistent with the current theory that laquinimod acts primarily through modulating astrocyte function (Bruck et al., 2012). Laquinimod was used in human trials for Multiple Sclerosis, and it is considered safe for human use; however, its immunomodulatory efficacy is still debated (Thimmulappa et al., 2007). In sum, I was unable to demonstrate a shift in inflammation of YAC128 microglia using laquinimod; however, it is conceivable that
laquinimod targets other immune pathways that were not detectable through the chosen end points.

Using MIND4-17, I was able to modulate the cytokine secretion phenotype. MIND4-17 acts through the NRF2 pathway, and I identified a limited specificity to genotypic effects at low concentrations of MIND4-17, effecting YAC128 microglia without changes in WT microglia secretion. While these experiments do not conclusively prove that MIND4 or the NRF2 pathway is involved in mHTT-driven, enhanced immune response, they illustrate that this phenotype can be altered by more than one immuno-modulatory pathway. In turn, this suggests a possible future for conventional inflammatory therapies in the treatment of this aspect of HD.

The last experiment involved reducing overall HTT levels by ASO treatment and measuring the effect on microglia function. Excitingly, this treatment produced a specific effect on the enhanced cytokine secretion phenotype despite causing only a 50% reduction in the levels of overall HTT. Indeed, using this treatment, I was able to completely ameliorate the secretion phenotype in mHTT containing microglia, lowering the cytokine secretion levels down to WT levels. This experiment is particularly relevant when considering current trials aimed HTT lowering. An HTT-lowering trial is currently conducted with an ASO that lowers both WT HTT and mHTT. Interestingly, virtually no information is yet available on the potential role of WT HTT in microglia or on the role HTT in normal inflammation. My work using ASOs illustrates that directly lowering mHTT can have specific and significant effects on the function of microglia. An exciting future question will be whether mHTT enhanced immune
response has an actual effect on HD progression? This is again particularly relevant when considering the ongoing human therapeutic trials using ASOs.

In this chapter, I also demonstrated that the cannabinoid pathway can be used as a potential therapeutic pathway to modulate the effect of mHTT on inflammation in HD. Specifically, I enhanced the levels of anandamide (an endogenous cannabinoid in the brain) by treatment with a compound that depletes the anandamide hydrolase FAAH, an approach previously used to modulate anandamide levels in vivo (Bisogno et al., 1998). By increasing endogenous anandamide production, I was able to skew the endocannabinoid system towards decreasing cytokine secretion; however, this effect did not differ between genotypes (i.e. was unrelated to mutant or WT HTT status). Nevertheless, I was able to reduce YAC128 microglia driven cytokine release to that of WT microglia. Interestingly, previous work has illustrated that cannabinoid receptors are some of the first receptors lost in HD (Ooms et al., 2014); however, it remains unclear whether this is a direct response to a stimulus or an effect of an altered immune state in the brains of HD patients.

Taken together, the data presented in this Chapter used multiple approaches to modulate the mHTT-enhanced cytokine secretion described in Chapter 2. The therapeutic approach taken with HD patients often targets the symptoms of the disease; future therapies may instead several phenotypes, including inflammation, as part of a combinatorial treatment strategy to modulate the age of onset and/or the severity of the disease. Interestingly, limiting mHTT in microglial cells was the most effective approach identified here, as it specifically inhibited the enhanced cytokine secretion phenotype caused by mHTT in microglia of YAC128 mice. As such, this
provides further support of the use of such therapies as a hopeful treatment strategy for HD.
4. Modulating Mutant Huntingtin-induced Immune Dysfunction in Mouse Models of Huntington’s disease

4.1 Introduction

In chapters 2 and 3 I showed that mHTT has a functional effect on the secretion of cytokines from microglia and that using pharmacological approaches I could modulate this phenotype. Next, I wanted to use a genetic system to study the interactions between mHTT and microglia, specifically using the Cannabinoid receptor 2 knockout (CB2KO) mice as well as monocyte specific HTT knockout by lysozyme-promoter driven Cre. Using these approaches, my objective was to understand the effect(s) of this enhanced cytokine secretion phenotype on the progression of HD.

In Chapter 3, I demonstrated an effect of AA-5HT on the microglial phenotype, and previous work within the HD field has demonstrated cannabinoid system alterations in HD (see Chapter 3). Notably, cannabinoid receptors disappear prior to the massive degeneration of cells in the striatum in the progression of HD, suggesting that cannabinoid signaling may play an early role in HD progression (Glass et al., 2000). Suppression of cannabinoid receptor 1 signaling accelerates the neurodegeneration seen in R6/2 mice (Meivis et al., 2011). Furthermore, studies using cannabinoids as therapeutics in R6/2 mice showed improved motor phenotypes, reduced brain lesions, ameliorated loss of synaptic density, and attenuation of
decreased BDNF levels (Glass et al., 2000). Finally, the clearest demonstration of the importance of CB2 in HD is an experiment on CB2KO animals injected with QA, a chemical commonly used to model HD (Palazuelos et al., 2004). In this experiment, CB2KO animals showed an increase in brain lesion volume after QA injection, suggesting a role for CB2 and cannabinoid signaling in microglia. Microglia cells express the rate-limiting enzyme IDO within the Kynurinine pathway (Mazarei, G., et al., 2013).

4.1.1 Cell Specific Expression of Huntingtin as HD models

Understanding the role of HTT in specific cell types is important to understand their role in the progression of HD. A model for astroglia-specific mHTT expression, specifically of an N-terminal fragment of mHTT driven by the GFAP promoter, has been described (Bradford et al., 2009). This mouse model displays body weight loss, neuronal function deficits, and a short life span. These phenotypes were attributed to the role that astrocytes play in glutamate uptake, as primary astrocytes isolated from these mice had decreased expression of glutamate transport proteins. As mentioned previously, the BACHD mouse model was also used to reduce HTT expression in specific neuronal populations in an effort to understand the roles of cortical and striatal crosstalk in the pathogenesis of HD (Wang et al., 2014). Little restoration of the HD phenotype was evident in both reduced expression in cortical and striatal neurons separately, however reduced expression in both cortical and striatal neurons
simultaneously was sufficient to reduce the phenotype and progression of HD in BACHD mice.

4.1.2 Mouse Models of Huntington’s disease

The YAC128 and BACHD mouse models of HD recapitulate several clinical features of HD. The symptoms and neuropathological features recapitulated in these animal models include impaired motor skills such as decreased latency to fall on the rotarod and striatal volume loss. Further studies in the YAC128 mice have demonstrated inflammation in the striatum with the presence of microglia (Fransciosi et al., 2012) and altered morphology in areas of neuronal loss (Sapp et al., 2001) and their presence is thought to precede disease onset (Tai et al., 2007; Bjorkqvist et al., 2008). Similarly, abnormalities in cerebrovasculature such as decreased cerebral blood flow in caudate have been reported from imaging studies in both symptomatic and presymptomatic HD patients (Harris et al., 1996; 1999). Furthermore, vessel lumen narrowing and increased angiogenesis in post-mortem HD brain tissue has been reported (Vis et al., 1998, Drouin-Ouellet et al., 2014).

Age-dependent changes in microglial morphology and neurovascular perturbations have been recently reported in the YAC128 animal model of HD (Fransciosi et al., 2012). A trend for increasing number and size of microglia occurs with increasing age with a significant decrease in the number of processes of
microglia in YAC128 at 12 months of age. Concurrently, significant alterations in the vasculature also occur with increasing age in the YAC128 with significant deposition of vascular basement membrane components at 6 months of age which continues to increase significantly at 12 months of age in YAC128 compared to WT (Fransciosi et al., 2012). Stereological assessment of vascular parameters indicate that vessel density, length and vessel wall thickness increase significantly in YAC128 compared to controls while vessel diameter decreases but not significantly (Fransciosi et al., 2012). These results indicate that vascular remodelling occurs in the YAC128 mouse model of HD and that these changes may play a role in microglial-mediated inflammatory processes and in HD pathogenesis.

In this chapter, I used genetic approaches to either amplify or suppress cytokine secretion from microglia expressing mHTT. First, I crossed the CB2KO into YAC128 mice; from previous studies on cannabinoid signaling, I expected the CB2KO to lead to increased cytokine secretion. Next, I crossed lyzosyme-Cre mice to BACHD mice, creating WT, BACHD, WT-lys-CRE (WT mice expressing Cre driven by the lysozyme promoter) and BACCre (expressing both mHTT and Cre driven by the lysozyme promoter).
4.2 Methods

4.2.1 Mice

YAC128 mice of mixed gender and their non-transgenic litter mates maintained on the B6 background were crossed to CB2KO mice to produce all genotypes required: WT, YAC128, CB2KO and CB2KO/YAC128. These mice were then used for microglial culture experiments. BACHD mice of mixed gender and their non-transgenic litter-mates were maintained on the FVB/N background (William Yang 2006), crossed to Lysozyme-Cre mice (the LysMcre knock-in allele, which has a nuclear Cre recombinase inserted into the first coding ATG of the lysozyme 2 gene (Lyz2) (Glasen et al., 1999); obtained from the Jackson Lab (B6.129P2-Lyz2tm1(cre)Ifom/J)) and maintained on a B6 background. Mice were bred to F1 FVB/B6 for these experiments. Mice were group housed with a normal 12 h light–dark cycle in a clean facility and given free access to food and water. All experiments were carried out in accordance with protocols approved by UBC Animal Care and Use Committee. Groups were as follows WT 15, BACHD 14, BACB6 12 and BACCre 21.

4.2.2 Accelerating Rotarod Assays

Accelerating rotarod testing was performed at 1 month intervals following 7 month baseline testing and conducted until the mice reached 12 months of age. Each testing day consisted of three trials each 2 hours apart. The accelerating protocol was
from 5rpm-40rpm over a period of 300 seconds. Mice that did not reach the maximum of 300 seconds were recorded and returned to their home cage.

4.2.3 Open-Field Experiments to Test Levels of Hypoactivity

The open field test consisted of one 10-minute trial at 12-months of age in a 50cm x 50cm x 20cm arena made of hard, white polyvinyl chloride (PVC). The center zone was defined as a square covering 16% of the total arena area (20cm x 20cm central square). Mice were placed in the center of the arena, which was well lit with overhead lighting. EthoVision XT 7.0 software (Noldus, VA) was used to record and score total distance moved and duration of time spent in the center zone.

4.2.4 Histology and Immunohistochemistry

Immunohistochemistry was performed on free-floating sections. The primary antibodies used were a rabbit polyclonal anti-IBA1 (1:1000; 019-19741 Wako Chemicals USA, Richmond, VA, USA). Sections incubated without primary antibody served as controls. Sections were blocked with Tris-buffered saline (TBS; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.6, 0.15 M NaCl)/0.1% Triton X-100/5% goat serum (TBS-TGS) for 1.5 h and the primary antibody was applied overnight in TBS-TGS at room temperature. Following washing in PBS for 1 h, immunofluorescence staining was detected by incubation with species specific Alexa Fluor secondary antibody conjugates (1:500; Invitrogen, Eugene, OR, USA) for 2 h in TBS-TGS/TBS-TDS.
Nuclei were counter-stained with 1 µg/ml 4′-6-diamidino-2-phenylindole (DAPI). After washing in PBS, sections were mounted on slides using Prolong Gold (Invitrogen).

4.2.5 Stereology for Striatal Microglia Cell Size and Volume

For analysis of microglia number, striatal volume parameters including vessel every eighth coronal section throughout the striatum from BACHD, Cre, BACCRe and WT mice was stained with Iba1 for microglia. The striatum was delineated using a stereology workstation, consisting of a Zeiss Imager M2 light microscope with a EC Plan Neofluar objective 2.5× (numerical aperture [N.A.], 0.075) to delineate brain regions and a EC Plan Neofluar objective 40× (N.A., 0.75; Zeiss, Göttingen, Germany) to count. For counting, a motorized specimen stage for automatic sampling (MAX6000; Ludl Electronics, Hawthorne, NY), CCD color video camera (CX9000; Microfire Digital CCD video camera) and stereology software (StereoInvestigator 9.0; MBF Bioscience, Williston, VT) were utilized. Striatal volume was determined using the Cavalieri principle where the area of each of the striatal contours was added up for each mouse and multiplied by the section thickness (25 µm) and sampling fraction (×8). Microglia number in striatum was determined using the physical fractionator probe with a grid size of 600 × 600 µm and counting frame of 100 × 100 µm. Microglial cell body size was determined from Iba1 positive cells using the nucleator probe of the stereology software. Number of primary microglial projections was determined visually as the number of primary processes emanating from the cell body using a Plan Apochromat objective 100x (N.A., 1.4 oil).
4.2.6 Statistical Analysis

Data are presented as mean ± the standard error of the mean (S.E.M.). Comparisons were made using Student's t-test for differences between YAC128 and WT, a two-way analysis of variance (ANOVA) with Newman-Keuls post-hoc test post hoc comparison test when multiple comparisons were made for all pairs among the groups when determining age-dependent changes in YAC128 with significance set at p < 0.05. Statistical tests were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

4.3 Results

4.3.1 Cannabinoid Receptor 2 loss weakly affects Microglial Cytokine Secretion in Primary Microglial Cultures

Cannabinoids are involved in mediating inflammation signals. Using my standardized ex vivo microglial stimulation model, I found that knocking out the Cannabinoid receptor 2 in the WT background had no effect on mRNA (Figure 4.1) or protein levels (Figure 4.2) of various cytokines. However, in YAC128 mice, loss of cannabinoid receptor 2 lead to enhanced cytokine release from microglia (Figure 4.3).
Figure 4.1 Increased expression of cytokines in both WT and CB2KO microglia.

mRNA Transcript analysis of cytokines in both WT and CB2KO. No differences were detected in either control or IFNγ for 1 hour followed by CSE (1HrP) treated conditions, however treatment was highly significant across all analysis. CD11b (Interaction F=0.002949 p=0.9601, Treatment F=31.8 p=0.011, Genotype F=0.00041 p=0.9851, TWO WAY-ANOVA). IL10 (Interaction F=1.912 p=0.2389, Treatment F=23.27 p=0.0085, Genotype F=1.141 p=0.3457, TWO WAY-ANOVA). TNFα (Interaction F=0.2208 p=0.6705, Treatment F=29.86 p=0.012, Genotype F=0.2528 p=0.0085, TWO WAY-ANOVA). IL1B (Interaction F=0.01666 p=0.9055, Treatment F=835.4 p<0.0001, Genotype F=1.084 p=0.7636, TWO WAY-ANOVA). IL12 (Interaction F=1.644 p=0.2899, Treatment F=97.37 p=0.0022, Genotype F=1.728
p=0.2801, TWO WAY-ANOVA). IL6 (Interaction F=0.4724 p=0.5413, Treatment F=190.5 p=0.0008, Genotype F=0.2894 p=0.628, TWO WAY-ANOVA).

Figure 4.2 No changes in cytokine secretions between WT and CB2KO.

Cytokine levels of both WT and CB2KO either untreated controls (CTRL) or treated for 1Hour with IFNγ prior to CSE treatment for 9 hours). No differences were detected in either control or CSE treated conditions, treatment was significant in all analysis. (L6 (Interaction F=0.59 p=0.4645, Treatment F=26.86 p=0.0008, Genotype F=0.198 p=0.6682, TWO WAY-ANOVA). IL10 (Interaction F=0.2084 p=0.6602, Treatment F=17.72 p=0.003, Genotype F=0.08262 p=0.7811, TWO WAY-
ANOVA). TNFα (Interaction F=0.5021 p=0.4987, Treatment F=16.65 p=0.0035, Genotype F=0.5529 p=0.4784, TWO WAY-ANOVA). IL1B (Interaction F=0.2714 p=0.6165, Treatment F=16.27 p=0.0038, Genotype F=0.000797 p=0.9782, TWO WAY-ANOVA). IL12 (Interaction F=0.000342 p=0.9857, Treatment F=29.39 p=0.0006, Genotype F=0.0000021 p=0.9989, TWO WAY-ANOVA). IL4 (Interaction F=1.713 p=0.227, Treatment F=8.89 p=0.0176, Genotype F=0.132 p=0.7258, TWO WAY-ANOVA).

Figure 4.3 IL6 secretion in WT, CB2KO, YAC128, and combined YAC128 and CB2KO mice.

Increased IL6 secretion from CB2KO/YAC128 microglia compared to YAC128, CB2KO, and WT microglia. Microglia were stimulated with IFNγ 1 hour prior to addition of CSE. The supernatant was collected 30 hours after CSE stimulation (n=5). The increased IL6 secretion from CB2KO/YAC128 microglia is not an additive affect of CB2KO with YAC128 as there is no significant difference in IL6 secretion between WT and CB2KO microglia. Data were assessed by one-way ANOVA followed by a Newman-Keuls post-hoc test.
4.3.2 Reduction of mHTT in BAC-Cre Primary Microglia ex vivo Normalizes Microglial Cytokine Release.

Next I wanted to test the effect of knocking down huntingtin and determine the effect on both the microglial secretion phenotype and the progression of HD. To do this I crossed BACHD mice with Lysozyme driven cre expression. Firstly I quantified the knockdown of mHTT in BACHD microglia compared to BACCre microglia by western blot (Figure 4.4A) and fluorescence resonance emission technology or FRET (Figure 4.4B). BACHD microglia cells stimulated for one hour with IFNγ and CSE show an increase in IL6 secretion compared to WT cells, reduction of microglial mHTT decreases the extracellular IL6 levels of BACCre mice to those of WT microglia.
Figure 4.4 Reduction of mHTT ameliorates the enhanced cytokine secretion of primary microglia expressing mHTT.

Immunoblot of huntingtin demonstrated a decrease in mHTT levels in BACCre compared to BACHD, with no overall changes in levels of Hdh, calnexin was used as the loading control (Figure 4.4A), Graphed quantification of total huntingtin levels also demonstrate a decrease in the levels of Hdh in BACCre compared to BACHD (Figure 4.4B). BACHD stimulated for one hour with IFNγ and CSE show an increase in IL6 compared to WT. Microglial specific reduction of mHTT (BACCre mice) decreases the extracellular IL6 levels to those of WT or WTCre (Figure 4.4B) (Two-way ANOVA, Cre p>0.0001, Genotype p>0.0001, Interaction p>0.0001).
4.3.3 Alterations in Microglia in vivo with Expression of Lysozyme Driven Cre.

Under normal resting and surveillance conditions, microglia display a spindle shaped ramified morphology, but when activated microglia, they contract their processes and transform into larger, ameboid-shaped cells that migrate to the site of injury (Hanisch and Kettenmann 2007, Moller 2010). Previous work assessed microglial cell numbers in WT and YAC128 mice in basal conditions and following peripheral LPS injections (Fransciosi et al., 2012). No changes in the motor or neuropathologic phenotype were identified in YAC128 mice in response to LPS stimulated activation of microglia. We used the same methodology to test the effect of reducing microglial huntingtin, firstly, I quantified the number of microglia in WT, BACHD, Cre, and BACCre animals, detected finding no change in overall microglia number. Additionally, I detected no difference in microglia process number between WT and BACHD mice, reducing the levels of microglial huntingtin had no effect; however, alterations in process number were identified in the presence of Cre expression (Figure 4.5). Lastly, I assessed microglia cell volume and cell area, and found that reducing the levels of microglial huntingtin had no effect, any change was solely in the presence of Cre expression (Figure 4.6).
No changes in microglial cell counts were identified between genotypes. Reduction in mHTT had no effect on processes per cell.

No changes in microglial cell counts were observed with or without the presence of mHTT (Figure 4.5A) (Two-way Anova, Interacyion 0.2963, Cre 0.2750, BAC 0.0683). No effect of reducing mHTT in processes per cell, however, a decrease in number of processes in the presence of Cre was evident (Figure 4.5B) (Two-way Anova, Interaction 0.0166, Cre < 0.0001, BAC 0.9701)
No effect of reducing mHTT in cell area, however, a decrease in cell area in the presence of Cre was evident (Figure 4.6A) (Interaction 0.2637, Cre<0.0001, BAC 0.5956). No effect of reducing mHTT in microglial cell volume, however, a decrease in microglial cell volume in the presence of Cre was evident (Figure 4.6B) (Interaction 0.2772, Cre<0.00001, BAC 0.05789).
4.3.4 Mild effect of mHTT Deletion in Microglia on the BACHD Phenotype

Next, I wanted to assess the effect of mHTT on motor phenotype, using rotarod ability as readout. As previously described (Pouladi et al., 2013), BACHD mice aged 7-12 months perform significantly worse in this assay, as seen in YAC128 mice. I found that monocyte-specific reduction of mHTT led to marginal improvement of performance in BACCre mice compared to BACHD mice (Figure 4.7).

BACHD and YAC128 mice are also hypoactive as assessed with the open field trial assay. Using this assay I confirmed that BACHD mice move significantly less than WT mice (total distance covered in a 10 minute open field trial); however, this knockdown of monocyte-specific mHTT did not cause any change in this phenotype (Figure 4.8).

Next, I assessed brain weight changes, another hallmark of HD in mouse models. Both BACHD and YAC128 mice display decreased whole brain and forebrain weight. However, monocyte-specific reduction of mHTT in BACCre mice did not reverse brain weight differences (Figure 4.9). I further looked at neuropathology, specifically striatal and cortical volumes. Striatal, but not cortical volume was significantly reduced in BACHD mice. Again, however, microglial deletion of mHTT in BACCre mice did not reverse the striatal volume loss seen in these mice (Figure 4.10).
Figure 4.7 BACHD mice display decreased performance on the Rotarod that is improved by microglial deletion of BACHD.

BACHD mice perform significantly worse on the rotarod from 7-12 months of age. Microglial deletion of mHTT leads to a moderate improvement in rotarod performance (Two-way ANOVA Time $p=0.001$, Genotype $p=0.0001$, Interaction $p=0.6221$)
Figure 4.8 No alteration in hypoactivity detected with reduction of mHTT in immune cells display a hypoactive phenotype in the open field.

BACHD mice cover significantly less distance in a 10 min open field trial compared to WT mice. Hypoactivity is not reversed by microglial deletion of mHTT in BACCre mice. (Two-way ANOVA BAC Transgene p= 0.0029, Cre p=0.819, Interaction p=0.9057)
Figure 4.9 No effect of reducing expression of mHTT in monocytes on brain weight.

Wholebrain and forebrain weights are significantly reduced in BACHD mice. Microglial deletion of mHTT in BACCre mice does not reverse brain weight differences observed in these mice. (Wholebrain: Two-way ANOVA BAC transgene p=0.0011, Cre p=0.8467, Interaction p=0.6679 Forebrain: Two-way ANOVA BAC transgene p=0.0001, Cre p=0.9556, Interaction p=0.78 )
Figure 4.10 No effect of reducing expression of mHTT in monocytes on striatal volume.

Striatal but not cortical volume is significantly reduced in BACHD mice. Microglial deletion of mHTT in BACCre mice does not reverse brain weight differences observed in these mice. (Striatum: Two-way ANOVA BAC transgene p=0.001, Cre p=0.7966, Interaction p=0.86)
4.4 Discussion

In this chapter, I utilized the knowledge gained from the previous two chapters to assess whether the immune hyperactivation in microglia expressing mHTT has an effect on HD pathogenesis, i.e. aiming to identify potential effects of this microglial phenotype on HD progression. Overall, I found that repairing the microglial over-secretion phenotype does not alter the disease progression, reducing huntingtin in monocyte cells did not alter the pathogenesis of HD, or alter the microglia assessed in-vivo.

Cannabinoid receptor research has focused on the beneficial (obesity, pain, inflammation) effects of activating the endo-cannabinoid system, the endogenous stimuli and receptors already present in the brain and across the body (Kogan, N. M. 2007). Several cannabinoid receptors exist. The two principal receptors are referred to as CB1 (Cannabinoid receptor 1) and CB2 (Cannabinoid receptor 2), although increasing attention has recently been paid to other cannabinoid receptors like GPR8 or GPR55. CB1 is expressed predominantly in the brain, although some expression has been observed in other tissues. CB2 is expressed in immune cells, and its function in immune responses has been thoroughly investigated (Mazarei et al., 2014; Merighi, S., et al., 2012). Activation of CB2 receptors decreases inflammatory responses (Munro et al., 1993), and enhanced signaling through this pathway prevents artherosclerosis in mice by dampening cytokine secretion (Steffens et al., 2005). While my studies were in progress, Bouchard et al., 2012 studied the effect of CB2KO on HD using the BACHD mice. They found that CB2KO accelerated the motor phenotype in the presence of mHTT, a worsening of HD progression.
Here, I demonstrate CB2KO mice can modulate the immune dysfunction, worsening it in microglial cells containing mHTT. These data corroborate the data presented in a trial of CB2 knockout mice crossed to BACHD mice, where the onset of HD was enhanced, indeed, serum IL6 levels were also increased in the presence of CB2KO (Bouchard et al., 2012). Indeed, CB2 agonists are protective in mouse models of several diseases (Pryce et al., 2003; Zhang et al., 2007; García et al., 2011; Martín-Moreno et al., 2012). CB2 levels are also increased in post-mortem HD brains of humans and HD mice, CB2 deletion exacerbates the disease in another model of HD (Palazuelos et al., 2009). Moreover, administration of a CB2 receptor agonist extended the lifespan and suppressed the motor deficits, synaptic loss, and CNS inflammation in BACHD, whereas a peripherally restricted CB2 receptor antagonist blocked these effects (Bouchard et al., 2012).

In a previous study, mHTT containing peripheral immune cells of R6/2 mice were replaced by bone marrow transplant and this work suggested that peripheral monocytes play a role in HD (Kwan et al., 2012). In this study, R6/2 mice transplanted with bone marrow expressing no mutant huntingtin showed a modest benefit, indicating that inflammation ss at least having disease-altering if not disease-causing effects. Taken together, these studies implicate myeloid cells in HD progression; however, these studies used mice expressing HTT fragments (e.g. R6/2 mice), which are known to have widespread transcriptional changes (Luthi-Carter et al., 2000). The BACHD model was also used to knockdown HTT in specific neuronal populations such as of cortex and striatum (Wang et al., 2014). In this study, expression of mHTT in both cellular regions led to the most severe degeneration, identifying distinct and
overlapping roles of mHTT in HD pathogenesis. The BACHD mouse model contains LOXP sites, I crossed BACHD mice with mice expressing Cre driven by the Lysozyme promoter. Noted that this would delete mHTT in both peripheral and CNS monocytes lineage cells. I found that this knockdown of monocyte specific huntingtin had no effect on the endpoints in this study, however the BACHD immune phenotype (microglial cell number, cells size and volume were unchanged compared to WT) was not detectable not similar to the YAC128 immune phenotype. My study reduced the levels of mHTT in both peripheral and central cells of the monocyte lineage, different to previous studies targeting solely peripheral immune cells. This study contradicts this previous studies, here I have demonstrated no effect of inflammation on the pathogenesis of HD. It is probable that using the BACCre model could result in stronger HTT knockdown in both peripheral and CNS immune cells. Throughout these experiments the immune modulation effect of having an absence of mHTT in monocyte cells is minimal in the BAC HD model. Though this model is not an absolute deletion of monocyte mHTT, the reduction of mHTT did completely ameliorate the secretion phenotype I was using as a primary endpoint of these inflammation studies (Chapters 2 and 3).

Cre expression in microglial cells did have multiple effects on the microglial-specific end points cell processes, cell area and cell volume. Taken together these do not necessarily demonstrate a more or less activated cell as previously described in the literature (Hovens et al., 2014).. It is evident from my experiments that Cre expression alone is altering these cells in a non-specific manner. Similar Cre effects have been demonstrated in the brain, e.g. nestin-driven Cre causes metabolic
alterations (Harno et al., 2013; Qiu et al., 2011). This demonstrates the importance of using Cre expressing WT mice as controls, which allows us to conclude that although Cre is affecting these cells, the deletion of monocytic mHTT in BACCre mice does not effect the immune alterations. Using this approach, we were unable to detect the alterations that have previously described in the YAC128 model of HD in BACHD mice, both microglial size and primary process number data (Fransciosi et al., 2012). I conclude that the microglial or neuroinflammatory phenotype is not present in BACHD mice to the same extent as YAC128 mice. However the cytokine secretion phenotype was present in both YAC128 and BACHD mice, I have demonstrated that the intrinsic secretion phenotype does not impact the progression of HD in BACHD mice.

Future directions of this research include GFAP specific mHTT knockout, which would delete mHTT from astrocytes and allow determining the roles of these cells on the progression of HD. This model could be used to understand the roles of cellular crosstalk and perturbations in this system in the presence or absence of mHTT. Systems like this where specific mHTT reduction in different subsets of cells will allow assessing the role of each cell type on MSN degeneration in HD.

Studies using astrocyte or oligodendrocyte-specific HTT deletion, need to be conducted to more fully understand the role of the immune system on HD progression. My study does not necessarily imply that microglia are not involved in HD, but rather shows that reducing monocyte mHTT in BACHD mice did not have a large effect on phenotypes investigated here.
5. Discussion

5.1 Study Objectives

At the start of my thesis, I set out to study three objectives: 1) to characterize the enhanced immune phenotype in primary microglia expressing full-length mutant human HTT, 2) to modulate the enhanced immune phenotype in microglia containing mHTT, and 3) to determine whether the enhanced immune phenotype in microglia affects the progression of HD in relevant mouse models. The overarching purpose of these objectives was to better understand the role of microglial dysfunction and neuroinflammation caused by mHTT. Ultimately, knowledge gained from such a study might assist the implementation of translational research aimed at curing or alleviating the symptoms of HD. Here, I further discuss the findings of my study, as well as future directions for this work.

Firstly, I will address the concerns that the ex vivo model of inflammation used for the experiments presented in Chapter 2 may not fully reflect in vivo interactions of these cells in the intact brain. The primary readout I used for my ex vivo studies, IL6 modulation, was chosen from a previous study (Bjorqvist et al., 2008) and was selected not because of the effect that it has in HD, but because it was successfully used to illustrate immune alterations in HD (Bjorkqvist et al., 2008; Trager et al., 2014).
Previous work on inflammation in HD primarily used imaging methods to assess microgliosis in brains of HD patients (Pavese et al., 2006; Tai et al., 2007). Additionally, some studies in mice suggested that the immune system was altered in the presence of mHTT, although a number of these studies utilized “fragment models” of HD that display widespread transcriptional alterations not seen in HD patients (Bjorkqvist et al., 2008; Trager et al., 2014).

The first demonstration of microglial activation in HD by Sapp et al., was based on morphological changes; reactive microglia were evident in all grades of HD pathology, and the number of immunostained microglia increased with increasing grades. Microglial morphology was also increased in the striatum and cortex. Additionally, PET imaging studies demonstrated microglial activation in HD as discussed in the introduction chapter. Along with data obtained in the YAC128 mouse model, these data contrast with the data I obtained in Chapter 4. Specifically, I did not find any immunocytochemical or morphological evidence for an altered microglial phenotype in the brains of BACHD mice, unlike the changes previously outlined in brains of aged YAC128 (Fransciosi et al., 2012). My work here builds on previous work in the Leavitt lab that identified alterations in YAC128 mice (Bjorkqvist et al., 2008), such as changes in microglial cell numbers and cell size in vivo as well as BBB changes (see introduction). Instead of studying mice in vivo, I studied isolated microglia expressing mHTT, aiming to identify how mHTT might alter microglia function. This ex vivo model allows examining microglia isolated at an age preceding the onset of HD symptoms. This is an important aspect of the ex vivo system, as it
suggests that mHTT can drive alterations in glial and neuronal cells prior to any neurodegeneration due to the presence of mHTT.

Altered cytokine levels occur in both human HD patients and in mouse models of HD. For example, Silverstroni et al., 2009 demonstrated an altered inflammatory profile from HD post-mortem brain tissue (Silverstroni et al., 2009). IL1B and TNF-a were increased in striatum, consistent with microglial activation (Silverstroni et al., 2009). Similarly, alterations in cytokines from activated mouse and human monocytes containing mHTT (Bjorkqvist et al., 2012). In my thesis, I used exacerbated cytokine release as an indication of inflammation and modulated it in vivo to determine its effect on HD progression in the BACHD mouse model.

I used mice expressing full-length human HTT, but as with any experiment, caveats exist for these models. Specifically, all mice I used express both endogenous WT mouse and human mHTT. Expression of Cre in these cells did have an effect on some of the endpoints both in the presence and absence of mHTT. Endpoints of this study were to determine the effect on the progression of HD, and my endpoints were not specifically tailored towards assessing inflammation onset or inflammation-driven neurodegeneration throughout the progression of HD.

Previous work using the R6/2 model demonstrated that myeloid cell modulation using a peripheral KMO inhibitor improved the HD-like phenotypes and prevented neurodegeneration (Zwilling et al., 2011). This study modulated the peripheral kynurinine pathway, which resulted in changes in mice IBa1 levels in vivo. The KMO study implicates the kynurinine pathway as a mechanism in the progression of HD.
However previous studies knocking out IDO, the rate-limiting enzyme of this pathway, resulted in only modest alterations in the progression of the HD phenotype of YAC128 mice (Zwillling et al., 2011). Knockdown of mHTT in peripheral immune cells of the R6/2 by bone marrow transplant studies suggested that peripheral monocytes play a role in HD (Kwan et al., 2012), as this study showed a reversal of the adverse phenotypes of R6/2 mice. Taken together, these studies implicate myeloid cells as central contributors to HD progression. However, these studies both examined “fragment models” of HD, such as R6/2 mice, which display widespread transcriptional changes (Luthi-Carter et al., 2000). These studies discussed above are examples of research that contradicts both other studies and the work laid out in this thesis, the monocyte lineage reduction of mHTT is a complete study with large numbers, clearly demonstrating no effect of mHTT reduction on the progression of HD in BACHD mice.

During my research, I decreased mHTT expression in microglia of BACHD mice using lysozyme promoter-driven Cre expression. Utilizing the R6/2 and Hdh171 mouse models, microglia-specific mHTT expression promoted cell autonomous pro-inflammatory signaling through transcriptional activation (Crotti et al., 2014). Mechanistically, the myeloid lineage-determining factors PU.1 and C/EBPs were driving these changes in genome transcription. There findings suggest a cell-autonomous mechanism for microglia reactivity, which in turn may alter cell non-autonomous HD pathogenesis. Other in vitro work found altered NF-kB signalling in microglia from HD mice (Hsiao et al., 2013) and human cells (Trager et al., 2014).

As outlined in chapter 4, there are other caveats in relation to Cre expression in microglia on the phenotype and characterization of the cells. This model may have
had an effect on the endpoints that we observed. Data presented in this thesis indicate that the immune alterations, microglial cell size, and primary projection are not altered in the BACHD mouse model, different to what was demonstrated in YAC128 mice (Bjorqvist et al., 2012). Both BACHD and YAC128 share a number of phenotypic changes in relation to HD; however, they have different insertion sites, number of insertions and expression levels could be the determining factor of altered microglial cells in the presence of mHTT.

5.2 Cre expression as a Model for mHTT reduction

The model I used to reduce HTT levels in myeloid cells (macrophage and microglia) was previously used to study IKKβ depletion in AD (Saura et al., 2003). In any disease state, compensatory changes may occur, and it is probable that there is some level of compensation in the HD models used here. To identify potential compensation mechanisms, it would be interesting to examine where mHTT is active in conditionally expressing mice; perhaps, a nestin-Cre crossed to BACHD (reducing huntingtin in astrocytes and neurons) would lead to microglia being the major support cell in the brain expressing mHTT. Such a model would address whether microglia have a negative effect on neurodegeneration. Potentially, these mice would perform like WT mice because microglia potentially have no input on the progression of HD, as explained by my data; alternatively, they might have mild HD phenotypes due to microglial expression of mHTT in brain. My model demonstrates an effect that occurs
very early in the development prior to the onset of HD-like symptoms. This suggests that mHTT expression can have an effect on the development of astrocytes and neurons in the brain potentially influencing the pathogenesis of the disease. Additional compensatory mechanisms may exist as astrocytes, neurons, and microglia may combinatorially combat the effects of mHTT. Astrocytes are the other major support cells in the brain. It is conceivable that they are at least as, if not more so, than microglia. Previously described astrocyte-specific mHTT expression demonstrated a HD-like progression (Bradford et al., 2009); this was a HTT “fragment model", often described to have transcriptional dysfunction not seen in human patients (Luthi-Carter et al., 2000).

5.3 HTT Post-translational Modification

In this thesis, I assessed mHTT that was natively processed in mouse microglial cells; I have not investigated the effect of the protein state, full-length, cleaved fragments or mHTT inclusions, it is unknown how mHTT is processed in a human microglia cell, this may be altered compared to mouse mHTT. mHTT can be processed in several different ways, some of which may be activated by inflammation (Qin, Z.H. & Gu, Z.L. 2004). HTT can be cleaved by proteases, including caspases (Graham et al., 2006; Wellington et al., 2002). It is still unclear whether HTT cleavage is required in microglia for the observed enhanced immune response. Some studies
indicate that HTT processing and/or modification can have an effect on HD (Steffan et al., 2004; Arrasate et al., 2012).

It is conceivable that in the model of HTT studied here, we are unable to detect the benefit of reducing inflammation, potentially because partial reduction of HTT causes imbalances in HTT protein fragments. Added weight to this argument comes from the R6/2 studies on which demonstrated altered immune profiles in the past that prevent progression by reducing mHTT (Kwan et al., 2012). Although the enhanced microglial phenotype was ameliorated by knockdown, or perhaps other actions of the microglia, could be altered due to the presence of mHTT in astrocytes and neurons.

5.4 Function of WT HTT in Microglia and Inflammation

In my thesis, I demonstrated that mHTT expression causes alterations in cytokine secretion from microglia ex vivo. I also conducted experiments in microglia from YAC18 mice that that over-express human WT HTT and found no effect on cytokine release. However, WT mouse HTT and even WT human HTT could have a role in inflammation. This would be particularly relevant to pan-HTT reduction studies. Perhaps, there is no alteration in inflammation with increased human HTT expression, whereas limiting the amount of endogenous HTT in cells might have an effect. Wolf-Hirschhorn syndrome occurs is a chromosomal deletion syndrome; individuals with this syndrome often have only one functional copy of the HTT gene (Battaglia et al.,
The length of the deletion can vary and this deletion also leads to the deletion of other genes, and only one report of any inflammatory phenotypes has yet been reported, the presence of an Inflammatory Myofibroblastic Tumor in the bladder (Marte et al., 2013).

My thesis explores the alterations in microglia expressing full-length mHTT, and demonstrates that these cells have an altered cytokine secretion phenotype. I further investigated whether this phenotype impacted HD progression by reducing mHTT levels in monocytes, ameliorating the phenotypes. Limiting mHTT expression in monocytes should have a greater effect than limiting HTT in microglial cells. Inflammation genes are increased in the polyglutamine expansion disease SCA3 (Kieling et al., 2007). This may indicate some role for polyglutamine expansion in immune dysfunction. Indeed, the data I presented here suggests that inflammation in HD is driven by intrinsic mechanisms of monocytes, but overall playing a relatively small role in HD progression.

The research presented here fills a void in the HD field and highlights the need to address the specific effects of mHTT in other cell types and to assess whether these alterations impact the progression of HD. Indeed, in another mouse model of HD or in humans with HD, immune dysfunction may play a greater role in the progression of the disease than in the BACHD mouse model I used. As mentioned previously BACHD mice differ from YAC128 mice, nuclear inclusions and mRNA changes of key striatal enriched genes are unchanged. In my studies, I addressed whether microglial mHTT influences HD progression in this specific full-length mouse model of HD. It remains possible that mHTT knockdown in astrocytes, neurons, or
oligodendrocytes, or a combination thereof, might lead to decreased microglial cytokine hypersecretion. In sum, our understanding of specific impact of each neuronal cell type on the selective degeneration of MSNs in HD remains incomplete. Nevertheless, the experiments presented here improve our understanding of the roles of microglia and monocytes in HD.

5.5 Implications for Huntington’s disease Research

Although the defined genetics of HD initially suggested that pathogenesis would be relatively easy to understand, the critical mechanism of action of mHTT protein remain obscure even now, 20 years after the discovery of the gene. Most current HD therapies were developed to target the symptoms of the disease. The HTT protein is thought to be involved in a plethora of cellular functions, such as vesicle transport and immune cell migration (Kwan et al., 2012) (Morton et al., 2001), and is essential for development, as knocking out the gene is embryonic lethal in mice (Dragatsis 1998).

Recent studies have focussed on transneural propagation of mHTT and how this may contribute to non-cell autonomous pathology (Pech-Vrieseling et al., 2014). Evidence from drosophila studies indicate mHTT transfer directly between neuronal cells (Babcock & Ganetzky 2015). These important findings may have implications on
HD research in the future, it is probably that mHTT transmission through microglia may play a role in the pathogenesis of HD.

Over the last five years, ASOs have been developed as a potential cure for HD (Kordasiewicz et al., 2012), representing a promising therapeutic approach. However, ASOs are non-specific way, leading to the reduction of both WT and mHTT. It is imperative that we understand the mechanism of cellular dysfunction in HD as well as understanding the causal changes that affect the disease progression. Additionally, we must also identify the alterations that do not change disease progression.

5. Concluding Statements

I have successfully accomplished the objectives set out at the beginning of my thesis. The resulting body of work has highlighted one potential mechanism thought to be involved in HD. I have demonstrated that at least some mouse models expressing full-length mHTT show an enhanced immune response in microglia. Building on this discovery, I explored whether modulating this phenotype with various therapeutic agents could lead to an ex vivo model, which in turn might be useful in screens for novel therapeutic HD agents. Finally, I explored the role of monocyte mHTT plays in the phenotypic progression, using the BACHD mouse model of HD. This research explores the concept of inflammation in HD, although this does not completely rule out inflammation in HD as outlined in the discussion. This is strong evidence that the cell-
intrinsic dysfunction caused by mHTT expression in monocytic cells does not directly contribute to HD progression in BACHD mice.
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