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Investigating the Role of Physiological Systems Outside the Central Nervous System on Huntington's Disease Pathogenesis

by

Jill Larimore

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Jill Larimore

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For my mom and dad, who offered me unconditional support

over the years and who both continue to inspire

me to pursue my dreams.

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Author Contributions

Jill Larimore and Jennifer Truong performed the mouse behavioral and genetic experiments in chapter 2. Data was analyzed by Jill Larimore using matlab code written by Kristofer Bouchard. Paul Muchowski directed and supervised the research. Jill Larimore wrote this chapter.

Jill Larimore and Jennifer Truong performed the mouse behavioral and genetic experiments in chapter 2.1. Both Jill Larimore and Kristofer Bouchard using matlab code written by Kristofer Bouchard analyzed data. Jill Larimore wrote this chapter.

Chapter 3 of this dissertation will be published on December 12, 2012 in the Journal of Neuroscience. Jill Larimore and Jennifer Truong performed all behavior and pharmacological drug study experiments. Diana Dunkelberger assisted with sectioning brains for neuropathological analysis and Jill Larimore performed all neuropathology and analysis, and quantitative PCR studies. Saliha Moussaoui and Sandrine Desrayaud performed pharmacokinetic analysis for the mouse drug studies. Paul Muchowski directed and supervised the research. Nephi Stella, Kristofer Bouchard and Sarah J. Tabrizi both assisted in editing this chapter. Paul Muchowski and Jill Larimore co-wrote this chapter together.

Abstract

Investigating the Role of Physiological Systems Outside the Central Nervous System on Huntington's Disease Pathogenesis

By Jill Larimore

Huntington's disease (HD) is a devastating neurodegenerative disease for which there is no cure. Classically, HD is thought to result from central nervous system (CNS) dysfunction because its hallmark pathology is neurodegeneration of brain regions important for controlling movement. However, co-occurring pathologies, including severe weight loss and peripheral immune system activation, might contribute to HD progression. Given that the mutant Huntingtin protein (mHtt) is ubiquitously expressed and directly affects cells outside the CNS, an integrated understanding of mHtt-induced pathogenesis is needed. In this dissertation, I sought to understand how both central nervous and peripheral physiological systems contribute to HD pathogenesis by taking advantage of the endocannabinoid system, in which the two main cannabinoid receptors, CB_1 and CB_2, have distinct roles. In chapter 2, I investigated the role of CB_1, which is primarily expressed in the CNS and whose expression is one of the earliest genetic changes to occur in HD-vulnerable neurons, on HD progression. We found that early genetic deletion of CB_1 receptors in R6/2 mice accelerated the onset of symptoms and further decreased lifespan compared to CB_1-receptor-expressing R6/2 mice, suggesting CB_1 plays a functional role in HD progression. In chapter 2.1, I investigated the effects

of animal weight on HD progression in BACHD mice, an overweight HD model. Specifically, using ANCOVA as a robust statistical tool, I found that changes in weight induced by deletion of CB_1 expression significantly influenced HD behavior. In chapter 3, we investigated the peripheral immune system, showing that genetic deletion of CB_2 in a slow-progressing HD mouse model accelerated the onset and increased the severity of motor deficits. Treatment of mice with a CB_2-receptor agonist extended lifespan and suppressed motor deficits, synapse loss, and CNS inflammation, while a peripherally restricted CB_2-receptor antagonist blocked these effects. CB_2 regulates blood interleukin-6 levels, and interleukin-6-neutralizing antibodies partially rescued motor deficits and weight loss in HD mice. These findings support a causal link between CB_2-receptor signaling in peripheral immune cells and the onset and severity of HD-induced neurodegeneration. Together, these results demonstrate that mHtt can significantly impact peripheral physiological systems, and identify novel therapeutic approaches for treating HD.

TABLE OF CONTENTS

Title Page……..…i

Copyright Page…….…...ii

Dedication……...iii

Acknowledgements…….iv

Author Contributions……..vi

Abstract…….....vii

List of Tables and Figures……...…...xi

Chapter 1: Introduction and Background…….….1

Chapter 2: Analysis of genetic loss of the CB_1 receptor in HD mouse models

Abstract……..10

Introduction……12

Results……15

Discussion……..19

Materials and Methods……...24

Chapter 2.1: Weight gain in BACHD mice is a major contributor to rotarod motor deficits

Abstract……..……27

Introduction……..……..29

Results……....31

Discussion……..…38

Materials and Methods……...……43

Chapter 3: Cannabinoid receptor type 2 signaling in peripheral immune cells modulates disease onset and severity in mouse models of Huntington's disease

Abstract……..44

Introduction……..……..45

Results……..……..47

Discussion……..62

Materials and Methods……...68

Chapter 4: Conclusions……..…74

References……..79

UCSF Library Release……103

LIST OF TABLES AND FIGURES

Chapter 2

Figure 2-1. Genetic deletion of CB_1 receptors accelerates the onset of motor symptoms, death, and weight loss in R6/2 mice……..16

Figure 2-2. Genetic deletion of CB_1 receptors does not exacerbate behavioral phenotypes in BACHD mice…….18

Chapter 2.1

Figure 2.1-1 BACHD mice are impaired on the rotarod at 3 and 6 months of age……..32

Figure 2.1-2 BACHD mice are heavier than littermate controls……..33

Figure 2.1-3 Linear regression trend line for BACHD mice……34

Figure 2.1-4 Combined regression trend lines for all genotypes……..35

Table 2.1. Comparison of statistical outcomes of behavioral performances on the rotarod for different genotypes……...38

Chapter 3

Figure 3-1. Genetic deletion of CB_2 receptors accelerates the onset of motor symptoms and increases their severity in a slowly progressing mouse model of HD……49

Figure 3-2. CB_2 mRNA and the CB_2 receptor antagonist SR2 are detected at low levels in brain tissue from HD mouse models……..51

Figure 3-3. Enhanced CB_2 receptor signaling in peripheral immune cells suppresses behavioral deficits and increases survival in R6/2 mice……54

Figure 3-4. Enhanced CB_2 receptor signaling in peripheral immune cells suppresses neurodegeneration and reduces CNS inflammation in R6/2 mice…….57

Figure 3-5. CB_2 receptor signaling in peripheral immune cells suppresses neurodegeneration and CNS inflammation in late stage R6/2 mice……..59

Figure 3-6. CB_1 receptors control IL-6 levels, which contribute to disease phenotypes in R6/2 mice……...61

Chapter 1: Introduction

Huntington's disease (HD) is a devastating genetic neurodegenerative disease that affects 5–10 of every 100,000 individuals in the developed world (Bates et al., 2002)**.** It is inherited in an autosomal dominant fashion, whereby children born to an HD individual have a 50/50 chance of developing the disease. The major neurological symptoms of HD include irrepressible, repetitive dance-like movements known as chorea, psychiatric disturbances, and cognitive impairments that are usually preceded by dementia (Martin and Gusella, 1986). The onset of symptoms usually occurs between 35–50 years of age, and death typically ensues 15 years after the initial presentation of symptoms (Menalled and Chesselet, 2002). HD is caused by a mutation in the *IT-15* gene, in which an expanded CAG tract yields a polyglutamine stretch in the protein product of this gene, known as Huntingtin (Htt) (Group, 1993). There is no cure or effective therapeutics to stop disease progression.

Importantly, mutant Htt (mHtt) is expressed in every cell in the body (Li et al., 1993), yet an integrated understanding of mHtt-induced pathogenesis across physiological systems is lacking. In the central nervous system (CNS), the major neuropathological features of HD are found in the basal ganglia, namely the neurodegeneration of striatal medium spiny neurons (Vonsattel et al., 1985), which are important for initiating and terminating movement (Albin et al., 1989). For this reason, HD has classically been considered to be a disease caused by CNS dysfunction. However, numerous studies have documented direct effects of mHtt on many cell types outside the CNS (van der Burg et al., 2009). For instance, the most common peripheral pathology is severe weight loss that cannot be explained by decreased food intake or an increase in movement (Sanberg et al.,

1981; Djousse et al., 2002; van der Burg et al., 2009), but instead by an increased metabolic rate (Seong et al., 2005; Aziz et al., 2008). Other commonly observed peripheral symptoms include skeletal muscle atrophy (Hamilton et al., 2004; Turner et al., 2007) and cardiac dysfunction (the second leading cause of HD deaths) (Chiu and Alexander, 1982). Interestingly, in blood from HD patients, there is widespread activation of the innate immune system, which can be detected years before disease onset (Leblhuber et al., 1998; Dalrymple et al., 2007; Bjorkqvist et al., 2008; Wild et al., 2011). Whether altered metabolism or inflammatory responses contribute to the clinical manifestations of HD, such as weight loss and skeletal muscle atrophy or even CNS pathologies, is poorly understood. At the very least, these studies suggest that understanding the contributions of other physiological systems will be important for understanding HD pathogenesis. Perhaps more importantly, targeting small molecules to cells outside the CNS, which is easier than cells within the CNS, could represent a therapeutic advantage for HD patients.

These general observations give rise to the central hypothesis of my thesis:

<u>Physiological systems outside the CNS contribute to clinical manifestations of HD.</u>

The endocannabinoid system mediates a number of important physiological processes throughout the CNS, the peripheral immune system, and the metabolic system. In the brain, engaging the endocannabinoid system in the basal ganglia leads to inhibitory effects on movement through modulation of neurotransmitter release (Kreitzer and

Malenka, 2007)**.** These effects are mediated through the cannabinoid receptor type-1 (CB_1). Endocannabinoids and CB_1 receptors are also involved in energy homeostasis through actions on both peripheral and central physiological systems (Gomez et al., 2002; Quarta et al., 2010). Importantly, the loss of CB_1 receptors in HD vulnerable brain regions is one of the earliest effects of mHtt on gene transcription (Glass et al., 1993; Richfield and Herkenham, 1994; Van Laere et al., 2010), suggesting that CB_1 receptors may be an early pathophysiological event.

In the peripheral immune system, endocannabinoids act through the cannabinoid receptor type-2 (CB_2) to dampen inflammatory responses by reducing pro-inflammatory cytokine levels (Munro et al., 1993; Ashton and Glass, 2007), especially those that are elevated in HD (Bjorkqvist et al., 2008). A recent study showed that global genetic loss of CB_2 worsens HD progression (Palazuelos et al., 2009), supporting the hypothesis that inflammation is functionally important in HD. However, these studies did not assess the contribution of CB_2 within the peripheral immune cells where it is highly expressed (compared to the brain, where CB_2 is almost undetectable).

The restricted spatial expression patterns of CB_1 and CB_2 are highly advantageous for investigating their individual contributions to HD progression within the CNS, metabolic, and peripheral immune systems. To investigate these issues, my dissertation consisted of 3 chapters, each addressing a different question raised by my hypothesis that physiological systems outside the CNS contribute to clinical manifestations of HD:

Chapter 2. Does the loss of CB_1 receptor expression affect the progression of HD within the CNS?

Chapter 2.1. How does weight contribute to the HD phenotype?

Chapter 3. Does the peripheral immune system have a role in HD progression?

Summary of Chapters

In chapter 2, we investigated the role of CB_1, which is primarily expressed in the CNS, in HD. To determine whether the loss of CB_1 expression would result in earlier and more severe HD symptoms, we generated HD mice in which CB_1 was genetically deleted. We found that genetic deletion of CB_1 receptors in R6/2 HD mice accelerated the onset of symptoms and further decreased lifespan compared to CB_1 receptor–expressing R6/2 HD mice. Surprisingly, these effects were not observed in BACHD mice, a comparatively slow-progressing HD mouse model. Rather, we found that BACHD mice, which tend to be overweight/obese relative to R6/2 and wildtype mice, were significantly thinner in the absence of CB_1. The results using the R6/2 mice support previous work showing that CB_1 loss in the striatum is a critical event mediated by mHtt in the CNS. In the BACHD studies, loss of CB_1 does not further exacerbate the HD phenotype, an effect that may be masked due to the presence of the covariate, weight, which is investigated in chapter 2.1 of this thesis.

In chapter 2.1, we show that BACHD mice are significantly heavier than wildtype mice and that, across different genotypes, there is a significant effect of animal weight on behavior. Using an analysis of covariance (ANCOVA), we show that after statistically

removing the effect of weight on performance, the behavioral deficits associated with BACHD mice on the rotarod are less severe and, in some cases, no longer significantly different from controls. Our results highlight the importance of accounting for animal weight on behavioral performance, and provide a statistical tool (ANCOVA) that can do so. More importantly, they demonstrate that mHtt can significantly impact physiological systems outside the CNS, suggesting that major clinical manifestations of HD may involve systems other than the CNS.

In chapter 3 we investigated a third physiological system—the peripheral immune system—using genetic and pharmacological manipulations. We show that the genetic deletion of CB_2 receptors in a slow-progressing HD mouse model accelerates the onset of motor deficits and increases their severity. Treatment of mice with a CB_2 receptor agonist extends lifespan and suppresses motor deficits, synapse loss, and CNS inflammation, while a peripherally restricted CB_2 receptor antagonist blocks these effects. CB_2 receptors regulate blood interleukin-6 (IL-6) levels, and IL-6 neutralizing antibodies partially rescue motor deficits and weight loss in HD mice. These findings support a causal link between CB_2 receptor signaling in peripheral immune cells and the onset and severity of neurodegeneration in HD, and they provide a novel therapeutic approach to treat HD.

Background

Huntington's Disease Mouse Models

With the identification of mutant huntingtin (mHtt) as the causative mutation responsible for HD, numerous mouse models have been created to study HD-related pathology (Menalled et al., 2009). The two transgenic HD mouse models used throughout this dissertation are R6/2 and BACHD mice. R6/2 mice (Mangiarini et al., 1996), which contain a 1.9kb human genomic fragment including the mHtt exon-1 (150 CAG repeats) with endogenous promoter sequences, and BACHD mice (Gray et al., 2008), expressing a 170kb human htt locus with exon-1 (97 CAG repeats) under control of endogenous htt regulatory machinery, develop different aspects of HD pathology. R6/2 mice develop a very rapid and robust set of neurological and neuropathological symptoms, including motor and cognitive dysfunction, inclusion bodies (aggregates of mHtt), neuronal loss, severe weight loss, and muscle atrophy (Mangiarini et al., 1996) (Li et al., 2005) (She et al., 2011). For these reasons, the R6/2 mouse model is commonly used to test potential HD therapeutics (Li et al., 2005). In contrast, BACHD mice have a slower disease progression, characterized by progressive motor and cognitive deficits, and exhibit pathology closer to that of the late stage of HD because of the longer time course and more selective neuropathology (Gray et al., 2008). Thus, because these two models exhibit complementary aspects of HD pathology, together they provide insight into if, and how, physiological systems outside the CNS contribute to HD pathogenesis.

The Endocannabinoid System

The endocannabinoid system (ECS), first discovered as being responsible for mediating the bioactive effects of marijuana, has emerged as a complex, endogenous lipid-signaling system involved in physiological functions throughout the body. Given that the ECS operates in nearly every major tissue it is not surprising that this system plays a role in a variety of different processes, including but not limited to**:** mechanisms underlying hippocampal-mediated memory formation, inhibitory effects on movement through the basal ganglia, central and peripheral analgesia, energy balance and homeostasis, and immunosuppression (Elphick and Egertova, 2001).

The two most common endocannabinoids in the body are 2-arachidonoyl glycerol (2-AG) (Stella et al., 1997) and anandamide (AEA) (Devane et al., 1992). Both 2-AG and AEA are derived from lipids and generated on demand primarily by phospholipase-D enzyme (PLD) and 1,2-diacylglycerol lipase (DAG lipase), respectively. Soon after their generation, endocannabinoids readily diffuse back into the cell through both passive and facilitated diffusion mechanisms. Anandamide is then hydrolyzed into arachidonic acid and ethanolamine by fatty-acid amide hydrolase while 2-AG degradation occurs via the actions of a monoacylglycerol lipase (Piomelli, 2003). Most of our knowledge of endocannabinoid metabolism comes from studies in the CNS, where their generation and release is dependent on neuronal activity. Outside the CNS, endocannabinoids are present in a variety of tissues, including testis, spleen, blood, and gut; however, their generation and release characteristics are not well understood, although they are known to be sensitive to changes in calcium (Felder et al., 1996; Felder and Glass, 1998) and are produced by PLD and DAG lipase (Pacher et al., 2006). The two main cannabinoid

receptors responsible for transducing the effects of endocannabinoids are the cannabinoid receptor type-1 (CB_1) (Matsuda et al., 1990) and cannabinoid receptor type-2 (CB_2) (Munro et al., 1993), both of which are inhibitory G-protein coupled receptors. The downstream cellular effects most often associated with cannabinoid-receptor activation are changes in cAMP levels through inhibition of adenylate cyclase, gene-expression changes through effects on kinase activity (e.g. mitogen-activated proteins), and changes in membrane excitability through changes in the permeability of ion channels, most notably in the brain (Piomelli, 2003).

In the CNS, the CB_1 receptor is expressed at high levels in the basal ganglia, cortex, hippocampus, and cerebellum (Herkenham et al., 1990; Westlake et al., 1994; Glass et al., 1997). Subcellular localization of CB_1 is generally confined to presynaptic axon terminals and/or axons of both excitatory and inhibitory neurons. In the basal ganglia, the region most affected in HD, CB_1 is expressed on presynaptic cortical neurons, and in the striatum, on inhibitory interneurons and the presynaptic terminals of medium spiny neurons (Herkenham, 1991; Herkenham et al., 1991; Hohmann and Herkenham, 2000; Gerdeman and Lovinger, 2001). The endocannabinoid system is best known for regulating synaptic transmission by modulating levels of neurotransmitter release (Freund et al., 2003), an effect attributed to CB_1 in light of its presynaptic localization and ability to modulate calcium and potassium concentrations. The CB_2 receptor, on the other hand, is not normally detected in healthy brains except in a small subset of neurons in the hypothalamus (Van Sickle et al., 2005). The functional role of hypothalamic CB_2 expression is unclear; however, many studies claim that CB_2 is expressed in the brain's

immune cells, microglia, and is important in modulating microglia-mediated inflammation (Carlisle et al., 2002; Ehrhart et al., 2005; Ashton and Glass, 2007).

Outside the CNS, CB_2 receptors are present at high levels in a variety of different immune cells and tissues (Galiegue et al., 1995; Schatz et al., 1997). Among these, CB_2 is expressed at high levels in B cells, natural killer cells, monocytes, macrophages, the tonsils, and in the spleen, while to a lesser extent in T cells and the thymus (Munro et al., 1993). In the peripheral immune system, the ECS exerts immunomodulatory effects through the CB_2 receptor by inhibiting chemotaxis (Miller and Stella, 2008) and decreasing the proinflammatory responses mediated through cytokines . The role of CB_2 in decreasing inflammation is supported by numerous studies utilizing CB_2-/- mice, showing that in a number of diseases where inflammation is a causative factor, the absence of CB_2 leads to a worsening of disease progression (Tschop et al., 2009; Engel et al., 2010; Singh et al., 2012).

Chapter 2. Analysis of Genetic Loss of the CB_1 Receptor in HD Mouse Models

ABSTRACT

In Huntington's disease (HD), it is unclear how mutant Htt, a ubiquitously expressed protein, leads to the selective vulnerability and eventual loss of striatal medium spiny neurons. To investigate this issue, one experimental approach has been to study gene-expression changes in the brains of HD patients and HD mouse models, as many of the dysregulated genes hypothesized to be important in HD progression are highly expressed in the striatum. Among the earliest and most robust gene-expression changes in the HD brain has been in the expression of cannabinoid receptor type 1 (CB_1), an inhibitory G protein–coupled receptor. In the striatum, CB_1 modulates synaptic plasticity and ultimately influences motor coordination. Also, CB_1 receptor activation is known to be neuroprotective and to significantly impede neurodegeneration. However, no behavioral studies have investigated whether the absence of CB_1 expression influences HD progression. To determine whether the loss of CB_1 expression would result in earlier and more severe HD symptoms, we generated HD mice in which CB_1 was genetically deleted. We found that genetic deletion of CB_1 receptors in R6/2 HD mice accelerated the onset of symptoms and further decreased lifespan compared to R6/2 HD mice expressing CB_1 receptors. Surprisingly, these effects were not observed in BACHD mice, a comparatively slow-progressing HD mouse model. Rather, we found that BACHD mice, which tend to be overweight/obese relative to R6/2 and wildtype mice, were

significantly thinner in the absence of CB_1. In light of these confounds, standard statistical analysis of genetic deletion of CB_1 on BACHD mice are inconclusive. Nonetheless, results of CB_1 loss in R6/2 mice are in agreement with the results of previous studies of CB_1 effects in HD mice.

INTRODUCTION

Huntington's disease is a devastating neurodegenerative disease characterized by progressive chorea, cognitive impairments, and emotional disturbances (Martin and Gusella, 1986). While the disease is caused by expansions in the CAG repeat region of the huntingtin (Htt) gene, it is not well understood how this mutation triggers the selective death of medium spiny neurons in the striatum and, to a lesser extent, in other brain regions (Walker, 2007). It is known that mutant Htt (mHtt) protein expression disrupts multiple cellular processes (Nucifora et al., 2001; Lin and Beal, 2006). Of these, alterations of gene expression, illustrated through large screen arrays in both human and mice, are thought to be key to understanding disease pathogenesis (Luthi-Carter et al., 2000; Luthi-Carter et al., 2002; Kuhn et al., 2007; Becanovic et al., 2010; Seredenina and Luthi-Carter, 2012). A thorough understanding of how these gene-expression changes influence disease progression could reveal potential disease-ameliorating therapies.

The endocannabinoid system is dysfunctional in many neurological diseases, including HD (Glass, 2001; Maccarrone et al., 2007). In fact, changes in the endocannabinoid system were among the earliest to be observed in the HD-affected brain. For example, multiple groups have shown a selective loss of CB_1 receptors in the basal ganglia of HD brains across varying Von Sattel grades of progression (Glass et al., 1993; Richfield and Herkenham, 1994) (Vonsattel et al., 1985). More recently, positron emission tomography studies showed that loss of CB_1 receptor expression in the basal ganglia of HD patients preceded the onset of motor symptoms (Van Laere et al., 2010). Furthermore, autoradiography and quantitative PCR studies have found that mRNA and protein levels of the CB_1 receptor are significantly decreased in many HD transgenic

mouse models (Denovan-Wright and Robertson, 2000; Lastres-Becker et al., 2002b; Lastres-Becker et al., 2002a; Naver et al., 2003; Glass et al., 2004; McCaw et al., 2004; Dowie et al., 2009). These results provide correlative evidence that dysregulation of CB_1 is a key molecular pathway through which mHtt exerts its disruptive behavioral effects.

In the central nervous system, the endocannabinoid system (ECS) is important in pain modulation (Hohmann and Suplita, 2006), motor control (Kreitzer and Malenka, 2007, 2008), and is responsible for the mood-altering effects of THC (Matsuda et al., 1990), the active ingredient in marijuana. This system consists of two main endocannabinoids, 2-arachidonoyl glycerol and anandamide, both of which are generated on demand and activate the two primary cannabinoid receptors, CB_1 and CB_2. There are also associated enzymes, such as fatty acid amide hydrolase and monoacylglycerol lipase, which are important for the degradation and breakdown of cannabinoids (Piomelli, 2003). While CB_2 is expressed at high levels in the peripheral immune cells (Munro et al., 1993; Galiegue et al., 1995), the CB_1 receptor is the most widely expressed G protein–coupled receptor in the brain, with high levels of expression detected in cortical projection neurons, medium spiny neurons, and 'fast-spiking' interneurons of the basal ganglia (Herkenham, 1991; Herkenham et al., 1991; Hohmann and Herkenham, 2000; Gerdeman and Lovinger, 2001). At the cellular level, activation of the CB_1 receptor, via retrograde signaling of cannabinoids, modulates synaptic transmission by reducing neurotransmitter release (Wilson and Nicoll, 2001) (Freund et al., 2003). Alternatively, studies show that CB1 receptor activity promotes the release and activity of BDNF (Khaspekov et al., 2004; Butovsky et al., 2005), a neurotrophic factor important in plasticity and survival of striatal medium spiny neurons (Ivkovic et al., 1997). Therefore, reduction of CB1 could

result in increased synaptic activity in the basal ganglia, a circuit intimately involved in motor coordination, as well as reduced levels of BDNF; these changes that might contribute to the motor deficits seen in HD patients.

Activation of the ECS is suggested to play a functional role in HD pathogenesis. For instance, recent pharmacological studies showed that treatment of R6/2 HD mice with Δ^9-tetrahydrocannbinol (THC), the bioactive component in marijuana that targets CB_1, ameliorates HD-like symptomatology by reducing motor deficits, improving neuropathological correlates of neurodegeneration, and reversing the molecular profile of neuronal markers lost in R6/2 mouse brains relative to WT controls (Blazquez et al., 2011). Additionally, studies have shown that the decrease in CB_1 expression in HD mice is due to mHtt's effects on transcription. For instance, the rate at which CB_1 mRNA is lost correlated with the length of CAG expansion and expression levels of mHtt (McCaw et al., 2004; Blazquez et al., 2011). Interestingly, housing HD mice in enriched environments improves multiple HD behavioral phenotypes and leads to an upregulation of CB_1 receptor levels in striatal output nuclei (Hockly et al., 2002). Combined, these studies highlight how CB_1 may be playing a functional role in HD and that the effect of mHtt on CB_1 transcription is an important event that contributes to disease onset and severity.

While strongly correlative, these studies fall short of establishing a casual link between CB_1 loss-of-function/expression and HD pathogenesis. To address this issue directly, we evaluated the contribution of CB_1 loss on motor behavior and overall survival in R6/2 and BACHD mice, two well-established transgenic mouse models of HD. We found that early genetic deletion of CB_1 receptors in R6/2 mice negatively influenced

disease progression by accelerating the onset of behavioral symptoms. In contrast, the effect of CB_1 loss in BACHD mice did not overtly worsen the HD phenotype but instead lead to increased leanness, confounding statistical conclusions.

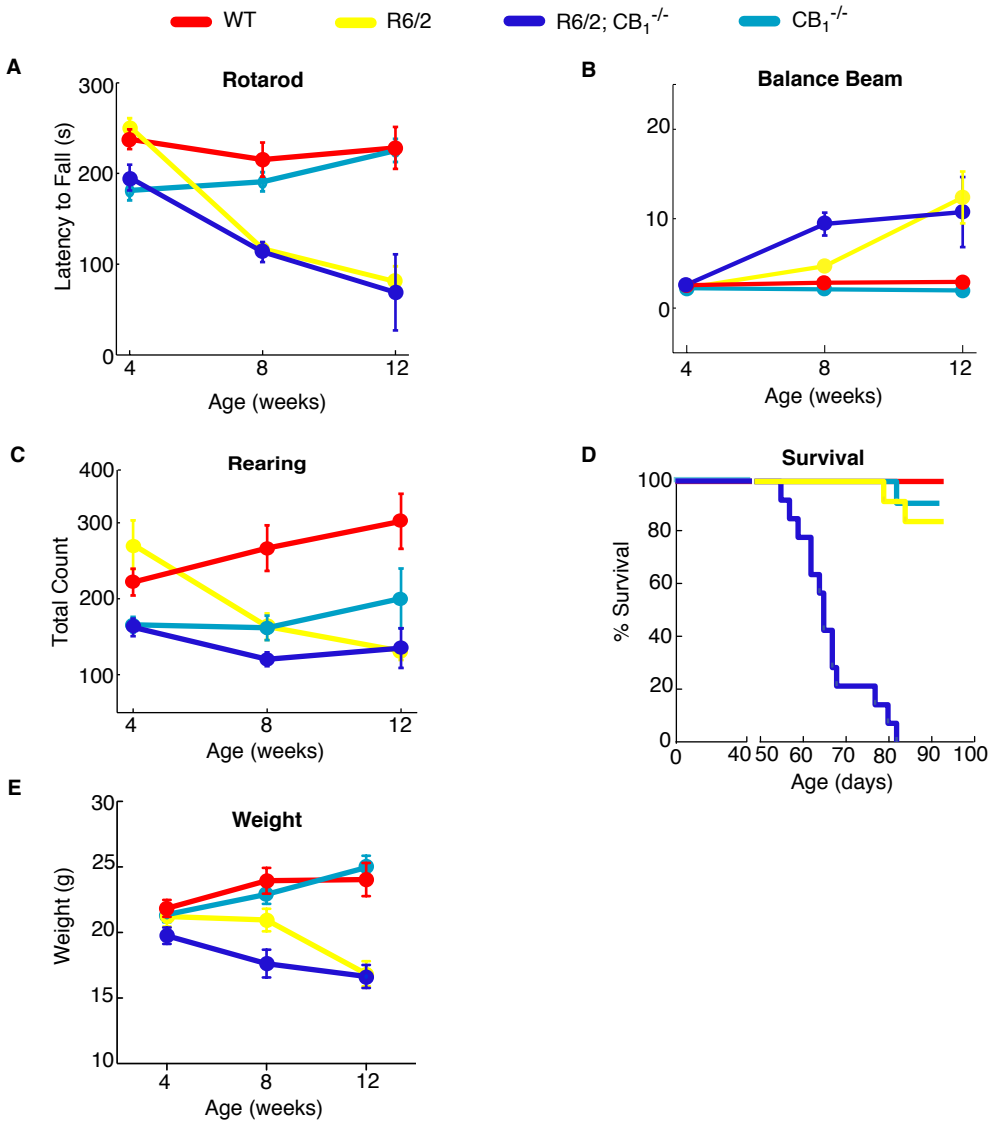
RESULTS

Genetic Deletion of CB_1 Receptors Accelerates Disease Onset and Exacerbates Symptoms in R6/2 Mice

To determine if the loss of CB_1 receptors influences HD progression, we first crossed $CB_1^{-/-}$ mice, with R6/2 mice (Mangiarini et al., 1996), which express the first exon of the human Htt gene (*IT-15*) containing a large CAG repeat expansion under the control of the *5′* end of *IT-15*. We measured the motor behaviors of these R6/2;$CB_1^{-/-}$ mice on the balance beam and rotarod, two measures of motor coordination, and in open field assays, a measure of locomotor activity. At 8 weeks of age, R6/2 mice stayed on the rotarod for significantly less time than WT controls (**Figure 2-1A** at 8 weeks; $p<0.001$), as reported (Blazquez et al., 2011). Interestingly, both $CB_1^{-/-}$ and R6/2; $CB_1^{-/-}$ mice exhibited these deficits much earlier, beginning at 4 weeks of age (**Figure 2-1A** at 4 weeks; $p<0.05$), suggesting that these effects were due to CB_1 receptor loss alone. However, $CB_1^{-/-}$ mice eventually performed equally well as WT mice by 8 and 12 weeks of age, an effect not seen in R6/2;$CB_1^{-/-}$ mice (**Figure 2-1A**).

On the balance beam, R6/2; $CB_1^{-/-}$ mice tended to perform worse at 8 weeks than WT and R6/2 mice, but this observation did not reach statistical significance after *post-hoc* tests (**Figure 2-1B**). In the open field test, R6/2 mice lacking CB_1 reared 25% less at 4 weeks than WT (**Figure 2-1C**; $p<0.05$) and R6/2 (**Figure 2-1C**; $p<0.001$) mice and

Figure 2-1 Genetic Deletion of CB_1 Receptors Accelerate the Onset of Motor Symptoms, Death, and Weight Loss in R6/2 Mice



(A) Deletion of CB_1 receptors increase the latency to fall of R6/2 mice in a rotarod assay at 4-weeks of age. (4 weeks, R6/2 vs. $CB_1^{-/-}$ ***, R6/2 vs. R6/2; $CB_1^{-/-}$ *; 8 weeks, WT vs. R6/2, R6/2; $CB_1^{-/-}$ ***; 12 weeks, WT vs. R6/2, R6/2; $CB_1^{-/-}$ **)

(B) Deletion of CB_1 receptors does not effect the latency of mice to cross the beam on a beam traversal assay.

(C) Deletion of CB_1 receptors decreases rearing activity in R6/2 mice. Spontaneous locomotor activity was quantified in an automated open field and the number of rearings was scored for 30 min. Data represents the sum of total rearings in 30 min. (4 weeks, R6/2; $CB_1^{-/-}$ vs. WT *, R6/2; $CB_1^{-/-}$ vs. R6/2 ***; 8 weeks, WT vs. R6/2; $CB_1^{-/-}$ ***; 12 weeks, WT vs. R6/2 **)

(D) Kaplan-Meier survival analysis shows that deletion of CB_1 receptors decreases survival in R6/2 mice. (Log-rank (Mantel-Cox) test, ***)

(E) Deletion of CB_1 receptors increases weight loss in R6/2 mice at 8-weeks of age. (4 weeks, ns; 8 weeks, R6/2; $CB_1^{-/-}$ vs. WT **, R6/2; $CB_1^{-/-}$ vs. R6/2 *; 12 weeks R6/2 and R6/2; $CB_1^{-/-}$ vs WT **)

Data at each time point were analyzed for significant differences using a one-way ANOVA in conjuction with Bonferonni *post hoc* analysis (*<0.05, **<0.01, ***<0.001)

nearly 50% less by 8 weeks (**Figure 2-1C**; $p<0.001$) when compared to WT mice (**Figure 2-1C**). By 8 weeks of age R6/2 and R6/2 mice lacking the CB_1 receptor were not significantly different from each other in the open field. These deficits are consistent with earlier reports (Blazquez et al., 2011).

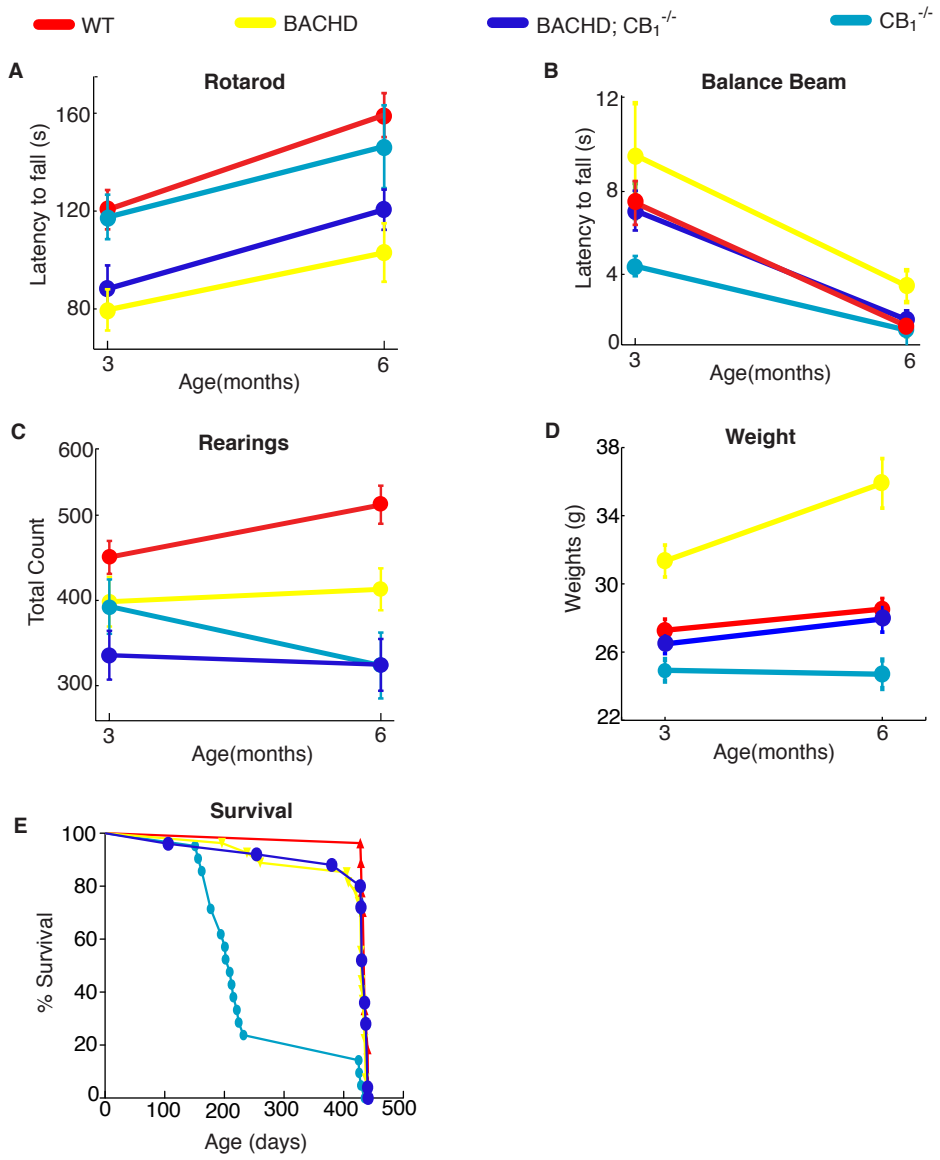
Kaplan-Meier survival analysis and analysis of weight in R6/2 mice revealed that the loss of CB_1 receptor expression significantly influenced overall survival and weight. Specifically, we found that R6/2;$CB_1^{-/-}$ mice lived for significantly less time than controls (**Figure 2-1D**; $p<0.0001$) and lost more weight at earlier time points than WT and R6/2 mice with CB_1 (**Figure 2-1E**; $p<0.001$, 4weeks and $p<0.001$, 8 weeks).

These results show that, in the absence of CB_1 receptors, the HD phenotype is worse in R6/2 HD mice, supporting the hypothesis that early reduction of CB_1 receptor expression in HD brains contributes to the onset and severity of HD symptoms.

Genetic Deletion of CB_1 Receptors Does Not Influence the HD Phenotype but Significantly Contributes to the Weight Phenotype in BACHD mice

We next sought to determine if CB_1 loss would influence disease progression in BACHD mice (Gray et al., 2008), which express full-length Htt under the control of its endogenous promoter and regulatory sequences, and generally display a milder HD phenotype. To determine if loss of CB_1 receptors influences disease progression, we generated BACHD; $CB_1^{-/-}$ mice and littermate controls and measured their motor behaviors in the balance beam, rotarod, and open field assays described above. In both the rotarod and balance beam assay, the BACHD transgene significantly affected behavior, as expected (**Figure 2-2A**; $p<0.01$ and **Figure 2-2B**; $p<0.05$) (Gray et al.,

Figure 2-2 Genetic Deletion of CB_1 Receptors Does Not Influence Behavioral Phenotypes n BACHD Mice



(A) Deletion of CB_1 receptors does not increase the latency to fall of BACHD mice in a rotarod assay at 3 and 6 months of age. (3 and 6 months, WT vs. BACHD and BACHD; $CB_1^{-/-}$ **)

(B) Deletion of CB_1 receptors does not increase the latency to cross of BACHD mice in a balance beam traversal assay at 3 months of age.

(C) Deletion of CB_1 receptors decreases rearing activity in BACHD mice. Spontaneous locomotor activity was quantified in an automated open field and the numbers of rearings were scored for 30 min. Data represent the sum of total rearings in 30 min. (3 months, WT vs. BACHD; $CB_1^{-/-}$ *; 6 months, WT vs. BACHD; $CB_1^{-/-}$ ***)

(D) Deletion of CB_1 receptors rescues the BACHD weight phenotype. (3 months, BACHD vs.WT **; 6 months, BACHD vs WT ***)

(E) Kaplan-Meier survival analysis shows that deletion of CB_1 receptors does not influence survival in BACHD mice.(Log-rank (Mantel-Cox) test, ***)

Data at each time point were analyzed for significant differences using a one-way ANOVA in conjuction with Bonferonni *post hoc* analysis (*<0.05, **<0.01, ***<0.001)

2008). Deletion of CB_1 in BACHD mice did not further influence the BACHD phenotype in either of these motor behavioral assays (**Figure 2-2A and B**). There was, however, a significant effect of CB_1 deletion in BACHD mice compared to WT in open-field rearing; specifically, BACHD;$CB_1^{-/-}$ mice reared significantly less than WT mice and 3 and 6 months of age (**Figure 2-2C**; $p<0.05$, 3 months and 6 months.

The survival curves of BACHD and BACHD; $CB_1^{-/-}$ mice were not significantly different, indicating that deletion of CB_1 did not influence overall survival in BACHD mice. Interestingly, deletion of CB_1 alone in WT mice significantly affected survival time (**Figure 2-2E**; $p<0.0001$).

As has been previously described, expression of the BACHD transgene leads to significant weight gain (**Figure 2-2D**; $p<0.001$) (Gray et al., 2008; Menalled et al., 2009; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012). The most profound effect in these studies was the effect of CB_1 deletion on the BACHD weight phenotype. Most notably, we observed that BACHD mice lacking CB_1 were no longer significantly heavier than WT mice (**Figure 2-2D**). This effect of CB_1 loss on weight hinders the accurate analysis and interpretation of the effects of CB_1 loss in BACHD mice. This will be discussed more in sub-chapter 2.1.

DISCUSSION

A dramatic decrease in CB_1 receptor levels is one of the first genetic changes to occur in the striatum of HD brains, and prior to the onset of any overt symptoms. Here, we show that genetic loss of CB_1 receptor signaling in R6/2 mice advances the onset of disease and exacerbates symptoms. These results are consistent with previous studies

(Blazquez et al., 2011; Mievis et al., 2011) showing that genetic deletion of CB_1 in R6/2 and N171-82Q mice lowers their performance on multiple motor readouts such as rotarod and open field activity. In our studies we also found that early, ubiquitous deletion of CB_1 reduced R6/2 mouse lifespan by 20% **(Figure 2-1D).** These results suggest that early loss of CB_1 receptor expression is an important event in disease pathogenesis.

Since the identification of mHtt, numerous mouse models have been generated to study HD-related pathology, since no single model recapitulates all aspects of the disease (Menalled et al., 2009). Here, we examined loss of CB_1 receptor signaling in two different HD mouse models, BACHD and R6/2. We found that deletion of CB_1 in R6/2 mice worsened HD progression while the loss of CB_1 in BACHD mice led to little, if any, overt phenotype. The divergence in behavioral outcomes resulting from CB_1 deficiency in suggests important differences between these models.

BACHD mice express the full-length human mHtt gene and exhibit more slowly progressing HD-like symptoms than R6/2 HD mice, which express a heavily CAG-expanded mHtt fragment and develop a very robust and rapid HD phenotype. Research suggests that the R6/2 mouse model resembles the juvenile form of HD, characterized by long CAG repeats and a rapid and robust onset of symptoms (Ona et al., 1999; Cepeda-Prado et al., 2012). Conversely, BACHD mice, because of their delayed phenotype, are thought to more accurately mimic the adult-onset form of HD (Gray et al., 2008)**.** Based on these results, it is tempting to speculate that loss of CB_1 expression may be more relevant in the juvenile form of HD. Studies investigating whether or not there is a relationship between the extent of CB_1 loss and juvenile HD in humans are therefore warranted, especially since juvenile HD patients traditionally have much longer CAG

repeats than their adult counterparts (Duyao et al., 1993; Snell et al., 1993) and CAG repeat length negatively correlates with CB_1 loss (McCaw et al., 2004; Van Laere et al., 2010). Alternatively, the divergence of behavioral outcomes in R6/2 and BACHD mice imply that one model may be more relevant to studying CB_1 receptor–mediated effects on HD pathogenesis than the other, especially in light of our observations that endogenous CB_1 receptor levels are differentially changed between these transgenic HD lines. For instance, in R6/2 mice, CB_1 levels are decreased to 70% of WT levels at 4 weeks of age (unpublished data, Nephi Stella). In contrast, CB_1 receptor mRNA levels in BACHD mice are increased 2-fold with no change in CB_1 protein levels (unpublished data, Nephi Stella). These data suggest that the mechanisms leading to mHtt-mediated CB_1 downregulation are different between R6/2 and BACHD mice; if so, the effects of CB_1 loss in HD mice may therefore depend on which model recapitulates the genetic features of HD.

More importantly though, the accurate interpretations of the effects of $CB_1^{-/-}$ loss on the BACHD phenotype were confounded by two major factors: the phenotype of the $CB_1^{-/-}$ mouse and animal weight. First, loss of CB_1 alone conferred a phenotype that with respect to many behaviors was worse than those observed in BACHD and BACHD;$CB_1^{-/-}$ mice (**Figure 2-2**). Most striking was the effect of CB_1 loss on the survival of WT mice, whereby all $CB_1^{-/-}$ were dead by the 6 months time point (**Figure 2-2**). Surprisingly, there was no effect of CB_1 deletion in BACHD mice on survival, questioning the physiological relevance of CB_1 signaling should the BACHD phenotype somehow confer protection against death. However, the observed protection conferred by the BACHD transgene to $CB_1^{-/-}$ mice may not be related to HD, and instead, may be due to the

influence of genetic background. The same phenotype is not observed in $CB_1^{-/-}$ mice on a C57/BL6 background, suggesting that the phenotype of CB_1 mice on the FVB background is due to genetic modifiers or traits that are unique to the background strain, unique to the gene of interest (CB_1), and/or potentially due to an interaction between the background and gene of interest (CB_1) (Linder et al., 2006). Similar effects have been observed in other studies of genes on different backgrounds (Carson et al., 1997; Roth et al., 2002; Coleman and Hummel, 1973). Second, expression of the BACHD transgene caused significant weight gain in BACHD mice that did not occur in BACHD mice lacking the CB_1 receptor (**Figure 2-2D**). Since the weights of BACHD and BACHD;$CB_1^{-/-}$ mice were changed in opposite directions, it is difficult to determine if BACHD;$CB_1^{-/-}$ mice would have performed worse than BACHD mice had their weights been equal across genotypes. For instance, on the rotarod—a very common behavioral readout for motor coordination and balance in HD—both the BACHD and BACHD;$CB_1^{-/-}$ mice performed significantly worse than WT littermate controls. Their means, however, were not different from each other, which would imply that loss of CB_1 does not further exacerbate the BACHD rotarod phenotype (**Figure 2-2A**). However, since weight has been shown to significantly influence motor behaviors (McFadyen et al., 2003; Brown and Wong, 2007), especially on the rotarod, we cannot discern between the effects of weight and genotype with standard statistical measures. Moreover, since heavy mice are known to perform worse on the rotarod when compared to thinner mice (McFadyen et al., 2003; Brown and Wong, 2007), one could imagine that the phenotype of BACHD mice in this motor task is heavily associated with animal weight and thus is masking the potential motor deficits between BACHD mice and BACHD mice lacking CB_1 receptors.

As a result of the weight variance across genotypes and the potential for misinterpretation, these findings prompted a more complete analysis of the weight effect. These studies will be presented in sub-chapter 2.1.

One last possible explanation for the divergent outcomes could be because one mouse model is more relevant than the other to understanding the role of CB_1 on HD pathogenesis. For instance, from a genetic standpoint the BACHD mouse model, which express the full-length human mutant huntingtin gene, has more face validity than the R6/2 mouse model that only express the CAG-containing expansion in exon 1. In addition, the promoter sequences controlling BACHD transgene expression are more complete in the BACHD mouse than R6/2 and thus may be expressed in a fashion that more accurately mimics the human disease. Importantly though while the BACHD mouse model more accurately mimics the genetics of human HD when compared to the R6/2, the R6/2 mouse has been shown to mimic many of the features of HD. For example, profiling data from R6/2 mice compare well with human data, suggesting that the expanded CAG region in exon 1 is sufficient to recapitulate the transcriptional phenotype seen in humans. While it is possible that one mouse model is more relevant to understanding the role of CB_1 on HD pathogenesis it is difficult to compare the relevance of R6/2 and BACHD mice using the CB_1 receptor because of the confounding effects observed.

While the molecular events that are affected by the absence of CB_1 receptor signaling and contribute to disease progression are not completely understood and likely to be complex, experimental evidence suggests a potential link between CB_1 receptor signaling and brain derived neurotrophic factor (BDNF) (De Chiara et al., 2010). BDNF is an

important neuronal growth factor that shapes neuronal plasticity (Jia et al., 2010) and promotes survival of striatal medium spiny neurons (Altar et al., 1997; Baquet et al., 2004), and its levels are decreased in HD brains (Ferrer et al., 2000; Zuccato et al., 2001) and in several of the HD mouse models, including R6/2 (Blazquez et al., 2011). Studies show that activation of CB_1 receptors increases BDNF expression (Butovsky et al., 2005; D'Souza et al., 2009), while inhibiting CB_1 has the opposite effect (Aso et al., 2008), implying that in the context of HD, the loss of CB_1 receptors negatively affect the levels and protective function of BDNF. Studies supporting this hypothesis show that the neuroprotective effects of endocannabinoids in the striatum are mediated by BDNF secretion from cortical neurons (Khaspekov et al., 2004). These data, combined with studies showing that overexpression of BDNF in cortical neurons or astrocytes of HD mice ameliorates disease progression (Perez-Navarro et al., 2000; Gharami et al., 2008; Xie et al., 2010; Giralt et al., 2011), warrant further investigation. For instance, if a protective effect is observed after BDNF treatment in R6/2;$CB_1^{-/-}$ mice, this could suggest that loss of CB_1 expression contributes to HD progression by causing a decrease in striatal BDNF levels.

MATERIALS & METHODS

Animals and Breeding Strategy

Experiments involving mice were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Mice were bred and maintained in compliance with National Institutes of Health guidelines. R6/2 mice (Mangiarini et al., 1996) were obtained by breeding R6/2 ovarian transplants expressing

160 CAG-repeat-length mHtt (002810 Jackson) with CBA WT males. BACHD mice (Gray et al., 2008) were obtained from Dr. William Yang (UCLA) and were maintained by breeding to WT FVB/NJ males. $CB_1^{-/-}$ mice on C57/BL6 background were obtained from the Feldman lab at UC Berkeley (Marsicano et al., 2002). Using speed congenics, $CB_1^{-/-}$ mice were backcrossed for 10 generations onto a FVB/NJ background prior to breeding to BACHD mice. To test if loss of CB_1 worsened HD progression, we crossed the $CB_1^{-/-}$ mice to BACHD mice. Progeny from these litters that were BACHD; $CB_1^{+/-}$ were then bred to each other to generate BACHD; $CB_1^{-/-}$ mice and all littermate controls.

Genotyping

Mouse-tail DNA was analyzed by PCR to determine the genotypes. The BACHD transgene was identified as described (Gray et al., 2008). $CB_1^{-/-}$ mice were genotyped as described . The R6/2 transgene was identified as described (Hockly et al., 2002).

Behavioral Analysis

An accelerating rotarod was used to analyze motor coordination and balance. For R6/2 mice, baseline behavior was performed at ~4 weeks, and mice were placed into balanced cohorts based on behavior, sex, and genotype. Mice were trained three times at a constant speed of 16 rpm for maximum of 300 sec. During testing, mice were subjected to beam acceleration of 4–40 rpm for a maximum of 300 seconds, three times per session for a total of three sessions. Performance was quantified by measuring the latency to fall off of the rotarod apparatus. For BACHD studies, mice were trained and tested as above but at 3 and 6 months of age.

The balance beam was also used to assess motor coordination and balance. This test consisted of three sessions with three trials in each session: one session of training, one session of testing on a large diameter beam, and one session of testing on a medium diameter beam. Motor performance was assessed by measuring the time it took for the mouse to traverse the beam. Data was averaged across each session and then sessions averaged together.

Rearing activity was assessed in an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments). Before testing, mice were acclimated in the testing room for 1 h. Mice were then placed in open field chambers with two 16x16 photobeam arrays to detect horizontal and vertical movements. Rearing behavior (the number of times the mouse stood erect on its hind legs) was measured over 30 min. Chambers were cleaned with 70% ethanol before and after each mouse.

Survival in R6/2 mice was defined as the time when the animals either died spontaneously or had lost more than 20% of their peak body weight. For all studies the experimenter was blind to the genotype and/or treatment group.

Statistics

All data are expressed as the mean +/- SEM. Unless otherwise indicated, a one-way ANOVA with Bonferroni's *post-hoc* test was performed to determine levels of significance between experimental groups at each time point.

Chapter 2.1. Weight Gain in BACHD Mice Is a Major Contributor to Rotarod Motor Deficits

ABSTRACT

The BACHD transgenic mouse model expresses a full-length variant of mutant huntingtin and develops Huntington's disease (HD) with a slowly progressive phenotype. It is commonly used to study HD because it is thought to mimic adult HD symptoms in humans, the phenotype for which is often associated with disruption of the central nervous system. However, it has been reported that these mice have a significant increase in weight compared to wildtype mice. Numerous studies have documented direct effects of animal weight in behavioral phenotypes, especially in locomotion and coordination. However, behavioral studies utilizing BACHD mice have failed to statistically account for the interaction of genotype and weight on behavioral performance. Furthermore, the issue of weight can be exacerbated by other genetic factors, making it difficult to know whether or not the behavioral phenotypes associated with these mice result from the increased weight or a deficit in the central nervous system, or some combination of the two. Here, we show that BACHD mice are significantly heavier than wildtype mice and that, across different genotypes, there is a significant effect of animal weight on behavior. Using an analysis of covariance (ANCOVA), we show that, after statistically removing the effect of weight on performance, the behavioral deficits associated with BACHD mice on the rotarod are less severe and, in some cases, no longer significantly different from controls. Together our results highlight the importance of accounting for animal weight on behavioral performances, and suggest a

statistical tool (ANCOVA) that can do so.

INTRODUCTION

In genetic studies, a primary goal of behavioral phenotyping is to determine whether experimentally induced mutations produce changes in distinct behaviors, in order to elucidate the possible biological significance of a gene product (van der Staay and Steckler, 2002). However, many genetic manipulations commonly employed in mice are not limited to one physiological system or cell type. This lack of specificity in genetic targeting can sometimes lead to unwanted physiological changes and can ultimately confound the interpretation of results (Picciotto and Wickman, 1998). Therefore, analytic methods should be employed to statistically dissociate the effects of co-occurring physiological phenotypes on the behavioral measure assayed experimentally. In HD, this is especially important since, to date, there is no cure or effective treatment. Therefore, correctly identifying HD phenotypes in animal models and assigning causal roles to physiological systems will be critical for better-informed drug discovery efforts.

Numerous mouse models expressing different forms of mHtt have been generated to study HD pathogenesis (Menalled et al., 2009). The BACHD mouse, which expresses full-length mHtt under the control of the endogenous htt regulatory machinery, is one of the more recent additions to the repertoire of HD animal models. Characterized by progressive motor deficits, synaptic dysfunction, and late-onset selective neuropathology, the BACHD mouse is thought to more closely mimic the adult form of HD (Gray et al., 2008). However, unlike human HD, in which patients suffer from severe weight loss (van der Burg et al., 2009), BACHD display significant weight gain relative to wildtype controls (Gray et al., 2008). Studies have shown that expression of the bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) containing either wildtype

huntingtin or mHtt lead to an increase in animal weight, suggesting that this effect is due to increased levels of either protein but not a pathological effect of mHtt (Van Raamsdonk et al., 2006; Pouladi et al., 2010).

Some studies suggest that the increased weight BACHD mice carry either does not influence motor behaviors or they claim that it does, but provide no statistical analysis supporting these claims (Gray et al., 2008; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012) (Pouladi et al., 2010). However, published studies show that weight negatively correlates with performance on motor assays. In fact, it has been shown that performance on the rotarod, a commonly used motor assay to measure balance and motor coordination in HD mice, is very sensitive to weight variations (McFadyen et al., 2003; Brown and Wong, 2007). For instance, differences in performance between various mouse strains were attributed to weight differences across strains, whereby mouse strains that weighed more spent less time on the rotarod compared to strains that weighed less (McFadyen et al., 2003; Brown and Wong, 2007). Despite the intuitive nature of this effect and the evidence demonstrating it experimentally, numerous HD studies fail to account for the effects of weight on behavior (Gray et al., 2008; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012) (Pouladi et al., 2010).

Here, we examined the contribution of weight on rotarod performance in BACHD mice and other strains (WT, BACHD; $CB_1^{-/-}$). We found that, prior to accounting for weight, BACHD mice are significantly impaired on the rotarod compared to WT controls at 3 and 6 months of age, as shown (Gray et al., 2008; Abada et al., 2012). Surprisingly, in the absence of the CB_1 receptor, BACHD mice were no longer heavy, revealing a potentially significant confound. Here, we propose a statistical method, Analysis of

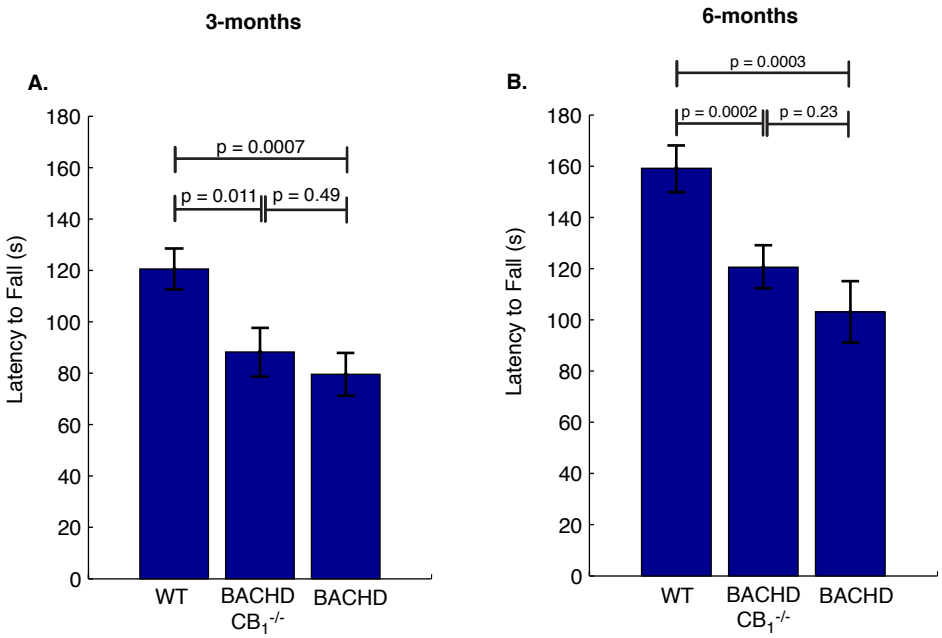
Covariance (ANCOVA), to assess the contributions of covariates (such as weight) to an observed effect. Using ANCOVA, we show that after appropriately controlling for weight across mice, the contribution of mHtt in CNS pathology was dramatically reduced and in some cases no longer significantly different from controls. These data show that weight does indeed influence the BACHD rotarod phenotype and demonstrate that by not accounting for this confounding effect, statistically incorrect conclusions can be reached. This study is a cautionary tale that reinforces the importance of accounting for all seemingly unrelated phenotypic changes on a behavior of interest.

RESULTS

BACHD Mice Are Impaired on the Rotarod Compared to WT Controls: Raw Data

Studies of BACHD mice reported an early-onset, statistically significant behavioral phenotype that progresses as the mouse ages (Gray et al., 2008; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012) (Pouladi et al., 2010). To recreate these effects, we tested BACHD mice and littermate controls at two different time points on the rotarod and measured the time to fall. The rotarod is commonly used as a measure of balance and motor coordination (Bogo et al., 1981). In line with previous studies (Gray et al., 2008; Abada et al., 2012) (Pouladi et al., 2010), we found that BACHD mice are impaired on the rotarod at 3 and 6 months of age compared to littermate controls **(Fig. 3-1)**. Analysis of this data with a one-way ANOVA followed by Bonferroni *post hoc* analysis revealed that mean BACHD mouse rotarod performance was significantly lower (3 months, p value = 0.0007; 6 months, p value = 0.0003) compared to WT littermate

Figure 2.1-1 BACHD Mice Are Impaired On The Rotarod At 3 And 6 Months Of Age



(A) In the absence of accounting for weight, BACHD mice perform worse on the rotarod compared to WT mice at 3 months. Deletion of CB_1 receptors does not worsen the BACHD rotarod performance at either time point.

(B) same as in (A) but at 6 months

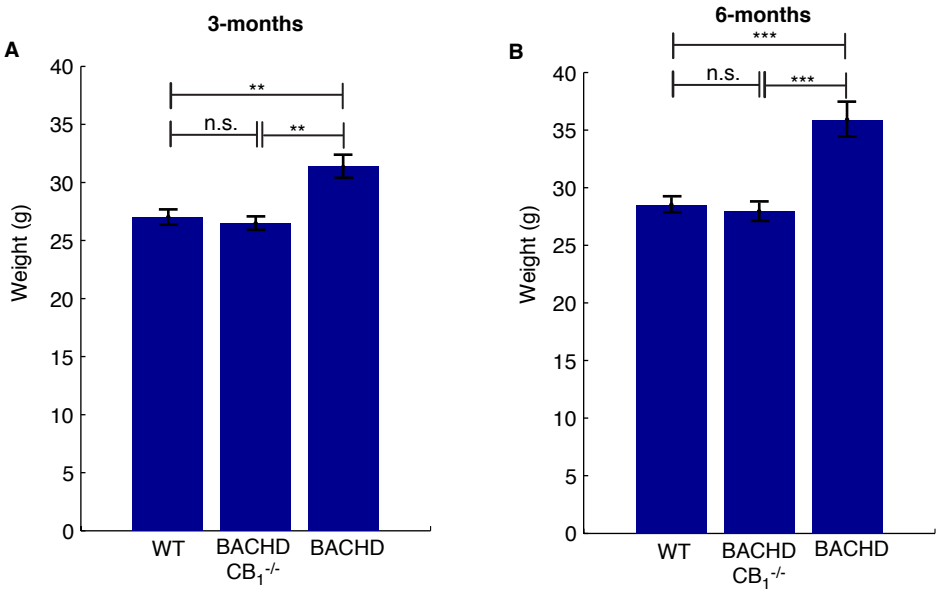
Values are means ± SEM. Statistics shown are results of a one-way ANOVA followed by Bonferonni *post hoc* tests. n = 25-30 mice/genotype

controls. Therefore, without controlling for the effects of weight, we would conclude that there is an effect of genotype on performance. Because HD is commonly thought to primarily affect behavior through effects on the CNS, we might therefore be tempted to interpret this result as evidence for a CNS effect on behavior. However, the validity of these conclusions and interpretation depends on the assumption that there are no co-occurring physiological phenotypes that also affect the behavioral phenotype.

BACHD Mice are Significantly Heavier Than Littermate Controls

Studies have shown that BACHD mice weigh significantly more than WT controls due to increase adipose accumulation (Hult et al., 2011). Indeed, we also found that

Figure 2.1-2 BACHD Mice Are Heavier Than Littermate Controls



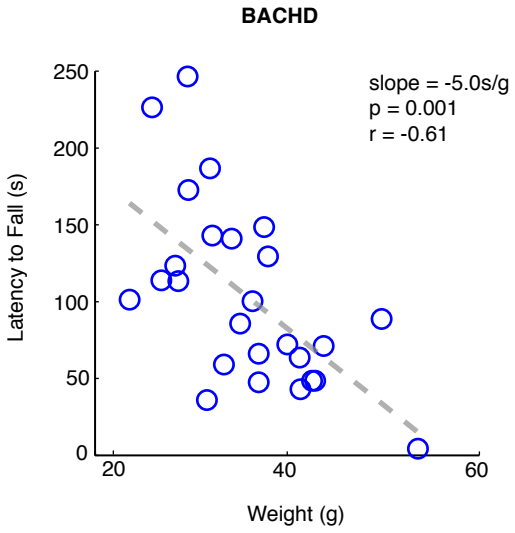
(A) Weight analysis of BACHD mice at 3 months shows expression of the BACHD transgene causes significant animal weight gain. Deletion of CB_1 reverses the effect of the BACHD transgene on weight gain.

(B) same as (A) but at 6 months

For each timepoint a one-way ANOVA was performed in conjuction with Bonferonni *post hoc* tests to determine significance between groups.
Values are means ± SEM. n = 25-30 mice/genotype $p<0.05$ *; $p<0.01$**; $p<0.0001$ *** ns, not significant

BACHD mice are significantly heavier at 3 and 6 months of age compared to wildtype mice **(Fig. 2.1-2)**. Interestingly, deletion of CB_1, a G protein–coupled receptor shown in several other HD mice to exacerbate rotarod performance (Blazquez et al., 2011; Mievis et al., 2011), reversed the effects of the BACHD transgene on weight gain, whereby BACHD; $CB_1^{-/-}$ mice were indistinguishable in weight from wildtype controls **(Figure 2.1-2).** These data demonstrate that BACHD transgene expression leads to significant weight gain that no longer occurs in BACHD mice lacking the CB_1 receptor. Since weight has been shown to correlate with motor performance in mice (McFadyen et al., 2003; Brown and Wong, 2007), these results prompted us to further investigate the effects of weight on BACHD behavioral phenotype.

Figure 2.1-3 Linear Regression Trend Lines for BACHD Mice

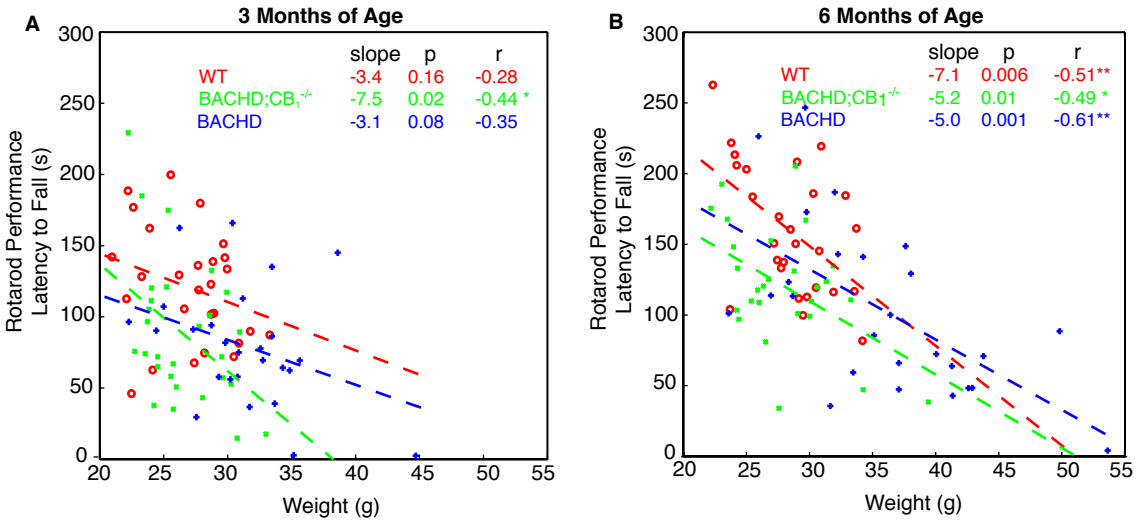


Rotarod performance (given in latency to fall (s))
correlates significantly with animal weight in BACHD mice
(r = -0.61, p=0.001, r2 = 0.36, n = 26, age: 6months)

Performance On Rotarod Is Negatively Correlated With Weight.

To examine the effect of animal weight on performance, we plotted the weight of individual mice (x-axis) and their corresponding average performance on the rotarod (y-axis) at 3 and 6 months of age. Contrary to published studies (Gray et al., 2008; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012) (Pouladi et al., 2010), we found that there was a negative linear relationship between weight and performance in BACHD mice, as indicated by a slope of -5.0 sec/gram and an r-coefficient of -0.61, implying that performance decreases when animal weight increases. The correlation coefficient of -0.60 indicates that 36% ($R^2 = 0.36$) of the variance in rotarod performance can be explained by weight. This relationship was significant at 6 months ($p = 0.001$) and was nearly significant at 3 months ($p = 0.08$) (**Figure 2.1-3**). In Figure 3-4, the

Figure 2.1-4 Combined Regression Trend Lines For All Genotypes



(A & B) Rotarod performance (given in latency to fall (s)) negatively correlates with animal weight across genotypes at 3 and 6 months of age. n = 20-25 mice/genotype

regression trend lines across all groups were compared, illustrating that by 6 months there was a significant effect of weight across all genotypes on rotarod performance, although at 3 months this relationship was not quite significant for wildtype mice ($p = 0.18$, $R = -0.28$). BACHD; $CB_1^{-/-}$ mice at both time points showed a negative linear relationship (3 months: $p = 0.02$; 6 months: $p = 0.01$).

These data and analysis demonstrate that there is a significant negative correlation between animal weight and rotarod performance **(Figure 2.1-3),** revealing a non-trivial relationship between these two variables. Indeed, by 6 months of age across genotypes, 25-30% of the variability in rotarod performance could be attributed to the effect of weight, as quantified by the coefficient of determination (R2). Therefore, because BACHD mice are significantly heavier than wildtype controls, these observations raise the question: is the phenotype of BACHD mice on the rotarod due, in part, to their increased weight?

BACHD Mice Are Not Significantly Impaired on the Rotarod Compared to WT After Accounting For The Effect Of Weight

To determine whether controlling for weight affected the significance of the deficit in performance in BACHD mice, we utilized an Analysis of Covariance (ANCOVA), a linear model that blends ANOVA with linear regression (Howell, 2013). While similar to the ANOVA in that it tests statistical differences in the means between groups, the ANCOVA is used when there is another factor (e.g. weight) that is co-varying across groups (e.g. genotypes) and that is also affecting the dependent variable (e.g. rotarod performance) (Sheean et al., 2011). Before comparing the means across genotypes, the ANCOVA adjusts the dependent variables (rotarod performance) to values that are linearly independent of weight by finding the best linear relationship between the covariate and the dependent variable. This is done by linearly regressing the covariate against the dependent variable. This linear relationship is then removed from the dependent variable, and the distributions of residuals (values of dependent variable after removing the effect of the covariate) are analyzed to test if the means are statistically different, as in an ANOVA. The statistical values given by these analyses are the 1) F-statistic and a 2) p-value associated with comparing the means of the de-trended data across groups. The F statistic, which is the variance between groups divided by the variance within groups, reflects the effect of the independent variable (i.e. the smaller the F-statistic, the more likely the mean difference is due to natural variation within a group). In contrast, the p-value, calculated from the F-statistic, determines whether or not the null hypothesis is rejected at a particular false-positive rate (Keppel, 1991; Howell, 2013).

Comparing the results of the ANOVA and ANCOVA for performance in BACHD

versus wildtype mice, we found that the F-statistic decreased from 13 to 4.66 at 3 months of age, indicating that after correcting for an increase in animal weight the effect of genotype is dramatically reduced. Associated p-values were consequently changed from 0.0007 to 0.0358. Similar differences are also present at 6 months, whereby the F-statistic was decreased from 14.94 to 1.31 and the corresponding p-value from 0.0003 to 0.2574. These results confirm that after accounting for weight in BACHD mice, the effect of genotype is dramatically reduced at 3 months and is no longer significant at 6 months. On the other hand, when BACHD; $CB_1^{-/-}$ mice were compared to wildtype controls and weight was accounted for, the effect of the genotype and corresponding p values were increased at both ages tested, as shown by the increase in the F statistic from 6.43 to 9.24 (p values: 0.0111 to 0.0037) and 10.55 to 16.01 (0.002 to 0.0002) at 3 and 6 months, respectively **(Table 2.1)**. This last finding suggests that CB_1 deletion somehow "unmasks" the lowered motor performance, thus validating the power of the analysis.

Table 2.1-1. Comparison Of Statistical Outcomes Of Behavioral Performances On the Rotarod For Different Genotypes. Listed p-values and F-statistics are from two-way ANOVA or ANCOVA. Groups included in analysis: WT, BACHD, and BACHD; $CB_1^{-/-}$. n = 25–30 mice/genotype.

		3-months of age		6-months of age	
Genotype	Statistical Test	*F statistic*	*p-value*	*F statistic*	*p-value*
WT vs. BACHD; $CB1^{-/-}$	*ANOVA*	6.43	0.0111	10.55	0.002
	ANCOVA	9.24	0.0037	16.01	0.0002
WT vs. BACHD	*ANOVA*	13	**0.0007**	14.94	**0.0003**
	ANCOVA	4.66	**0.0358**	1.31	**0.2574**
BACHD; $CB1^{-/-}$ vs. BACHD	*ANOVA*	0.47	0.4949	1.54	0.2199
	ANCOVA	0.87	0.3555	2.45	0.124

DISCUSSION

The behavior of an organism is dependent upon the complex interaction of several physiological systems, which are themselves constructed from cellular components that share a common genetic code. For example, the simple act of running on a rotating rod is dependent upon the peripheral and central nervous system for sensation and motor control, on the musculoskeletal system for movement of the legs, the digestive system and cellular metabolism for proper energy intake and processing, the circulatory system for distribution and transportation of cellular nutrients and byproducts, and the immune

system for general animal health. Deficits in anyone of these systems could, in theory, affect locomotor behaviors. How the many physiological systems that give rise to an animal interact under normal conditions is poorly understood. Therefore, interpreting how a global manipulation like a pan-cellular genetic knockout or transgenic expression affects each individual physiological system as well as the interaction of systems across an organism is fraught with peril.

Researchers often assign a behavioral phenotype to one physiological system, and indeed to particular sub-systems, without considering the integrity of the rest of the organism. For instance, the aberrant response of mutant flies to repellent odorants might imply causality to olfaction impairments; however, motor deficits could also be responsible for the phenotype (Anholt and Mackay, 2004). Similarly, reduced burrowing behavior in mice could be explained by deficits in motor control or to a lack of motivation (Deacon, 2006a, b), or some combination thereof. In order to make claims about the causality of experimental manipulations on behavior, ruling out other potential contributions to the behavior are necessary. For instance, an experimenter could easily assess the motor abilities of flies or test mice on assays that measure affect in order to determine if multiple physiological systems are contributing to an observed behavioral phenotype. By not performing the correct statistical analysis or running control experiments, interpretations of the data can be misleading or even factually incorrect.

In the context of HD, our study addresses the issue of correctly assigning behavioral phenotypes—resulting from genetic manipulations—to specific physiological systems. Previous studies claim that the weight phenotype in BACHD mice does not correlate with and/or contribute to motor deficits (Gray et al., 2008; Southwell et al., 2009; Abada et al.,

2012; Gafni et al., 2012) (Pouladi et al., 2010), despite other studies showing that weight can affect motor performance (McFadyen et al., 2003; Wong and Brown, 2007). Our results show that the weight phenotype in BACHD mice does in fact contribute significantly to the behavioral symptoms observed on the rotarod. Without appropriately statistically correcting for weight, studies using this mouse model may inadvertently draw incomplete and even incorrect conclusions, and ultimately hinder the progress of HD research.

Our analysis clearly shows that the relationship between weight and motor performance is non-trivial and needs to be statistically accounted for when evaluating data from BACHD studies **(Table 2.1)**. Using linear regression, we showed a strong negative correlation of weight on motor performance of BACHD mice **(Fig. 2.1-3)**, demonstrating that, in addition to any potential mHtt effects on the CNS, weight may be contributing to the rotarod phenotype. Further analysis deploying an ANCOVA showed that after statistically removing the contribution of weight, the effect of mHtt on performance was dramatically reduced, and in some cases no longer significantly different from wildtype. Furthermore, the effects detected by the ANCOVA were observed across multiple time points, illustrating that despite the many physiological changes occurring over time, the ANCOVA detects consistent effects across time.

Some studies claim that removing from analysis those BACHD mice that fall outside two standard deviations of the mean weight of wildtype mice should overcome any contribution of weight on performance (Kernie et al., 2000; Gray et al., 2008), and justify the use of an ANOVA. This analysis assumes that the effect of weight on the dependent variable is the same across genotypes. Contrary to this assumption, we show that the

effect of weight on performance is not the same across groups, indicating the changes in weight may affect each group differently **(Fig. 2.1-4)**. Together, our results illustrate that the weight phenotype of BACHD mice contributes significantly to the rotarod phenotype, and that after correctly removing the weight effect, there is a much reduced—and in some cases no longer significant—effect of mHtt on performance.

Our data and analysis suggest that previous studies using BACHD mice should be reevaluated. In particular, it has been claimed that BACHD mice exhibit robust and progressive motor deficits on the rotarod that can be detected as early as 2 months. We believe this claim is misleading, since we show with ANCOVA that these deficits are not statistically significant once the effect of animal weight on behavior has been removed **(Table 2.1)**. More generally, our results demonstrate that studies utilizing BACHD mice and their behavior on the rotarod as a readout to investigate the contribution of specific genes on HD progression need to statistically account for animal weight before interpreting the results. For instance, genetic deletion of caspase-6 improves BACHD performance on the rotarod, implying that inhibiting caspase-6 may be a potential therapeutic treatment. However, as with CB1 deletion, the deletion of caspase-6 in these BACHD mice reversed the effect of the BACHD transgene on weight (Gafni et al., 2012), which was not accounted for in their analysis. However, as is commonly found in studies using BACHD mice, the weight gain is either reported and dismissed as a contributor to rotarod phenotype, or is shown to negatively correlate with rotarod performance, leading researchers to claim that weight is a partial contributor to the phenotype (Gray et al., 2008; Southwell et al., 2009; Abada et al., 2012; Gafni et al., 2012). Notably, these claims are not supported by any statistical analysis of the data. Making either one of

these claims needs to be supported by the correct statistical measures.

Previous studies evaluating the rotarod performance in BACHD mice utilize t-tests or ANOVA. However, in the presence of other changing variables, such as weight, these tests do not provide accurate statistical assessment of the relationship between the independent and dependent variables of interest, because they ignore covariance and attribute variability across groups solely to the independent variable (Howell DC 2009). In contrast, the ANCOVA is specifically designed to adjust statistical inferences in the presence of a covariate and, importantly, proper use of this method can confer several statistical advantages. First, deployment of an ANCOVA can reduce the statistical acceptance of false positives and the rejection of false negatives. For instance, the difference between BACHD and WT mice at 3 months (change of p-value = 0.0007 to 0.0358) and at 6 months (change of p-value = 0.0003 to 0.2574) was dramatically reduced after removing the effect of weight, and no longer significantly different from wildtype after correcting for multiple comparisons, demonstrating false positives. Additionally, by removing the confounding effect of weight, the effect of genotype was increased in the comparison of WT to BACHD; $CB_1^{-/-}$ mice at 3 (change from p value = 0.01 to 0.0037) and 6 months of age (change from p value = 0.002 to 0.0002), illustrating that, in principle, the ANCOVA can prevent false negatives as well. Second, an ANCOVA can increase the statistical power of a study. By statistically removing the effect of a covariate on behavior directly for all animals, utilizing an ANCOVA removes a source of 'noise' from the data and obviates the *ad hoc* removal of animals from the study.

METHODS

Behavioral Analysis

An accelerating rotarod was used to analyze motor coordination and balance. Mice were trained three times at a constant speed of 16 rpm for maximum of 300 sec. During testing, mice were subjected to beam acceleration of 4–40 rpm for a maximum of 300 seconds, three times per session for a total of three sessions. Performance was quantified by measuring the latency to fall off of the rotarod apparatus.

Statistics

All data are expressed as the mean +/- SEM. Unless otherwise indicated, a one-way ANOVA with Bonferroni's *post hoc* test was performed to determine levels of significance between experimental groups at each time point. Level of significance was set to $p<0.05$. Statistical analysis was performed using GraphPad prism and Matlab statistical programs.

Chapter 3. Cannabinoid Receptor Type 2 Signaling in Peripheral Immune Cells Modulates Disease Onset and Severity in Mouse Models of Huntington's Disease

ABSTRACT

Peripheral immune cells and brain microglia exhibit an activated phenotype in pre-manifest Huntington's disease (HD) patients that persists chronically and correlates with clinical measures of neurodegeneration. However, whether activation of the immune system contributes to neurodegeneration in HD, or is a consequence thereof, remains unclear. Signaling through cannabinoid receptor 2 (CB_2) dampens immune activation. Here, we show that the genetic deletion of CB_2 receptors in a slowly progressing HD mouse model accelerates the onset of motor deficits and increases their severity. Treatment of mice with a CB_2 receptor agonist extends lifespan and suppresses motor deficits, synapse loss, and CNS inflammation, while a peripherally restricted CB_2 receptor antagonist blocks these effects. CB_2 receptors regulate blood interleukin-6 (IL-6) levels, and IL-6 neutralizing antibodies partially rescue motor deficits and weight loss in HD mice. These findings support a causal link between CB_2 receptor signaling in peripheral immune cells and the onset and severity of neurodegeneration in HD, and they provide a novel therapeutic approach to treat HD.

INTRODUCTION

HD is an inherited and devastating neurodegenerative disease caused by a mutation in the *IT-15* gene, in which an expanded CAG repeat yields a polyglutamine stretch in the huntingtin (htt) protein (Group, 1993). Mutant htt expression in the striatum is neither necessary nor sufficient for neurodegeneration, implicating other cell types in HD (Gu et al., 2005; Gu et al., 2007). Mutant htt is expressed ubiquitously (Li et al., 1993), and although long overlooked, it's expression in non-neuronal cells might contribute to important disease phenotypes in HD. Skeletal muscle atrophy, increased cardiac failure, impaired glucose tolerance, gastrointestinal dysfunction, testicular atrophy, osteoporosis and weight loss have all been described in HD patients (van der Burg et al., 2009).

Mutant htt is also expressed at high levels in immune cells (Moscovitch-Lopatin et al., 2010), and abnormalities in immune responses have been reported in patients with HD. Plasma samples from HD patients exhibit increased levels of pro-inflammatory cytokines (Leblhuber et al., 1998; Dalrymple et al., 2007; Bjorkqvist et al., 2008) and chemokines (Wild et al., 2011) that correlate with disease progression, occurring years before the onset of chorea and other cognitive symptoms. Stimulated monocytes and macrophages from HD patients and mouse models produce elevated levels of these pro-inflammatory factors (Bjorkqvist et al., 2008). Bone marrow transplantation with wild-type cells normalizes levels of cytokines and chemokines, and partially suppresses behavioral and neuropathological deficits in HD mouse models (Kwan et al., 2012a). Based on these findings, we sought to identify signaling pathways in peripheral immune cells that contribute to neurodegeneration in HD which could be targeted by small molecules.

Activation of CB_2 receptors decreases inflammatory responses (Munro et al., 1993; Ashton and Glass, 2007), and enhanced signaling through them prevents atherosclerosis in mice by dampening cytokine secretion (Steffens et al., 2005). CB_2 receptors are critical for the host response to sepsis (Tschop et al., 2009) and colitis (Singh et al., 2012), and decrease production of pro-inflammatory cytokines interleukin 1ß (IL-1ß), tumor necrosis-α (TNF- α), and interleukin-6 (IL-6) (Klegeris et al., 2003; Rajesh et al., 2008; Su et al., 2011). Selective CB_2 receptor agonists are protective in mouse models of ischemia (Zhang et al., 2007; Murikinati et al., 2010), multiple sclerosis (Arevalo-Martin et al., 2003; Pryce et al., 2003), Parkinson's disease (Garcia et al., 2011), and Alzheimer's disease (Martin-Moreno et al., 2012). In HD, CB_2 receptors are shown to increase in postmortem brains and genetic deletion of CB_2 receptors in a mouse model of HD worsened motor symptoms and survival (Palazuelos et al., 2009). The central hypothesis tested in this study was that neuroprotection induced by CB_2 receptor signaling is mediated by direct effects on microglia, the resident immune cells in the brain. This interpretation is primarily based on immunohistochemical studies that show CB_2 colocalizes with the microglia marker Iba-1. However, because anti-CB_2 antibodies lack specificity, it is difficult to interpret these results without appropriate controls (Atwood and Mackie, 2010). Since CB_2 is expressed at high levels outside the CNS and is an important modulator of the immune system, we sought to determine its contributions to HD pathogenesis in the peripheral immune system.

Here we show that deletion of CB_2 receptors accelerates the onset of disease phenotypes and exacerbates behavioral deficits in a slowly progressing mouse model of HD. Treatment of a mouse model of HD with a CB_2–selective receptor agonist

suppresses neurodegeneration, and, surprisingly, is mediated by CB_2 receptor signaling not in parenchymal microglia, but rather in peripheral immune cells. Finally, enhanced CB_2 receptor signaling controls IL-6 blood levels and IL-6 inhibition partially suppresses pathogenesis in a mouse model of HD.

RESULTS

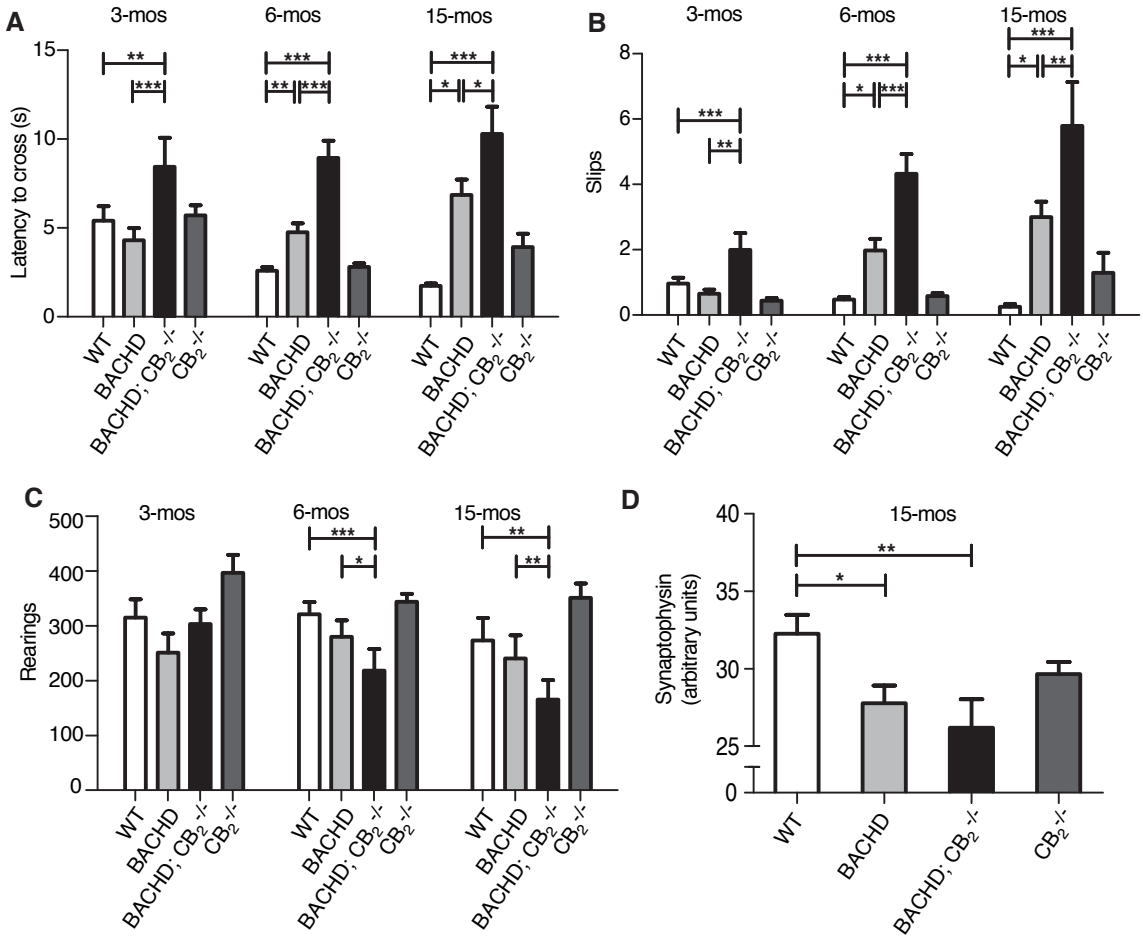
Genetic Deletion of CB_2 Receptors Accelerate Disease Onset and Exacerbate Severity in a Slowly Progressing HD Mouse Model

To determine if loss of CB_2 receptors influences disease progression in a slowly progressing HD mouse model, we crossed $CB_2^{-/-}$ mice with BACHD mice (Gray et al., 2008), which express full-length htt under the control of its endogenous promoter and regulatory sequences, and generated BACHD; $CB_2^{-/-}$ mice and littermate controls. We measured their motor behaviors on the balance beam and rotarod, two measures of motor coordination, and in open field assays, a measure of locomotor activity. BACHD mice took significantly longer than WT mice to traverse the balance beam at 6 months, but not 3 months of age, consistent with reported deficits of these mice in a rotarod assay (Gray et al., 2008; Menalled et al., 2009).

Remarkably, at 3 months of age, behavioral deficits were clearly detectable in BACHD mice lacking CB_2. Indeed, BACHD; $CB_2^{-/-}$ mice took significantly longer to traverse the beam than WT or BACHD mice. These deficits were even greater at 6 and 15 months (**Figure 3-1A**). Note that BACHD; $CB_2^{-/-}$ mice also had significantly more slips, and these deficits were also detected at 3 months of age, when BACHD mice are

phenotypically normal (**Figure 3-1B**). Loss of CB_2 alone had no effect on latency or slips at any time, indicating a specific genetic interaction between mutant htt and CB_2 that mediates the onset and severity of motor deficits in BACHD mice. BACHD;$CB_2^{-/-}$ mice performed worse on the rotarod when compared to WT and BACHD mice, but this difference did not reach statistical significance after accounting for differences in animal weight (data not shown).

Figure 3-1 Genetic Deletion of CB_2 Receptors Accelerate the Onset of Motor Symptoms and Increase Their Severity in a Slowly Progressing Mouse Model of HD



(A) Deletion of CB_2 receptors increase the latency to cross of BACHD mice in a balance beam traversal assay at 3, 6, and 15 months.

(B) Deletion of CB_2 receptors increase the number of slips of BACHD mice in a balance beam traversal assay at 3, 6, and 15 months.

(C) Deletion of CB_2 receptors decrease rearing activity in BACHD mice. Spontaneous locomotor activity was quantified in an automated open field and the numbers of rearings were scored for 30 min. Data represents the sum of total rearings in 30 min. Two-way ANOVA ($P<0.05$) was performed in conjunction with the Bonferonni post-hoc analysis multiple comparison test.

(D) Quantification of synaptophysin levels in the striatum at 15-months of age. One-way ANOVA was performed in conjuction with Dunnett's Multiple Comparison's test. Values are means ± SEM. n = 25-30/genotype. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

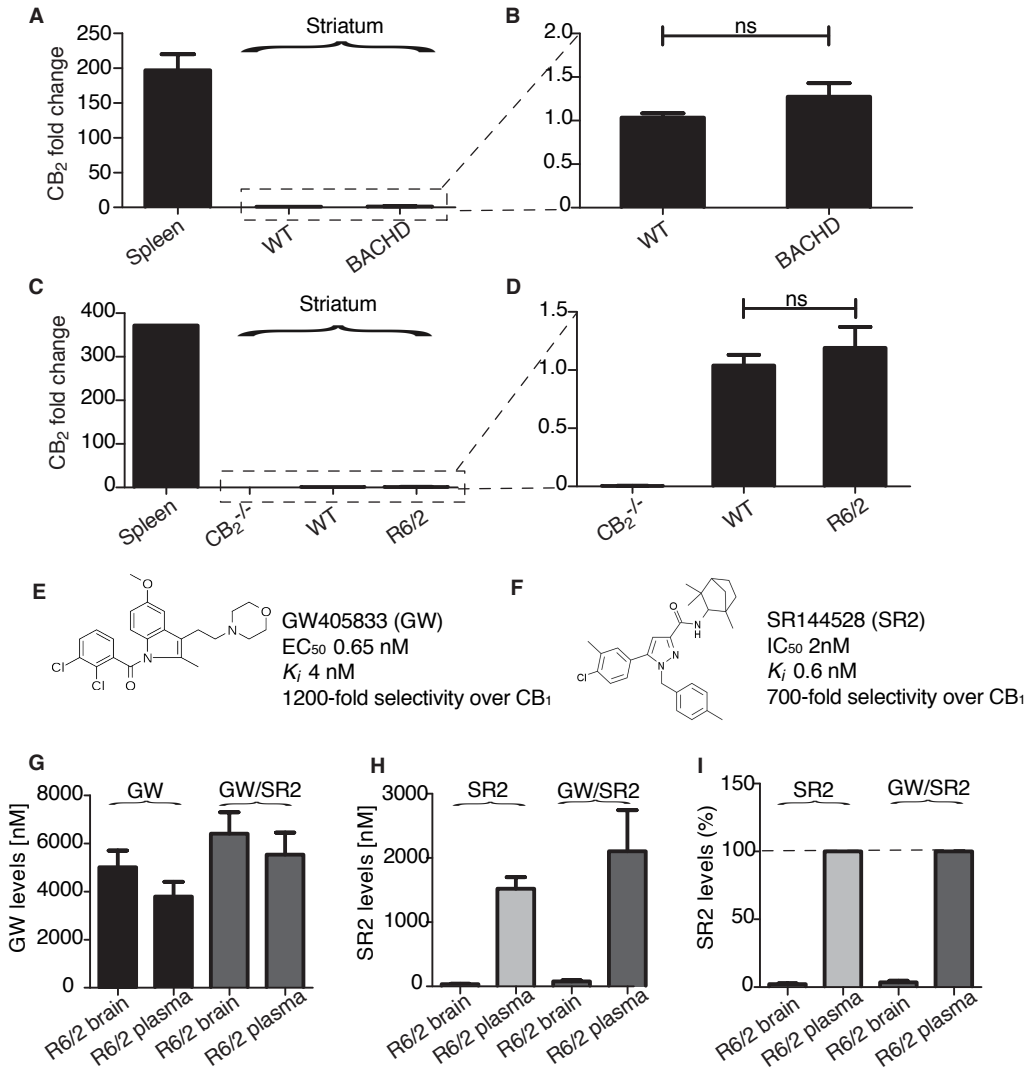
In the open field test, BACHD mice lacking CB_2 reared 30% less by 6 months and nearly 70% less by 15 months than WT and BACHD mice (**Figure 3-1C**). We could not detect an effect in BACHD or $CB_2^{-/-}$ mice alone, indicating that the loss of CB_2 in BACHD mice influences the onset and severity of spontaneous motor activity in the open field. These results demonstrate that loss of CB_2 receptor signaling accelerates behavioral phenotypes of disease progression in BACHD mice.

We next quantified levels of the presynaptic marker synaptophysin in our experimental cohorts. In agreement with a previous study (Kwan et al., 2012a), we observed that synaptophysin levels in 15-month-old BACHD striata were significantly lower than in WT controls (**Figure 3-1D**). Synaptophysin levels were further decreased in BACHD mice lacking CB_2; however, this decrease did not reach statistical significance. Similar results were observed in the cortex (data not shown).

CB_2 Receptor Signaling in Peripheral Immune Cells Suppresses Neurodegeneration in HD Mouse Models

We next sought to determine in which cells CB_2 receptor signaling is important for modulating behavioral phenotypes in BACHD mice. CB_2 receptors are expressed at high levels in peripheral immune cells (Munro et al., 1993; Galiegue et al., 1995; Schatz et al., 1997; Griffin et al., 1999) and under healthy conditions are thought to be expressed in the CNS only by specific neurons on the brain stem (Van Sickle et al., 2005). However, in some neurological diseases associated with strong inflammatory responses and immune cell infiltration into the CNS, such as multiple sclerosis, CB_2 receptor expression is dramatically increased (Maresz et al., 2005; Yiangou et al., 2006). Using qPCR, we

Figure 3-2 CB_2 mRNA and the CB_2 Receptor Antagonist SR2 are Detected at Low Levels in Brain Tissue from HD Mouse Models



(A and B) Relative levels of CB_2 mRNA in striatum from ~15-18 month old BACHD mice (n = 13) and WT littermates (n = 13), normalized to WT levels as determined by qPCR

(C and D) Relative levels of CB_2 mRNA in striatum from ~10-12 week old R6/2 mice (n = 6) and WT littermates (n = 6), normalized to CB_2 -/- levels as determined by qPCR

(E) Chemical structure and pharmacological properties of GW405833 (GW), a CB_2 receptor partial agonist

(F) Chemical structure and pharmacological properties of SR144528 (SR2), a CB_2 receptor antagonist.

(G) The CB_2 receptor agonist GW accumulates at high levels in plasma and brains from mice dosed with GW alone (20 mg/kg i.p.; n = 11) or GW co-administered with SR2 (2.1 mg/kg i.p.; n = 6). Blood and brains were harvested 1 h after i.p. administration, and GW levels were quantified by LC MS/MS

(H) The CB_2 receptor antagonist SR2 accumulates at high levels in plasma but not in brain tissues isolated from mice dosed with SR2 alone (2.1 mg/kg; n = 5) or SR2 co-administered with GW (20mg/kg; n = 6). Blood and brains were harvested 1 h after i.p. administration, and SR2 levels were quantified by LC MS/MS

(I) Concentration of SR2 detected in brains of mice expressed as a percentage of plasma levels in SR2 dosed mice (2.1 mg/kg; n = 5) or SR2 co-administered with GW (20 mg/kg; n = 6). ns, not significant. Values are means ± SEM.

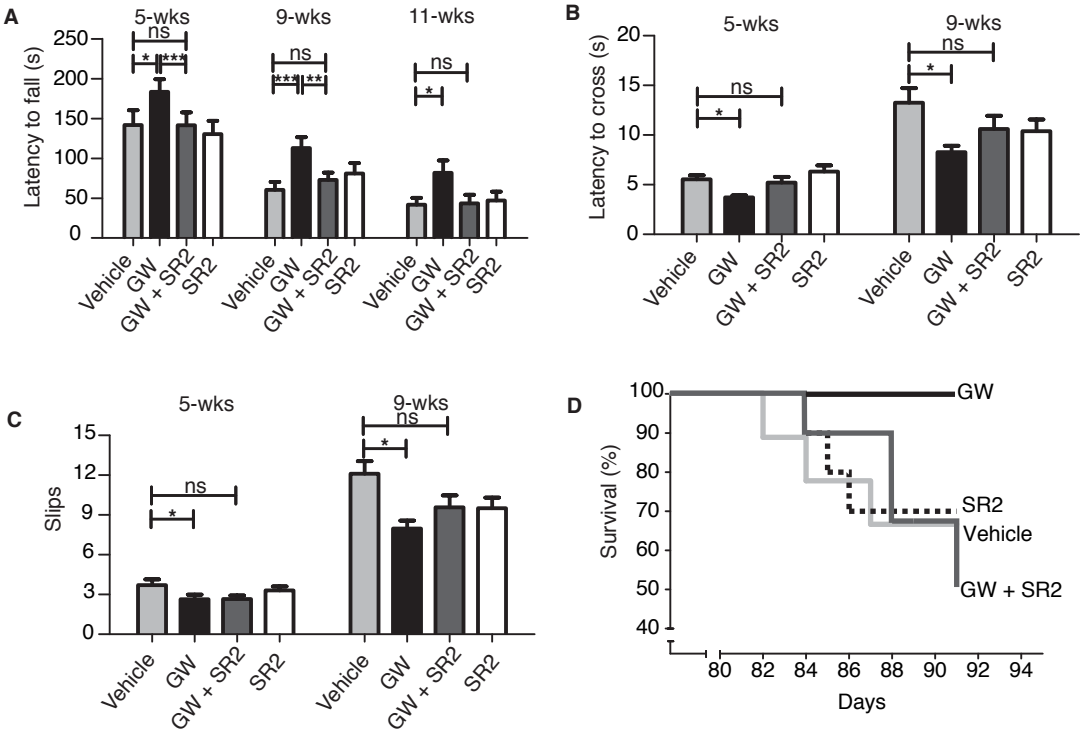
measured the levels of CB_2 mRNA transcripts in isolated striatal tissue from BACHD and WT littermates. In the striatum, CB_2 transcript levels were near the limit of detection in our assay (data not shown). In contrast, levels of the transcript were ~200-fold higher in the spleen (**Figure 3-2A** and **3-2B**). We also quantified CB_2 mRNA in R6/2 mice, which express the first exon of human htt driven by the human htt promoter and display a more rapid and severe disease progression than BACHD mice (Mangiarini et al., 1996; Menalled et al., 2009). Similar to BACHD mice, R6/2 mice had very low levels of CB_2 in striatal tissue and ~300-fold higher levels in the spleen (**Figure 3-2C** and **3-2D**). These studies are consistent with previous studies showing that CB_2 mRNA cannot be detected in rodent brains by *in situ* hybridization, Northern blot analyses or qPCR, but is readily detected by these methods in immune cells (Munro et al., 1993; Galiegue et al., 1995; Schatz et al., 1997; Griffin et al., 1999). Unfortunately, we could not use antibodies to quantify CB_2 protein levels in HD mouse models, since we found that several commercially available CB_2 antibodies stained brain sections from $CB_2^{-/-}$ mice, indicating a lack of specificity for CB_2 (data not shown).

Based on these results, we hypothesized that the action of CB_2 receptor signaling on BACHD progression was likely mediated through peripheral immune cells. If this hypothesis is correct, then treating HD mice with a CB_2 receptor-selective agonist should protect against HD phenotypes, and critically, coadministration with a CB_2 receptor antagonist that does not cross the blood brain barrier (BBB) should block this protective effect. For these drug studies, we used R6/2 mice (Mangiarini et al., 1996), a well-characterized and widely used genetic model of HD. In this mouse model, the first exon of the htt gene (*IT-15*) with a large CAG repeat expansion is expressed under the control

of the 5' end of human *IT-15*. R6/2 mice reliably develop progressive neurological phenotypes, including motor deficits, weight loss, and premature death. These features, along with the rapid progression of symptoms and the relatively short lifespan of the mice, have contributed to their popularity and utility for preclinical studies.

We treated R6/2 mice with GW405833 (GW) (**Figure 3-2E**), a high-affinity (K_i = 4 nM) and highly selective CB_2 receptor partial agonist with a half-life of ~4 h (Valenzano et al., 2005). To block this effect, we coadministered GW with SR144528 (SR2) (Rinaldi-Carmona et al., 1998) (**Figure 3-2F**), a highly selective CB_2 receptor antagonist that displays subnanomolar affinity (K_i = 0.6 nM). A previous study showed that oral administration of SR2 as a single bolus readily displaces *ex vivo* [^{3}H]-CP 55,940 binding to mouse spleen membranes (ED_{50} 0.35 mg/kg) for up to 20 h, but does not prevent binding to cannabinoid receptors expressed in mouse brain membranes (Rinaldi-Carmona et al., 1998), suggesting that SR2 does not cross the BBB. To confirm that SR2 does not cross the BBB, starting at 4 weeks of age, we treated R6/2 mice daily with GW alone (20 mg/kg/day i.p.), SR2 alone (2.1 mg/kg/day i.p.), or GW together with SR2 (20 mg/kg/day i.p. and 2.1 mg/kg/day i.p., respectively). At ~12 weeks of age, plasma and brains samples were isolated from R6/2 mice 1 h after the daily i.p. injections to quantify GW and SR2 levels. When administered alone, we found that GW accumulates at a high concentration in plasma (3.8 ± 0.6 μM) and brain (5.0 ± 0.7 μM) (**Figure 3-2G**). In contrast, although SR2 was also present at a high concentration in plasma when administered alone (1.5 ± 0.2 μM), only very low levels (34.8 ± 6.9 nM, or ~2% of plasma levels) were found in the brain (**Figures 3-2H** and **3-2I**). Similar findings were observed when GW was co-administered with SR2. Low concentrations of SR2 in the

Figure 3-3 Enhanced CB_2 Receptor Signaling in Peripheral Immune Cells Suppresses Behavioral Deficits and Increases Survival in R6/2 Mice



(A) A CB_2 receptor agonist (GW; 20 mg/kg/day i.p. starting at ~4 weeks of age) improves motor deficits in R6/2 mice on the rotarod, while co-administration of a peripherally restricted CB_2 receptor antagonist (SR2; 2.1 mg/kg/day i.p.) prevents this effect

(B and C) GW improves the latency of R6/2 mice to cross and the number of slips in a balance beam traversal assay, while co-administration of SR2 prevents this effect

(D) Kaplan-Meier survival analysis shows that GW increases survival in R6/2 mice, while coadministration of SR2 prevents this effect. Vehicle and GW-treated R6/2 mice were subjected to Logrank (Mantel-cox) test, $p = 0.041$; all groups compared, $p = 0.17$. n = 10-11 mice/group at beginning of study. The number of mice in each group when taken down for neuropathology at ~13-weeks of age: GW-treated mice, n = 11; all other groups; n = 5-7.

Values are means ± SEM. n = 10-11/treatment group for time points 5- and 9-weeks and n = 5-11/treatment group at 11-weeks. *p<0.05; **p<0.01; ***p<0.001. ns, not significant.

brain might reflect small amounts of residual blood that remained in brain preparations despite the saline perfusion. These results indicate that, while GW readily crosses the BBB, CNS penetration of SR2 is greatly limited.

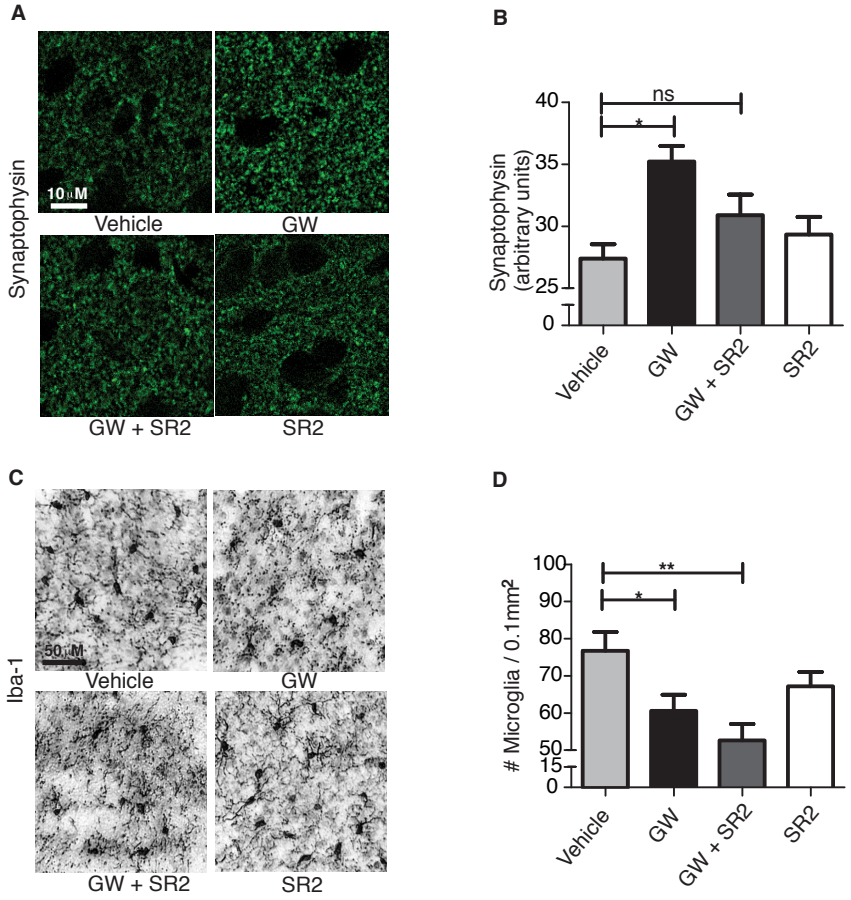
Using the agonist/antagonist strategy described above, we tested the hypothesis that the CB_2 receptor acts through the peripheral immune system. Confirming our hypothesis, administration of the CB_2 receptor partial agonist GW starting at 4 weeks of age, an early symptomatic stage in these mice (Menalled et al., 2009), ameliorated behavioral and neuropathological deficits in R6/2 mice, and this protective effect was negated when GW was coadmistered with the peripherally restricted CB_2 receptor antagonist SR2 (**Figure 3-3**). Indeed, after only ~1.5 weeks of treatment with GW (at ~5.5 weeks of age), GW significantly improved R6/2 mice performance on both the rotarod and balance beam, indicating that CB_2 receptor signaling in peripheral immune cells is important at early disease stages in R6/2 mice. On the rotarod, mice treated with GW performed better during training than vehicle-treated R6/2 mice (data not shown) and stayed on the accelerating rotarod longer during each individual testing session. The protective effect of GW in R6/2 mice in the rotarod assay was observed at all time points tested (5, 9 and 11 weeks) (**Figure 3-3A**). GW-treated mice also took significantly less time to traverse a balance beam and slipped less than controls (**Figure 3-3B** and **3-3C**). Notably, the protective effect of GW in these assays was not observed in mice treated with GW and SR2. Although genetic deletion of CB_2 receptors accelerated the onset and exacerbated the severity of behavioral phenotypes in BACHD mice (**Figure 3-1**), the CB_2 antagonist SR2 alone did not worsen phenotypes in R6/2 mice (**Figure 3-3**). This result is not unexpected, since SR2, similar to other receptor antagonists, does not provoke a

biological response itself upon binding to CB_2 receptors, but only blocks or dampens agonist-mediated responses (Rinaldi-Carmona et al., 1998). Kaplan-Meier survival analysis revealed that R6/2 mice treated with GW lived significantly longer than mice treated with vehicle alone (**Figure 3-3D**). Importantly, the survival curves of R6/2 mice treated with the combination of GW and SR2 were no longer distinguishable from vehicle-treated mice, as were the endpoint survival rates. These results support the two main tennets of our hypothesis: enhanced CB_2 receptor signaling is protective in mouse models of HD, and this effect is mediated by CB_2 receptor signaling in peripheral immune cells.

We next asked if there were neuropathological correlates of improvement in R6/2 mice treated with GW. R6/2 mice show marked neurodegeneration as measured by loss of the presynaptic marker synaptophysin in the cortex and striatum by 11–15 weeks (Zwilling et al., 2011), analogous to BACHD mice (Kwan et al., 2012a) at later times. Consistent with our behavioral results, synaptophysin levels were higher in the striatum **(Figure 3-4A** and **3-4B**) and cortex (data not shown) of ~12-week-old R6/2 mice treated with GW than in those treated with vehicle control. As observed in behavioral assays, coadministration of SR2 prevented the protective effect of GW against synapse loss. Therefore, CB_2 receptor signaling in peripheral immune cells protects against synapse loss in the brains of R6/2 mice.

Previous studies in R6/2 mice and HD brains showed increased Iba-1expression (Simmons et al., 2007; Zwilling et al., 2011), consistent with microglial activation and/or proliferation. R6/2 mice treated with GW had significantly fewer Iba-1-positive cells than vehicle-treated controls (**Figure 3-4C** and **3-4D**). Surprisingly, this effect was not

Figure 3-4 Enhanced CB_2 Receptor Signaling in Peripheral Immune Cells Suppresses Neurodegeneration and Reduces CNS Inflammation in R6/2 Mice



(A and B) A CB_2 receptor agonist (GW; 20 mg/kg/day i.p.) prevents synapse loss in the striatum of ~ 12-week-old R6/2 mice, while co-administration of a peripherally restricted CB_2 receptor antagonist (SR2; 2.1 mg/kg/day i.p.) prevents this effect

(C and D) GW reduces Iba-1 immunostaining in the striatum of ~12-week old R6/2 mice, but SR2 does not prevent this effect.

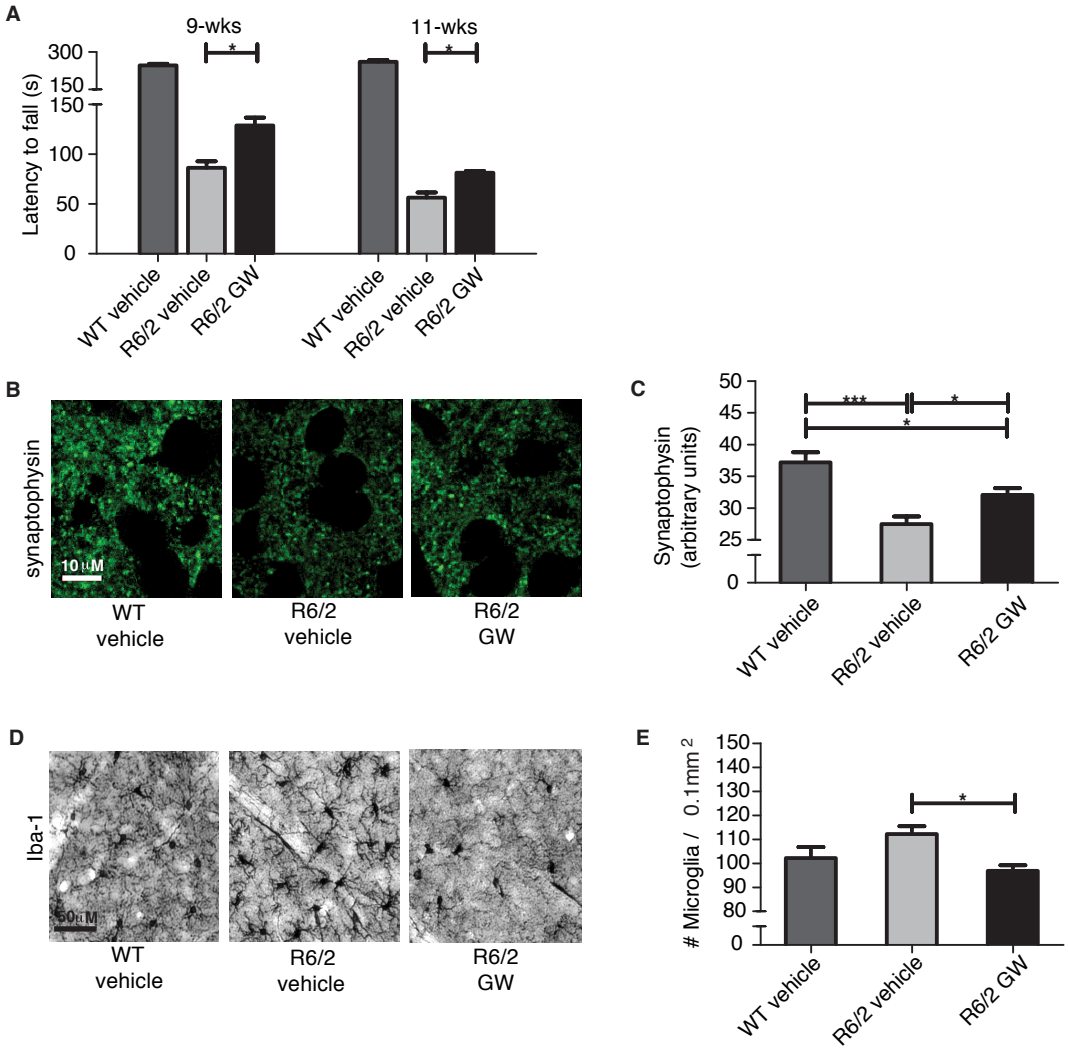
Values are means ± SEM. n = 5-10/treatment group. *p<0.05; **p<0.01; ***p<0.001. ns, not significant.

prevented by coadministration of GW with SR2. As the protective effects of CB_2 receptor signaling on behavioral deficits and synapse loss in R6/2 mice are antagonized by SR2, increased Iba-1 expression in the CNS *per se* probably does not contribute to neurodegeneration, at least under these experimental conditions. However, immunohistochemical levels of Iba-1 are not necessarily indicative of the functional state of microglia, and one cannot rule out the possibility that GW/SR2 treated microglia in R6/2 mice may still secrete higher levels of pro-inflammatory cytokines/chemokines or other mediators of neuronal toxicity. Nevertheless, this result provides further support that the protective effects of enhanced CB_2 receptor signaling are mediated primarily through peripheral immune cells and not through changes in Iba-1 levels in parenchymal microglia.

CB_2 Receptor Signaling in Peripheral Immune Cells Is Protective even when Dosing Is Initiated at Late Disease Stages in HD Mice

Given that treatment with GW prevented behavioral and neuropathological deficits in R6/2 mice when administered starting at an early symptomatic stage (~4 weeks), we next wanted to determine if this effect would still be observed if mice were treated at a late symptomatic stage (when the mice are severely impaired in behavioral assays) (Menalled et al., 2009). Here, treatment began at ~8 weeks of age (20 mg/kg/day, i.p.) and continued daily until mice were ~13 weeks when neuropathological indices were measured. When measuring the rotarod performance of these cohorts at 9 and 11 weeks of age, we found that GW treatment later in disease progression still confers significant benefits (**Figure 3-**

Figure 3-5 CB_2 Receptor Signaling in Peripheral Immune Cells Suppresses Neurodegeneration and CNS Inflammation in Late Stage R6/2 Mice



(A) A CB_2 receptor agonist (GW; 20 mg/kg/day i.p. starting at ~8 weeks of age) improves motor deficits in late-stage R6/2 mice (rotarod)

(B and C) GW (20 mg/kg/day i.p. starting at ~8 weeks of age) prevents synapse loss in the striatum of ~12-week-old R6/2 mice

(D and E) GW (20 mg/kg/day i.p. starting at ~8 weeks of age) reduces Iba-1 immuno staining in the striatum of ~12-week old R6/2 mice.

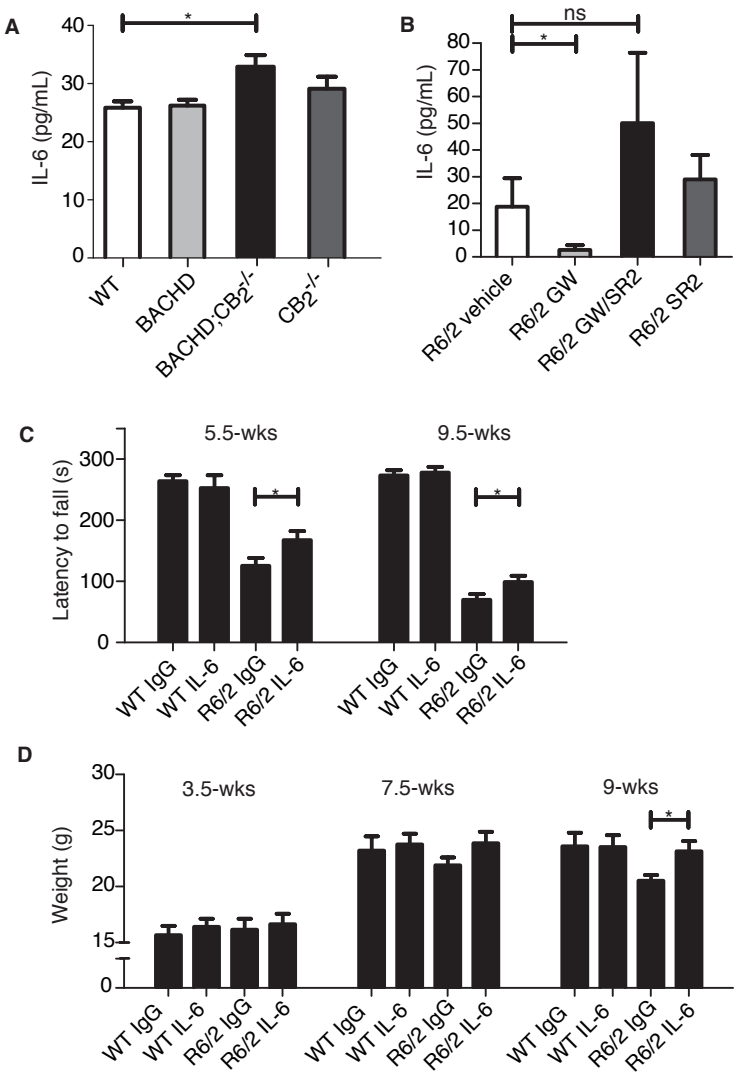
Values are means ± SEM. n = 12-17/ treatment group for (A), and 5-10 for (B-E). *p<0.05; **p<0.01; ***p<0.001. ns, not significant.

5A). Here also, GW partially prevented the loss of synaptophysin immunoreactivity and decreased Iba-1 levels in R6/2 mice (**Figure 3-5B**–**E**), indicating that increasing CB_2 receptor signaling in peripheral immune cells even at late stages of disease is still beneficial in R6/2 mice.

CB_2 Receptors Control IL-6 Levels That Contribute to Disease Phenotypes in HD Mice

Our results indicate that the beneficial effects of treatment induced by a CB_2 receptor agonist on behavioral deficits were elicited rapidly (within ~12 days of dosing), suggesting that perhaps soluble factors secreted from peripheral immune cells, such as cytokines and chemokines, might contribute to neurodegeneration. In an effort to identify potential factors downstream from CB_2 that might contribute to its effects on neurodegeneration, we next quantified levels of the pro-inflammatory cytokines IL-6 and TNF-α in the blood of HD mice from our studies, since CB_2 receptors regulate the production of these cytokines (Klegeris et al., 2003; Rajesh et al., 2008; Su et al., 2011) and that these immune mediators are elevated in HD patients and mouse models (Bjorkqvist et al., 2008). Genetic deletion of CB_2 receptors in BACHD mice led to increased blood levels of IL-6 (**Figure 3-6A**). Conversely, blood IL-6 levels were markedly lower in R6/2 mice treated with GW than in those treated with a vehicle control (**Figure 3-6B**). Importantly, coadministration of SR2 with GW prevented the GW-mediated decrease in IL-6 levels, suggesting that CB_2 receptor signaling in peripheral immune cells is the major source of increased IL-6 production in R6/2 mouse blood. Similar results were found with IL-1β (data not shown), which increases in R6/2 mice

Figure 3-6 CB_2 Receptors Control IL-6 Levels, Which Contribute to Disease Phenotypes in R6/2 Mice



(A) IL-6 serum levels are increased in ~9-10-month old BACHD mice lacking CB_2. Data was subjected to one-way ANOVA ($p<0.05$) followed by Tukey's post hoc analysis. n = 8-10/genotype.

(B) A CB_2 receptor agonist (GW) decreases IL-6 levels in ~12-week old R6/2 mice, and this effect is prevented by co-administration of a CB_2 receptor antagonist (SR2). *$p<0.05$ (student t-test, vehicle vs. GW); ns, not significant. n = 6-11 mice/cohort

(C) Treatment with a neutralizing IL-6 antibody partially rescues motor deficits in R6/2 mice on the rotarod. *$p<0.05$ (student t-test). n = 12-15 mice/group

(D) Treatment with a neutralizing IL-6 antibody prevents weight loss at late stages in R6/2 mice. *$p<0.05$ (student t-test). n = 12-15 mice/group.

Values are means ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

(Bjorkqvist et al., 2008). Levels of TNF-α were not significantly increased in R6/2 or BACHD mice and were not changed in R6/2 mice by GW treatment (data not shown).

These findings led us to hypothesize that IL-6 produced by peripheral immune cells might contribute to pathogenesis in R6/2 mice. To test this hypothesis, we administered an IL-6 neutralizing antibody (1.5 mg/kg i.p.) or a control IgG antibody (1.5 mg/kg i.p.) every other day for 30 days beginning at ~4 weeks of age to R6/2 and WT littermate controls. R6/2 mice treated with the IL-6 antibody performed significantly better than IgG-treated mice in a rotarod assay at each time point investigated (**Figure 3-6C**). Moreover, treatment with the IL-6 antibody prevented weight-loss at 9 weeks of age in R6/2 mice (**Figure 3-6D**). Treatment with the IL-6 antibody had no effect on rotarod performance or body weight in WT mice.

DISCUSSION

In HD, immune system activation is detected many years before the onset of clinical symptoms, persists chronically and correlates with clinical severity scores (Bjorkqvist et al., 2008). Here we show that genetic deletion of CB_2 receptors, that are expressed predominantly in peripheral immune cells, increases the severity of behavioral deficits in BACHD mice and dramatically accelerate their onset. Previous work has shown that the age of onset in HD patients with similar CAG repeat lengths varies widely, suggesting that environmental factors may influence disease onset (Wexler et al., 2004). It is therefore tempting to speculate that immune activation may be one important factor that influences disease onset and/or progression. For instance, polymorphisms in CB_2 or other

immune related molecules may be associated with variation in the age of onset. Moreover, our results suggest that immune system activation by bacterial and viral infections early during HD pathogenesis might be sufficient to trigger the onset of behavioral symptoms and/or accelerate/exacerbate neurodegeneration, hypotheses we are currently testing in HD mouse models.

Our results confirm a previous study in which genetic deletion of CB_2 receptors exacerbated behavioral and neuropathological deficits in R6/2 mice (Palazuelos et al., 2009). However, in this previous study it was argued that CB_2 receptor signaling in parenchymal microglia mediated these effects. Our data from the present study clearly show that a peripherally restricted CB_2 receptor antagonist (SR2) fully blocks all beneficial effects of a CB_2 receptor agonist (GW). Although low nanomolar concentrations of SR2 that were detected in R6/2 brains could conceivably mediate a local neuroprotective effect on microglia if CB_2 receptors were indeed expressed at functional levels on these cells, converging lines of evidence argue against this possibility. First, using qPCR we found that CB_2 mRNA is present at 300X higher levels in peripheral immune tissues relative to striatal tissue in R6/2 and BACHD mice, questioning the physiological relevance of the low levels of CB2 in the brain. Second, the ED_{50} of SR2 after oral administration required to displace *ex vivo* [^{3}H]-CP 55,940 binding to mouse spleen membranes being 0.35 mg/kg is most likely associated with peripheral drug concentrations >100 nM (around 350 nM, assuming a perfect dose-dependent PK linearity) (i.e., far above the brain concentration achieved after SR2 administration to R6/2 mice at 2.1 mg/kg, which is 35 nM in our experiment) (**Figure 3-2H**). Third, while treatment with a CB_2 receptor agonist (GW) decreased apparent microglial activation in

R6/2 brains (as determined by Iba-1 immunostaining), coadministration of GW with SR2 *did not* block this effect (i.e., although the number of Iba-1^{+} microglia in CNS was reduced, behavioral and neuropathological deficits still persisted). Together, these data strongly argue that the effect of CB_2 receptor activity in HD mouse models is primarily mediated by cells in the peripheral immune system, not on microglia in the central nervous system.

Previous genetic and pharmacological studies that report beneficial effects of CB_2 receptor signaling in animal models of neurodegeneration have interpreted these results as being mediated by parenchymal microglia (Arevalo-Martin et al., 2003; Pryce et al., 2003; Kim et al., 2006; Shoemaker et al., 2007; Zhang et al., 2007; Palazuelos et al., 2009; Murikinati et al., 2010; Garcia et al., 2011; Martin-Moreno et al., 2012). However, data from this study combined with methodological issues in these previous studies question the validity of that interpretation. Like Palazuelos *et al*., many of these studies used immunohistochemical techniques to show that levels of the CB_2 receptor are increased in disease states in the CNS. These results are not easily interpretable because commercially available antibodies do not selectively recognize CB_2 (i.e., all commercial antibodies described to date stain brain sections in $CB_2^{-/-}$ mice), and when they were used, the proper controls were not incorporated into their experimental design (e.g., $CB_2^{-/-}$ tissue). Unfortunately, the majority of previous studies in which CB_2 receptor agonists showed beneficial effects in animal models of neurodegeneration did not include pharmacokinetic measurements to establish the site of action for these compounds, and in general did not include an antagonist to demonstrate specificity for CB_2. In the absence of these controls, it is premature to conclude that the protective effects of CB_2 agonists

derive from CB_2 signaling in microglia, since compelling evidence for CB_2 receptor expression in these cells is absent. Moreover, CB_2 compounds may also bind to CB_1 and/or may exert nonspecific effects if inappropriate concentrations are used. Based on these caveats, our results suggest that the interpretation that CB_2 signaling in microglia mediates neuroprotection in mouse models of neurodegeneration needs to be re-evaluated. Importantly, CB_2 agonists restricted to the periphery would avoid potential side effects due to CB_1 agonism in the CNS.

The molecular mechanisms by which CB_2 receptor signaling in peripheral immune cells modulates neurodegeneration are likely to be complex and will need to be further explored. Several laboratories reported that CB_2 receptor agonists decrease the production of pro-inflammatory cytokines (Klegeris et al., 2003; Rajesh et al., 2008; Su et al., 2011), including those that are elevated in HD (Bjorkqvist et al., 2008). Consistent with these studies, we found that CB_2 receptors regulate IL-6 levels in HD mouse models, and that a neutralizing antibody to IL-6 partially ameliorates behavioral deficits in R6/2 mice. IL-6 crosses the BBB (Banks et al., 1994) and its functional signaling is important for brain development, learning and memory, and CNS responses to disease and injury (Bauer et al., 2007; Jankord et al., 2010). Increased CNS levels of IL-6 have been linked to impairments in memory performance and sickness behavior during inflammation (Bluthe et al., 2000; Sparkman et al., 2006), and transgenic mice bearing additional copies of the IL-6 gene under the control of a brain-specific promoter develop a marked cortical pathology, including severe alterations of the dendritic arborization of cortical neurons (Campbell et al., 1993). While these studies indicate that increased IL-6 levels are sufficient to promote behavioral changes and neurodegeneration in the CNS, the

detailed molecular mechanisms that mediate these events remain to be elucidated. For instance, IL-6 produced in the peripheral immune system may be crossing the blood-brain-barrier and exacerbating neurodegeneration through direct effects on neurons or indirect effects on microglia. However, given that treatment with an IL-6 antibody only showed a partial rescue of motor deficits in R6/2 mice, these results suggest that the beneficial effects elicited by CB_2 receptor signaling in HD mice are likely to be mediated by signals in addition to IL-6.

More broadly, our data are consistent with other recent studies suggesting that soluble signals derived from peripheral immune cells transduce signals into the CNS that contribute to cognitive dysfunction and/or neurodegeneration. For example, plasma from old mice, that has elevated levels of chemokines, impairs learning and memory in young mice (Villeda et al., 2011). Blocking peripheral immune molecules (e.g. CD40 and TGF-beta-Smad2/3) attenuates pathology in a mouse model of Alzheimer's disease (Tan et al., 2002; Town et al., 2008). Given that CB_2 receptor signaling also regulates immune cell migration (Miller and Stella, 2008), which is impaired in HD (Kwan et al., 2012b), the protective effects of CB_2 receptor agonists might be mediated by effects on immune cell migration coincident with dampening of pro-inflammatory cytokine production.

The development of peripherally restricted CB_2 receptor agonists holds promise for treating HD and other neurodegenerative diseases. The beneficial effects of enhancing CB_2 receptor signaling in HD mice occur rapidly after initial dosing and this therapeutic effect is still present in severely impaired mice. Elucidating the molecular mechanisms downstream from CB_2 receptors that mediate neuroprotection may have important implications for HD research. For example, transgenic expression of IL-6 causes muscle

degeneration which is prevented with an IL-6 antibody, and IL-6 might contribute to muscle degeneration and cachexia in HD. Given that an IL-6 antibody is an effective and well-tolerated treatment for arthritis (Maini et al., 2006), additional studies to investigate the therapeutic benefits of modulating levels of cytokines, such as IL-6, in HD are clearly warranted.

In summary, our results suggest CB_2 receptor signaling in peripheral immune cells has an important role in HD and other neurodegeneration disorders. Further elucidation of the molecular mechanisms that underlie these effects may lead to novel therapeutic strategies to treat these disorders.

MATERIALS & METHODS

Animals and Breeding Strategy

Experiments involving mice were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Mice were bred and maintained in compliance with National Institutes of Health guidelines. R6/2 mice were obtained by breeding R6/2 ovarian transplants with 160 CAG repeat length (002810 Jackson) with CBA WT males. BACHD mice (Gray et al., 2008) were obtained from Dr. William Yang (UCLA) and were maintained by breeding to WT FVB/NJ males. $CB_2^{-/-}$ mice (Buckley et al., 2000) on C57/BL6 background were obtained from Dr. Nancy Buckley. Using speed congenics, $CB_2^{-/-}$ mice were backcrossed for 10 generations onto a FVB/NJ background prior to breeding to BACHD mice. To test if loss of CB_2 worsened HD progression, we crossed the $CB_2^{-/-}$ mice to BACHD mice. Progeny from these litters that were BACHD; $CB_2^{+/-}$ were then bred to each other to generate BACHD; $CB_2^{-/-}$ mice and all littermate controls.

Genotyping

Mouse-tail DNA was analyzed by PCR to determine the genotypes. The BACHD transgene was identified as described (Gray et al., 2008). $CB_2^{-/-}$ mice were identified as described (Buckley et al., 2000). The R6/2 transgene was identified as described (Hockly et al., 2003).

RNA Extraction, Reverse-Transcription, Real-Time PCR Analysis

Brains from BACHD and R6/2 mice were collected after perfusing with 0.9% saline transcardially followed by snap freezing in dry ice and storage at –80 °C until use. On the day of RNA isolation, striatal tissue was collected and placed in 1 ml of Qiazol reagent. Brains were homogenized and 200 μl of chloroform was added and centrifuged at 4°C for 15 min. An equal volume of 70% EtOH was added to the supernatant and mixed together. Immediately after this step, samples were isolated with the Qiagen RNA Lipid Isolation Kit and their standard protocol and then treated with DNase (Qiagen) for 15 min and subsequent reverse transcription was performed with RT Superscript III (Invitrogen). qPCR reactions were performed on the ABI Prism Sequence Detector 7700HT with TAQMAN PCR master mix (Applied Biosystems). For qPCR data we used the delta CT method and each reaction was normalized against the expression of the ATP5B (4331182 Applied Biosystems). The primer sequences from 5' to 3' for CB_2 are forward primer, AAGGTCCTCGGTTACAGAAACA; reverse primer, GGAGTGAACTGAACGGACTTCT; taqman probe, 6FAM-TCCAGAACTCCAGGCTGCTCCAAC-TAMRA (Applied Biosystems).

Drug Studies

For the first experiment, R6/2 mice received either vehicle (10% EtOH, 20% Cremaphor EL (Sigma), 70% saline), SR2 antagonist (2.1 mg/kg), GW405833 (20 mg/kg), or SR2 and GW405833 (2.1 mg/kg and 20 mg/kg, respectively), once daily via i.p. Treatment began at ~4 weeks of age and continued throughout the experiment until mice either died or were euthanized. The methods for the second experiment were identical, except that dosing was initiated at ~8-weeks of age. All compounds were dissolved in vehicle.

Briefly, ethanol was added and each mixture was incubated for 5 min at 55°C. This was then followed by the addition of Cremaphor EL and another incubation at 55°C for 15–20 min. Saline was added once all compounds were dissolved. Drugs were formulated for 7 days at a time and stored at –20 °C until use.

Behavioral Analysis

An accelerating rotarod was used to analyze motor coordination and balance. Baseline behavior was performed at ~4 weeks, and mice were placed into balanced cohorts based on behavior, sex, and genotype. For R6/2 studies, mice were trained three times at a constant speed of 16 rpm for maximum of 300 sec. During testing, mice were subjected to beam acceleration of 4–40 rpm for a maximum of 300 seconds, three times per session for a total of three sessions. Performance was quantified by measuring the latency to fall off of the rotarod apparatus.

The balance beam was also used to assess motor coordination and balance. This test consisted of three sessions with three trials in each sessions: one session of training, one session of testing on large diameter beam, one session of testing on medium diameter beam. Motor performance was assessed by measuring the time it takes for the mouse to traverse the beam and the number of hind paw slips that occur in this process.

Rearing activity was assessed in an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments). Before testing, mice were acclimated in the testing room for 1 h. Mice were then placed in open field chambers with two 16x16 photobeam arrays to detect horizontal and vertical movements. Rearing behavior (the

number of times the mouse stood erect on its hind legs) was measured over 30 min. Chambers were cleaned with 70% ethanol before and after each mouse.

Survival in R6/2 mice was evaluated as the time when the animals either died spontaneously or had lost more than 20% of their peak body weight. For all studies the experimenter was blind to the genotype and/or treatment group.

Neuropathology

Mice were anesthetized with avertin (tribromoethanol, 250 mg/kg) and perfused with saline. Hemi brains were then drop fixed in 4% PFA for 48 h and sectioned into 40-μm sections with a Leica Vibratome. Free-floating sections were incubated in either 10% horse (synaptophysin) or goat serum (Iba-1) in 0.1% Triton X-100 for 1 h before incubation with primary antibodies at 4°C for 24 h. To investigate the effects of the CB_2 receptor agonist on microglial activation/accumulation, free-floating sections were immunostained with a mouse monoclonal antibody against Iba-1 (1:1000, DakoCytomation, CA), followed by biotinylated secondary antibody, avidin coupled to horseradish peroxidase, and reacted with DAB. The number of microglia per unit area of 0.1 mm^2 were assessed using Image J. For synaptophysin staining, sections were immunostained with anti-synaptophysin (1:200; Roche Diagnostics) and FITC-conjugated horse anti-mouse IgG (1:500; Vector Laboratories), and fluorescence images were obtained on the Spectral Confocal C1Si at the NIKON imaging center, UCSF. The percentage of fluorescent immunostaining was evaluated using the Image J software. For all neuropathological analysis, at least 3 sections were analyzed per mouse and for each section at least three fields of view were imaged randomly. For comparison across

genotypes we selected sections with similar neuroanatomical features, such as the hippocampus or striatum. For image quantification each image was assessed using similar microscopy settings and thresholding in Image J. For all studies the experimenter was blind to the genotype and/or treatment group.

Cytokine Analysis

Blood samples were collected from R6/2 mice at 12–13 weeks and BACHD mice at ~9–10 months of age. IL-6 plasma levels were assessed with the Mouse IL-6 Quantikine kit (R & D Systems) for R6/2 studies, and with the mouse chemokine 5-plex panel (Invitrogen) and the Luminex SD xMAP machine for BACHD studies.

IL-6 Antibody Treatment

R6/2 and WT mice were weaned and genotyped at ~3 weeks. Baseline behavior was performed at ~4 weeks, and mice were placed into balanced cohorts based on behavior, sex, and genotype. R6/2 and WT mice were given i.p. injections of a neutralizing antibody against IL-6 (anti-IL-6; rat IgG) (R & D Systems) dissolved in saline. Control mice were given injections of the rat IgG control antibody (R & D Systems) which is the same isotype as the anti-IL6 neutralizing antibody used in this study. Mice were injected with IL-6 neutralizing antibodies or control antibodies (1.5 mg/kg i.p.) every other day starting at ~4 weeks and continued until mice were sacrificed at ~9.5 weeks. Behavioral performance was assessed at ~5.5 and ~9.5-weeks of age using the rotarod assay. Mice were weighed biweekly.

Statistics

All data are expressed as the mean +/- SEM. Unless otherwise indicated, a one-way ANOVA with Bonferroni's post-hoc test was performed to determine levels of significance between experimental groups at each time point.

Chapter 4: Thesis Conclusions

In this thesis, I investigated three different physiological systems in the context of HD through manipulations of the endocannabinoid system. We found that physiological systems outside the CNS, including the peripheral immune and musculoskeletal systems, are significant contributors to HD pathogenesis. Since each chapter's results are discussed in depth, the conclusions presented here are devoted to larger implications and future directions.

Traditionally, it was thought that the CNS and the peripheral immune system were not in communication with one another. This concept of strict mutual exclusivity has been weakened by experimental evidence showing that the CNS is neither isolated nor passive in its interactions with the immune system. We now know that peripheral immune cells can cross the intact blood-brain barrier, and CNS neurons and immune cells, both centrally and peripherally, can influence several physiological processes (Carson et al., 2006; Quan, 2008) . Peripheral immune system dysfunction was observed in HD patients many decades prior to discovery of the underlying causative mutation (Beverstock, 1984). However, these changes have traditionally not been given much thought, as they were thought to stem from general sickness or the consequence of neurological dysfunction (van der Burg 2009). While we do not address what came first (i.e., peripheral immune system or CNS dysfunction), our studies demonstrate that regardless of origin, these pathological changes are not without functional consequences in HD. Furthermore, these results are the first evidence that changes observed in the peripheral immune system of HD patients are functionally relevant to disease progression. One can therefore imagine that dysfunction in the peripheral immune system, which can

be detected prior to overt CNS neuropathological changes, could provide a window into CNS dysfunction.

Perhaps more relevant to the HD community is the identification of both the CB_2 receptor and IL-6 as promising targets for disease-modifying therapies. Research has shown a potential role for the CB_2 receptor in brain microglia activation but failed to assess the contribution of CB_2 in the peripheral immune system, where its expression level is high compared to the CNS. Importantly, targeting the endocannabinoid system in the blood overcomes the dual challenges of getting disease-modifying therapies across the blood-brain barrier and the risk of inducing undesirable psychoactive effects through CB1 activation in the brain. Indeed, drugs similar to the CB_2 agonist GW are in clinical trials for other inflammatory diseases (clinical trial no. NCT00447486, GW842166), lending further support to the concept of CB_2 as a viable clinical target. We also found that neutralizing IL-6, a cytokine whose levels are significantly elevated nearly 20 years prior to disease onset, in HD mice conferred modest protection from motor deficits and suppressed weight loss, suggesting that lowering levels of IL-6 in presymptomatic HD patients may be beneficial. Neutralizing antibodies similar to IL-6 are used for treatment of inflammatory diseases (Nishimoto et al., 2004) and could easily be tested in HD patients. Combined, these studies provide a platform to further investigate the use of CB_2-selective cannabinoids that target the peripheral immune system and IL-6-lowering approaches as treatments for HD.

In HD, a critical relationship exists between CAG-expansion length in the huntingtin gene and age of disease onset (Duyao et al., 1993; Stine et al., 1993). However, there is large variability in disease onset for individuals with the same CAG-repeat length,

suggesting that other factors may be involved. In addition to known environmental factors and genetic modifiers (Wexler et al., 2004), our studies identify physiological systems outside the CNS as an additional contributor to HD progression. The relative importance of early immune activation in HD is unclear, but studies from our lab investigating the effects of different immune challenges on HD progression are underway. It is possible, for example, that an HD patient's history of bacterial or viral infections—acquired over their lifetime—may be priming their immune system and modulating disease onset and severity. Conceivably, doctors that treat HD patients could begin to inquire about an individual's history of infections/diseases, so that a more comprehensive view of each patient can be documented and potentially compared with other HD patient histories. Studies similar to these are underway in the United Kingdom (personal communication with Nancy Wexler), which is home to a large HD population.

Here, we show that the peripheral immune system can functionally contribute to the progression of HD. However, future experiments will be necessary to tease apart the discrete contributions of the two main arms of the peripheral immune system: the innate immune system, responsible for the non-specific response to infections, and the adaptive system, a more complex system based on antigen-specific responses. All types of infections lead to activation of the innate but not necessarily the adaptive immune system. While the evidence so far supports the presence of widespread innate immune–system activation (Bjorkqvist et al. 2008), whether adaptive immune–system dysfunction plays a role in HD progression has not been fully investigated. For instance, studies show that levels of standard IgG and IgM levels are not elevated in serum from HD patients, suggesting that the adaptive arm is not dysfunctional (Bjorkqvist et al., 2008). However,

as these molecules are short lived (Stanley, 2002), and long-term memory in the adaptive arm is endowed at the cellular level via memory B cells (Tangye and Tarlington, 2009), it is possible that instead of detecting baseline changes there may be a dysfunctional response during infection that activates the adaptive immune system. More recently, studies showed that there is a correlation between mHtt levels and disease progression (brain atrophy and disease burden score) in monocytes, cells important in the innate immune response. Similar observations were found in adaptive immune–cell types such as T cells, and to a lesser extent B cells, suggesting that the adaptive immune system may also be abnormal (Weiss et al., 2012). Additionally, mHtt affects signaling pathways that several immune cell types have in common; for example, the actin remodeling pathway necessary for cell migration (Kwan et al., 2012) or the NFκB pathway important for all immune cells (unpublished data, Sarah Tabrizi). Given that mHtt is found in every immune cell (Galiegue et al.), and its expression correlates with disease (Weiss et al., 2012), these signaling cascades that are dysfunctional in the innate immune system may also be abnormal in adaptive immune cells. Furthermore, while the CB_2 receptor has been mostly implicated as a mediator of inflammation, an innate immune response, studies show that CB_2 plays a role in B-cell function in the adaptive arm of the immune system (Pereira et al., 2009), suggesting that it may have a role there. Future experiments directed towards challenging the different arms of the peripheral immune system, and documenting immunological changes and history in human patients, will inform as to the influence of each individual arm on HD progression.

Lastly, HD mouse models are the first-pass *in vivo* system for identifying potential drug therapies in HD. Therefore, determining how specific experimental manipulations

alter the HD phenotype are important when determining whether or not further investigation of a treatment is warranted (e.g. in larger mouse studies or human clinical trials). In chapter 2 we clearly show that changes in the musculoskeletal system (i.e., weight) can influence the HD phenotype in BACHD mice. In fact, after accounting for these changes statistically, there is no longer an effect of mHtt on the CNS. The consequence of this finding cannot be emphasized enough. The ability of an organism to execute movement and motor control, a major clinical pathology of HD, is not just accomplished by the CNS but in fact relies on other physiological systems. By not accurately phenotyping the HD mouse, as shown in the BACHD mouse model, interpretation of data from such studies may be misleading and ultimately incorrect. Here, we provided a statistical tool, the ANCOVA, which will account for potential covariance across genotypes that may arise in studies with BACHD mice. Future studies will need to determine the effect of weight on all behaviors, not just the rotarod, as weight may be playing a role in other commonly used HD assays used to assess HD progression.

Together, the data presented in this dissertation underscore how studies of HD should transcend physiological systems, focusing on an integrated rather than solely CNS view of the disease. Doing so may provide a better understanding of HD as well as increase the potential for discovery of disease-modifying therapeutic targets that are desperately needed for HD patients. Future studies utilizing the CB_2 receptor will surely reveal the mechanistic details of how the peripheral immune system changes observed in HD influence disease progression.

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