Molecular Mechanisms Underlying Neuropathology in Mouse Models of Huntington’s Disease
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in Mouse Models of Huntington’s Disease

A dissertation submitted in partial satisfaction of the requirements
for the degree of Doctor of Philosophy in Neuroscience

by

Jeffrey Philip Cantle

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ABSTRACT OF THE DISSERTATION

Molecular Mechanisms Underlying Neuropathology in Mouse Models of Huntington’s Disease

by

Jeffrey Philip Cantle

Doctor of Philosophy in Neuroscience

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Professor Xiangdong William Yang, Chair

Huntington’s disease (HD) is the most common dominantly-inherited neurodegenerative disease and affects roughly 30,000 patients in the United States, with another 150,000 at risk for developing the disease. HD is caused by an expanded polyglutamine repeat in the Huntingtin protein, which is encoded by an expanded CAG repeat in the Huntingtin gene. HD causes severe and relatively selective death of striatal medium-sized spiny neurons and cortical pyramidal neurons, leading to the typically mid-age onset of a clinical triad of symptoms: motor dysfunction, cognitive decline, and psychiatric disturbances, although the age of onset is variable and inversely correlated with the length of CAG repeat. Much work characterizing mechanisms underlying HD pathogenesis has come from the development of genetic mouse models of the disease. This dissertation will detail work performed in characterizing the cellular and molecular determinants of HD pathogenesis in novel mouse models developed in the Yang lab. We have
used conditional genetics to define cells in the cortex and striatum as essential to developing HD phenotypes, then assessed molecular signatures in these cells that are dependent on the mutant huntingtin protein. Further, we have used mice in which the N17 domain, which is critical for exclusion of small, toxic huntingtin fragments from the nucleus, to assess the effects of nuclear mutant huntingtin on the development of disease-related phenotypes including behavior, neuropathology, and transcriptionopathy. Work in this thesis helps lead to the bases underlying the pathology of HD, as well as informing future studies aimed at developing therapeutic interventions.
The dissertation of Jeffrey Philip Cantle is approved.

Michael S. Levine
Kelsey C. Martin
Desmond Smith

Xiangdong William Yang, Committee Chair

University of California, Los Angeles
This dissertation is dedicated to my wife, Alexandra,

and parents, Peter and Cindy, for their unconditional love and support.
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Xiaofeng Gu, myself, Erin Greiner, Daniel Lee, Albert Barth, Fuying Gao, Chris Park, Zhiqiang Zhang, Susie Sandoval-Miller, Richard Zhang, Marc Diamond, Istvan Mody, Giovanni Coppola, and William Yang. Xiaofeng Gu generated the mice and led the phenotyping effort, to which I contributed behavioral, neuropathological, and transcriptional analyses. Erin Greiner performed all Western blots shown. Susie Sandoval-Miller and Richard Zhang quantitated CDL behavior, and Albert Barth and Istvan Mody performed the in vivo field recordings. Daniel Lee performed cell culture experiments. Bioinformatics were performed by Fuying Gao and Giovanni Coppola. Work by other authors is included in the published study, but not in this dissertation.
Vita

Jeffrey Philip Cantle

EDUCATION

Ph.D. Candidate, Neuroscience
University of California, Los Angeles; Los Angeles California
Expected Completion June 2015
Bachelor of Science, Animal Physiology and Neuroscience
University of California, San Diego; La Jolla, California

June 2006

RESEARCH

Graduate Student Researcher
Laboratory of Dr. X. William Yang, University of California, Los Angeles
June 2008-Present
Thesis title: Molecular Mechanisms Underlying Neuropathology in Mouse Models of Huntington’s Disease

• Perform behavioral and molecular assays on genetically engineered mice to determine genetic interactions that may perturb or ameliorate Huntington’s disease-like phenotypes
• Skills include brain microdissection, RNA sequencing, quantitative PCR, microarray, Western blotting, cloning, immunohistochemistry, microscopy, behavioral phenotyping of motor, cognitive, and psychiatric-like deficits in mice
• Data analysis using statistics software including Prism, SPSS, and R
• Extensive experience in proofreading and grant / manuscript editing, lab safety coordination, and UCLA material / intellectual property transfer protocols

Research Assistant
Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies
June 2006-September 2007

Cloned expression constructs and purified corticotropin releasing factor (CRF) receptor with specific mutations to be used in structural analysis

• Performed radioligand binding assays of neuroendocrine analogues and receptor mutants to validate structural observations, search for drug candidates
• Developed tissue autoradiography protocols to determine regions of CRF binding in mouse and rat brain

PUBLICATIONS AND PRESENTATIONS


Neuronal targets for reducing mutant huntingtin expression to ameliorate disease in a mouse model of Huntington's disease. Wang N, Gray M, Lu XH, Cantle JP, Holley SM,


Chapter 1
Introduction to Huntington’s Disease
1.1 A Brief History of Huntington’s Disease

George Huntington first described what is now known as Huntington’s disease (HD) in his 1872 article *On Chorea*, referring to it as “the hereditary chorea.” In the article, he contrasts his description of Sydenham’s chorea, which he seems to largely dismiss as “by no means a serious or dangerous affection,” with the sinister nature of the disease that would later bear his name:

> It begins as an ordinary chorea might begin, by the irregular and spasmodic action of certain muscles, as of the face, arms, etc. These movements gradually increase, when muscles hitherto unaffected take on the spasmodic action, until every muscle in the body becomes affected (excepting the involuntary ones), and the poor patient presents a spectacle which is anything but pleasing to witness. I have never known a recovery or even an amelioration of symptoms in this form of chorea; when once it begins it clings to the bitter end. No treatment seems to be of any avail, and indeed nowadays its end is so well-known to the sufferer and his friends, that medical advice is seldom sought. It seems at least to be one of the incurables. (Huntington, 1872)

Huntington described the disease briefly, but hit upon three salient aspects of the disease: the hereditary nature, which he accurately describes as never skipping a generation; the involvement of cognitive and psychiatric symptoms, which he describes as “a tendency to insanity and suicide;” and the adult-onset of movement disorder (Huntington, 1872).

HD is typically fatal within twenty years of the onset of clinical symptoms (Walker, 2007). The description of chorea and the genetic component made the disease of interest to the scientific and medical communities, and researchers quickly confirmed the dominant mode of inheritance (Jelliffe et al., 1913). While HD was known to be of genetic origins, it took a large-scale multi-year effort to track down the causal mutation. This collaboration, known as the
Huntington’s Disease Collaborative Research Project, used state of the art genomics techniques and a population of HD afflicted families in the region of Lake Maracaibo, Venezuela to map the approximate location of the Huntington’s mutation in 1983 (Gusella et al., 1983). Ten years later, the group announced that HD is caused by a trinucleotide CAG repeat in the huntingtin gene (HTT in human, Htt in mice) encoding an expanded polyglutamine (polyQ) repeat in the huntingtin protein (HTT or Htt, for human and mouse respectively) (Group, 1993). Further work has shown that CAG repeat lengths vary somewhat but are below 35 in the general population, while HD patients typically have repeat lengths in excess of 40. Additionally, there is reduced penetrance of HD in the 35-39 repeat range (Wasmuth et al., 1997), and CAG repeat lengths become unstable above 28 (MacDonald et al., 1993). The CAG expansion causes neurodegeneration of primarily the caudate / putamen and cerebral cortex (Mattsson et al., 1974), although other regions are affected (Vonsattel and Difiglia, 1998), and leads to the clinical triad of symptoms: movement disorder, cognitive decline, and psychiatric disturbances. With the discovery of the causal mutation in HD, genetic mouse models were quickly developed, first featuring N-terminal fragments of mHTT, and later with full-length mouse and human huntingtin (reviewed in Crook and Housman, 2011). Despite the known etiology and decades of focused research, there is yet no disease course-modifying treatment for HD.

1.2 HD Molecular Genetics

Huntington’s disease is caused by a trinucleotide (CAG) expansion in HTT (Group, 1993). Repeats between 35-39 show reduced penetrance for HD, while those over 40 typically confer a middle-age onset of HD’s motor, psychiatric, and cognitive symptoms (Orr and Zoghbi, 2007). However, there exists an inverse relationship between CAG repeat length and age of
onset (Stine et al., 1993), with particularly long CAG repeats (typically those over 60) leading to the onset of juvenile HD (Andresen et al., 2007). HTT is proposed to have multiple roles in cellular biology, having functions in multiple cellular compartments, localizing with multiple organelles, acting as a scaffold protein, in cellular transport, as well playing a distinct role in the nucleus (Cattaneo et al., 2005; Landles and Bates, 2004). Indeed, HTT has many binding partners determined by proteomics studies, with CAG length affecting a subset of these (Shirasaki et al., 2012).

The HTT gene encodes the long, 3144 amino acid huntingtin protein, which is ubiquitously expressed in the body (Sharp et al., 1995). The protein itself shares little homology with others. It features several notable domains, including the N-terminus 17 amino acid domain (N17) preceding the polyQ domain, followed by a polyproline (polyP domain) and HEAT repeats. HTT function is essential for development, with mouse Htt null mice exhibiting embryonic lethality (Duyao et al., 1995; Zeitlin et al., 1995), as well as for normal brain function, with conditional knockout of Htt in adult mice leading to forebrain degeneration (Dragatsis et al., 2000). Mutation of the HTT polyQ domain to greater than 40Q, while it may confer mild loss of normal HTT function, appears to primarily cause a dominant toxic gain of function (Landles and Bates, 2004). While normal huntingtin is protective, mutant huntingtin causes multiple pro-pathogenic pathways, including sequestration of transcription factors (Dunah et al., 2002), disruption of pro-survival signaling (Chaturvedi et al., 2012; Nucifora, 2001; Steffan et al., 2000), alterations to the ubiquitin proteasome pathway (Jana et al., 2001), toxic cortico-striatal signaling (Cepeda et al., 2007; Milnerwood et al., 2010), and loss of trophic support (Zuccato et al., 2001; 2005). Like other CAG repeat expansion diseases, such as the androgen receptor in spinal and bulbar muscular atrophy, there is evidence that mHTT translocation to the nucleus in
some way exacerbates cellular CAG length dependent pathology (Landles et al., 2010; Schilling et al., 2004; Wheeler et al., 2000).

A striking finding of HD neuropathology is the presence of mHTT aggregates (DiFiglia et al., 1997). While aggregates were initially assumed to be toxic, the further work has shown that aggregates do not correlate with neuronal loss (Gutekunst et al., 1999) and longitudinal studies of cells with mHTT aggregates suggest that they may actually be protective (Arrasate et al., 2004). A more complex story is likely, with the exact species of aggregates as well as the cellular localization playing critical roles in pathogenesis of HD (Arrasate and Finkbeiner, 2012; Ratovitski et al., 2009).

1.3 HD Neuropathology

A rich history of postmortem neuropathological studies informed the field’s early understanding of HD pathogenesis. Early studies found massive loss of brain weight and degeneration of the cortex and basal ganglia (Mattsson et al., 1974). Losses of 20% of cortical and 60% of striatal weight were found, with neuronal loss, astrogliosis, and microgliosis (Averback, 1980; Lange et al., 1976; Roos et al., 1986; Sapp et al., 2001). Of the volume lost, a large amount is due to the death of striatal GABAergic medium-sized spiny neurons, with cortical pyramidal neurons (CPNs) also being lost while interneurons are largely spared (Cudkowicz and Kowall, 1990; Ferrante et al., 1987; Vonsattel et al., 1985a). Interestingly, there seems to be preferential loss of MSNs that project to the globus pallidus externa (GPe), the so-called indirect pathway MSNs that express dopamine receptor D2 and enkephalin, compared to MSNs that project to the globus pallidus interna (GPi), the direct-pathway MSNs that express dopamine receptor D1 and substance P (Albin et al., 1992; Ferrante et al., 1985; Reiner et al.,
These findings speak of a critical issue in the study of HD pathogenesis: how the ubiquitously expressed mutant huntingtin (mHTT) protein causes relatively selective degeneration in certain brain cells, a question that largely drives the research in this thesis.

Loss of MSNs and CPNs, however, does not account for the entirety of neurodegeneration seen in HD patients. Postmortem tissues also show degeneration of the neuropil, as well as reduced neuronal volume in addition to cell loss. Other regions show evidence of degeneration, although not to the same degree as the caudate and cortex, including the amygdala (Vonsattel and Difiglia, 1998), hypothalamus (Kremer et al., 1991), thalamus (Heinsen et al., 1999), and cerebellum (Jeste et al., 1984). In addition, reductions of up to 30% of white matter tracts (la Monte et al., 1988; Mann et al., 1993), are found in HD patients. It is apparent that while HD pathogenesis overwhelmingly affects striatal MSNs and CPNs, pathology is also apparent in other regions and cell types. Determining the factors that make certain cells more vulnerable to toxicity elicited by mHTT remains a critical component of understanding HD pathogenic mechanisms.

With the development of noninvasive techniques, such as positron emission tomography (PET) and magnetic resonance imaging (MRI), imagers now have the ability to track neurodegeneration in living HD patients instead of relying on postmortem tissue (reviewed in Niccolini and Politis, 2014). When these techniques are combined with genotyping tests to determine whether an individual carries the HD mutation, researchers now have the ability to image the brain of both premanifest and manifest HD patients, tracking the progression of the disease, determining correlates of behavioral manifestations, and potentially developing clinical endpoints for therapeutic trials (Klöppel et al., 2009; Montoya et al., 2006).
Imaging studies have shown that HD neuropathology, as seen by loss of volume, precedes the onset of movement disorder by up to a decade, with patients exhibiting a 30-50% reduction in caudate volume prior to clinical diagnosis (Aylward et al., 2004; Rosas et al., 2003). Caudate appears to be the most vulnerable to degeneration in HD, with loss preceding that of cortex, and is also the best predictor of age of disease onset, however cortical and white matter volume changes also correlate (Dumas et al., 2012; Paulsen et al., 2010). These findings shed light on the appearance of sometimes subtle cognitive and behavioral manifestations of HD that occur before the onset of overt motor symptoms, suggesting that cortico-striatal dysfunction may drive these behaviors.

1.4 Modeling HD in Mice

Like most animal models of disease, HD mouse models can be characterized by their validity in three categories: construct validity, face validity, and predictive validity (Nestler and Hyman, 2010). Construct validity addresses to what degree the mouse model recapitulates the genetics of the disease in question. Face validity concerns itself with how well phenotypes, molecular, behavioral, etc., are recapitulated in the model. Predictive validity, perhaps the most difficult to address, regards whether treatments or interventions in the model will translate to the human disease. There are other criteria used in addressing which mouse model to use for any given study of Huntington’s disease. For example, FvB strain mice have large pronuclei that are afford relatively easy pronuclear injection of transgene DNA and have large litter sizes— excellent traits for mouse geneticists. The 129 mouse strain is also of particular use due to the relative ease of generating gene targeted mutations in it’s embryonic stem cells for knockin and knockout mice. The C57Bl/6 mouse strain, which is not the easiest for performing genetic
manipulations, performs well in behavioral tests, such as the water maze and fear conditioning. Mouse models, despite how they are developed, can be backcrossed into the desired background for the necessary tests or used in a 50-50 mix in an F1 generation. A further consideration is the ability to use conditional genetics in some mouse models of HD, allowing the genetic dissection of cells and circuits that influence HD pathogenesis. There is not one “best” mouse model of HD, and the large number of mouse models available to researchers offers a variety of molecular and behavioral phenotypes and severities to choose for a given study. As a result, different mouse models are commonly used for different purposes even within a single study, often yielding unique insights. A table of HD mouse models, including those used in this thesis is seen in Table 1, and for an excellent review of HD models and best practices, please see A Field Guide to Working with Mouse Models of Huntington’s Disease, a joint publication by CHDI, Jackson Laboratories, and PsychoGenics, Inc. (Menalled et al., 2014).

Still sometimes used, although increasingly rarely, initial mouse models of HD used chemical toxins to induce striatal neurodegeneration. Quinolinic acid injection (QA) was found to recapitulate the MSN toxicity found in HD while largely sparing striatal interneurons (Beal et al., 1986). 3-nitropropionic acid, a mitochondrial toxin, also produces relatively selective striatal lesions (Bossi et al., 1993). Addressing these models in terms of their validities, they have decent face validity (i.e. they show signs of striatal neurodegeneration), but are lacking in construct validity. While these models of striatal neurotoxicity contributed greatly to the early study of Huntington’s, genetic mouse models, made possible by the discovery and cloning of HTT, are typically preferred for their improved construct validities.

Genetic HD mouse models can be widely separated into three categories: (1) models expressing huntingtin fragments (e.g. R6/1, R6/2, N171), (2) full-length transgenic models that
express human huntingtin using the endogenous human promoter (e.g. YAC128, BACHD), and (3) Htt knock-in mice that express full-length mouse huntingtin (or a chimeric mouse-human huntingtin) from the endogenous mouse huntingtin locus (e.g. Q140, Q175).

Featuring improved construct validity compared to chemical toxin models, N-terminal HTT fragment models also exhibit excellent face validity, with earlier onset and more severe HD-like phenotypes compared to most full-length HTT models. The R6 lines (i.e. R6/1 and R6/2), expressing an exon1 fragment of huntingtin with 144 polyglutamines (although this is variable due to CAG repeat instability) from the human HTT promoter, are widely used in the HD field for their robust “HD-like” phenotypes (Mangiarini et al., 1996). These mice were crucial for the discovery of HTT N-terminal aggregates, but lack overt cell loss (Davies et al., 1997). In addition, these mice exhibit age-dependent brain atrophy and HD-like transcriptionopathy (Kuhn et al., 2007; Stack et al., 2005). Despite these benefits, they are also prone to seizures, have much more widespread neuropathology than that seen in HD patients, and exhibit very fast disease course that may better reflect the accelerated disease course seen in juvenile HD rather than adult onset (Lee et al., 2013). Another HTT fragment model is the N171 family, which expresses the N-terminal 171 amino acids from the murine prion promoter. These mice were made with 18, 44, and 82 polyglutamine repeats, allowing for the comparison of HD-like phenotypes between glutamine repeats that, in human, would confer HD (18Q), mid-age onset (44Q), and likely juvenile onset (82Q). Like the R6 mouse lines, the N171-82Q mice exhibit early pathogenesis, severe HD-like phenotypes, widespread HD-like neuropathology, and premature death (Schilling et al., 1999). In addition, derivative lines expressing N-terminal HTT from different promoters and with different polyQ lengths have been used to assess the impact of HTT fragment expression in certain cell types or regions, including astrocytes and striatal MSNs.
The RosaHD mouse line is a conditional HTT fragment model, using flanking LoxP sites surrounding an upstream STOP sequence to allow HTT expression of 103Q HTT exon1 in cells that express Cre recombinase. This allows researchers to “turn on” expression in particularly interesting cell types to assess the impact of mHTT expression. Gu and colleagues performed crosses with a pan-neuronal Cre line (Nestin-Cre), as well as cortical specific (Emx1-Cre) and striatal-specific (Dlx5/6-Cre), concluding that while aggregation appears to be cell-autonomous, mHTT expression in striatum or cortex alone is not sufficient for behavioral phenotypes and requires pathological non-cell-autonomous interactions (Gu et al., 2007; 2005).

Table 1. Commonly used genetic mouse models of HD.

<table>
<thead>
<tr>
<th>Name</th>
<th>Genetic Strategy</th>
<th>Mouse Strain</th>
<th>Behavioral Effects</th>
<th>Neuropathological</th>
<th>Transcription Data</th>
<th>Accession Number</th>
<th>Thesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6/2</td>
<td>Human HTT Human Exon 1 fragment</td>
<td>128 Adult</td>
<td>++ Selective</td>
<td>++</td>
<td>Yes, GEO</td>
<td>G059830, G059837</td>
<td>No</td>
</tr>
<tr>
<td>N171-IQ7</td>
<td>Mouse Prior N-terminus 171 amino acids</td>
<td>82 Early</td>
<td>+++ Widespread</td>
<td>+++</td>
<td>Yes, GEO</td>
<td>Microarray</td>
<td>G059830, G059837</td>
</tr>
<tr>
<td>RosaHDL</td>
<td>Rosa locus Human HTT, Flxed STOP exon 1</td>
<td>103 Promoter dependent</td>
<td></td>
<td></td>
<td>Promoter dependent</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Transgenic Full-length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YAC128</td>
<td>Human HTT Human HTT with few interspersed CAA in CAG repeat</td>
<td>128 Adult</td>
<td>++ Selective</td>
<td>++</td>
<td>Yes, GEO</td>
<td>Microarray</td>
<td>G059677</td>
</tr>
<tr>
<td>BACHeD</td>
<td>Human HTT Human HTT with flxed exon 1 and mixed CAA-CAG repeat</td>
<td>97 Adult</td>
<td>++ Selective</td>
<td>++</td>
<td>Generated in this thesis, not yet deposited</td>
<td>Microarray, RNA-seq</td>
<td>Yes</td>
</tr>
<tr>
<td>BACHeD-BN17</td>
<td>Human HTT Human HTT with deletion of amino acids 2-16, flxed exon 1, and mixed CAA-CAG repeat</td>
<td>Adult</td>
<td>+++ Selective</td>
<td>+++</td>
<td>Generated in this thesis, deposited at GEO</td>
<td>Microarray</td>
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<td>HdhQ10</td>
<td>Mouse Htt Full-length mouse Htt with hybrid human/mouse exon 1</td>
<td>80 Late Adult</td>
<td>+ Selective</td>
<td>+</td>
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<td>90 Late Adult</td>
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<td>109 Late Adult</td>
<td>+ Selective</td>
<td>+</td>
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<td>RNA-seq</td>
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<td>CAG140</td>
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<td>140 Late Adult</td>
<td>+ Selective</td>
<td>+</td>
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<td>RNA-seq</td>
<td>No</td>
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<tr>
<td>HdhQ10x50</td>
<td>Mouse Htt Mouse Htt</td>
<td>150 Late Adult</td>
<td>+ Selective</td>
<td>+</td>
<td>Yes, GEO</td>
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<tr>
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<td>~175 Early</td>
<td>+++ Selective</td>
<td>+++</td>
<td>Yes, HJHHD.org</td>
<td>RNA-seq</td>
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Dividing the full-length HTT models into two groups can be accomplished by looking at those that are gene-targeted vs. transgenic. Large transgenes, such as those needed for full-length huntingtin are accomplished by using bacterial or yeast artificial chromosomes (BACs and YACs, respectively), and there are HD mouse models engineered using both. These large
transgenes improve the construct validity of these models over the huntingtin fragment models, although they often result in reduced phenotypic severity. Additionally, care has to be taken to ensure proper expression (integration site and copy number can affect transgene expression), and multiple lines are necessary to confirm key phenotypes and rule out transgene integration site effects on endogenous genes. YAC HD mice are available in multiple polyQ repeat lengths, expressing full-length human huntingtin from the endogenous human promoter, with the most widely used being the YAC128 line (Slow et al., 2003). It should be pointed out that the transgenes used to make these mice include several CAA repeats, also encoding glutamine, into their CAG repeat region to avoid repeat length instability. These mice exhibit adult onset motor symptoms, selective neuropathology, and HD-like transcriptional dysregulation (Becanovic et al., 2010; Menalled et al., 2009). In addition, derivative strains have been used to test whether cleavage of full-length mHTT is required for pathogenesis (Graham et al., 2006). BAC HD mice were developed in the Yang lab and use a human HTT BAC to drive full-length huntingtin with 97Q from the human promoter (Gray et al., 2008). These mice use a mixed CAA-CAG repeat to avoid instability, and in the case of BACHD, argue against a toxic RNA hypothesis that had been proposed for CAG repeat disorders, at least in the case of HD (Griesche et al., 2013). BACHD mice are widely used in the field and exhibit progressive motor and psychiatric like deficits, as well as selective neuropathology (Gray et al., 2008; Menalled et al., 2012). Work in this thesis will describe the transcriptional dysfunction in these mice and derivative lines. Like YAC128 mice, BACHD mice exhibit later onset of disease symptoms compared to the fragment models, and do not exhibit any of the seizure susceptibility seen in the R6 models. BACHD derivative lines have been used to test several hypotheses related to the HTT N17 domain. First, to test the role of phosphorylation at serines 13 and 16, phosphomimetic aspartic acid mutations were
introduced at these amino acid residues (BACHD-SD line) as well as phosphoresistant alanine mutations (BACHD-SA line). This work demonstrated that BACHD-SD line rescues the behavioral and neuropathological phenotypes seen in the original BACHD line, while BACHD-SA mice have similar deficits seen in BACHD (Gu et al., 2009). To further test the role of the N17 domain in HD pathogenesis, a BACHD line expressing HTT with amino acids 2-16 of the N17 domain was created (BACHD-ΔN17). Description of this mouse line will occupy a large portion of this thesis, but I will briefly mention here that deletion of the N17 domain greatly accelerates disease symptoms compared to BACHD and leads to enhanced nuclear pathogenesis, with HD-like transcriptionopathy (Gu et al., 2015). Also of important note, the BACHD mice and derivative lines feature LoxP sites flanking exon1 of HTT, which includes the translational start codon. This allows the conditional removal of the transgene in mice that express Cre recombinase and allows the dissection of cell types and circuits for which HTT expression is necessary for pathogenesis (e.g. Hult et al., 2011; Wang et al., 2014). This topic will also be a larger part of this thesis. While the face validity of the full-length human transgene mouse models (i.e. YAC128 and BACHD) could be seen, in general, as reduced compared to that seen in the fragment models, these mice exhibit a slower disease course that is likely more reflective of the slowly progressive nature of HD in patients, with the symptoms often recapitulating early-stage HD symptoms (Lee et al., 2013). One notable exception, in regards to face validity of the full-length transgenic models compared to the fragment models, is that BACHD-ΔN17 mice recapitulate striking features of HD—adult onset movement disorder reminiscent of chorea, progressive neurological symptoms including falls and inability to groom, and selective overt degeneration of cortex and striatum that have never before been seen in an HD mouse model.
*Huntingtin* knockin mice use gene targeting to replace the mouse *Htt* exon1, which has a polyQ repeat of just 7Q, with typically a mouse-human hybrid exon1 containing a longer polyglutamine repeat and the human polyP region (one exception to the human polyP is the CAG150 mice, which feature mouse polyP) (Lin et al., 2000; Menalled et al., 2003; Wheeler et al., 2000; 1999). These mice have improved construct validity compared to the transgenic (fragment and full-length) models, expressing the murine Htt from the *Htt* locus with mouse regulatory elements intact. A downside of this, however, is that the knockin models have, until recently, shown very mild HD-like phenotypes. There are multiple knockin mice, with polyQ repeat lengths ranging from Q20 through Q175. Until the chance generation of the Q175 line (which occurred spontaneously through expansion of the CAG repeat region in Q140 mice), knockin mice exhibited reduced severity of disease compared even to the full-length transgene models (Menalled et al., 2009). The Q175 line has generated much interest in the research community due to its robust HD-like behavioral and neuropathological phenotypes (typically on par with those seen in the full-length transgenic models) while still maintaining the construct validity benefits of a knockin model (Carty et al., 2015; Heikkinen et al., 2012; Menalled et al., 2012). Of interest, recent efforts by CHDI have undertaken an ambitious strategy to perform RNA sequencing on *huntingtin* knockin mice in an allelic series—that is a set of knockin mice with varying CAG repeats of Q20, Q80, Q92, Q111, Q140, and Q175. These mice also underwent extensive behavioral phenotyping, and with tissue collected from multiple brain regions and peripheral tissues, this comprehensive dataset will likely yield incredibly rich insight into the polyQ-dependent molecular correlates of HD-like pathogenesis in mice.

In summary, there are myriad mouse models with which to study HD pathogenic mechanisms. The ones used for a given study need to be selected carefully based on the
experimental design and phenotypes being assessed. The above discussion focused on the face and construct validities of various mouse models. The degree to which any of these has predictive validity is yet to be determined, as there is yet no disease modifying therapy for HD. These mice have been invaluable, however, for dissecting circuitry, cellular, and molecular pathogenic mechanisms, and are promising tools for ongoing and future preclinical trials.

1.5 Proposed Pathogenic Mechanisms in HD

If ever there was a loaded topic, this would surely qualify. For such a seemingly simple mutation, there seem to be an endless number of pathogenic mechanisms at play in the HD brain. Decades of HD research, by some of the smartest and most dedicated minds in the field have developed innumerable hypotheses, identified dozens of signaling pathways, proposed countless molecules, and queried a plethora of cells and circuits that may contribute to toxicity in HD. The best of these have staying power in the field, and I will discuss a fraction of them here. When attempting a description of HD pathogenic mechanisms, an excellent framework is the two-step reductionist approach to define first the cellular determinants of disease and then the molecular pathogenic processes occurring in this defined set of cells. Those interested in further reading are directed to the numerous reviews on the subject, of which MacDonald and Gusella (MacDonald and Gusella, 1996), Lundles and Bates (Landles and Bates, 2004), and Orr and Zoghbi (Orr and Zoghbi, 2007), and the very recent (Bates et al., 2015) are of particular interest. My own review on the topic may also be of use (Cantle et al., 2012).

To begin the two-step reductionist approach, one needs to define the cells and circuits driving the disease. Decades of neuropathological studies on HD postmortem brains immediately implicate the cells of the basal ganglia and cortex, two highly connected regions
that see massive degeneration. These two regions are intimately connected, with the striatum receiving massive glutamatergic input from the cortex that is necessary for normal behavior (Kreitzer and Malenka, 2008). These are the vulnerable regions, but how does mHTT cause pathology that is largely selective to MSNs and to a lesser extent CPNs? One possibility is that mHTT acts in a solely cell autonomous manner, with these cells having some intrinsic vulnerability that sets them apart from other cell types which remain largely unaffected. A second possibility is that non-cell autonomous effects drive pathogenesis, perhaps through aberrant neuron-neuron or neuron-glia interaction. In addition, some combination of these mechanisms may be at play, with some amount of cell autonomous toxicity being further exacerbated by pathological cell-cell interaction.

1.5A Cells and Circuitry Involved in HD Pathogenesis

Mouse models have been invaluable for dissecting circuitry mechanisms at play in HD. Important work by Michael Levine’s lab and others have characterized cortico-striatal dysfunction that precedes degeneration and onset of motor phenotypes (reviewed in Cepeda et al., 2007). An oversimplification of the complex circuitry changes in HD is that early increases in excitatory input to striatum, preceding symptom onset, are later followed by reduced excitatory cortico-striatal signaling as the disease progresses (Cummings et al., 2010; Joshi et al., 2009). While CPNs and MSNs are the most affected cells in HD, there seems to be earlier loss of the D2 MSNs in HD (Albin et al., 1992), that could provide clues into what makes MSNs vulnerable in the disease. Previous work in the Yang lab also sought to address the contributions of the cortex and striatum in HD by using conditional mHTT expression in the RosaHD model system in a pair of papers. Mice expressing a mHTT exon1 fragment in cortex or striatum alone
do not exhibit HD-like behavioral phenotypes, however neuropathological examination found evidence of cell-autonomous aggregation in the absence of degenerating neurons (Gu et al., 2005; 2007). In addition, the pan-neuronal mHTT model, but not the CPN mHTT model, shows evidence of cortical interneuron dysfunction, suggesting that interneuron modulation of CPN activity may also play a part in altered cortico-striatal signaling (Gu et al., 2005). It is important to note that a different fragment model (N171) with transgene expression in the striatum and a small number of cortical cells developed HD-like behavioral and neuropathological phenotypes, however they were delayed and less severe compared to N171 mice with ubiquitous expression, despite the striatal line containing a further expanded polyQ domain (Brown et al., 2008; Schilling et al., 1999). Recently published work in the Yang lab, detailed in Chapter 2 of this thesis, took the converse approach to the RosaHD studies by genetically reducing transgene expression in the BACHD mouse line using Cre expression in cortex, striatum, or both cortex and striatum. Underscoring the importance of cortico-striatal signaling in HD pathogenesis, BACHD mice with genetic reduction of mHTT in the cortex rescued or partially rescued behavioral and neuropathological phenotypes while striatal mHTT reduction did not (Wang et al., 2014). These results indicate that, while mutant huntingtin expression likely plays a large role in cortical and striatal vulnerability, the full extent of HD pathogenesis is only seen in the context of a second insult from the cortex.

The above experiments are lacking an experimental cross that would answer critical questions about the roles of other brain cell types in HD: that is whether cortical striatal mHTT expression alone are sufficient to drive HD-like phenotypes in mice. This would require (ideally full-length) conditional mHTT expression mouse line with striatal and cortical Cre. In the absence of this cross, the question remains whether and to what extent other cell types contribute
to HD pathogenesis. Of particular interest is the role of astrocytes, which are activated in HD patient brains (Vonsattel et al., 1985b) and play critical roles in normal brain functions such as glutamate uptake (Bezzi et al., 2004), modulating synapses (Fiacco and McCarthy, 2004), neuronal metabolism (Brown and Ransom, 2007; Pfrieger and Ungerer, 2011), and neuroinflammatory responses (Carson et al., 2006; Glass et al., 2010). mHTT exon1 fragment expression in astrocytes alone can either drive the development of HD-like behavioral deficits, or at least confer enhanced glutamate sensitivity in mice and exacerbate symptoms when crossed with a pan-neuronal fragment line (Bradford et al., 2009; 2010). Another cell type that may play a role in the neurodegenerative process is microglia—the resident immune cells of the brain. Microglia activation is found in postmortem HD brains (Sapp et al., 2001), as well as in presymptomatic HD gene carriers, with the degree of activation correlating with striatal degeneration (Pavese et al., 2006; Tai et al., 2007). Whether microglia play a contributory pathogenic role in HD or are a response to the diseased state of the brain is yet unclear (Crotti et al., 2014; Moller, 2010).

In summary of this brief description of the cellular determinants of HD pathogenesis, there is excellent evidence of cell-autonomous vulnerability that is created by mHTT expression in the cells of the cortex and striatum, with the full brunt of toxicity being caused by aberrant interaction with other cell types in the diseased brain. With well-established roles for MSNs, CPNs, and even astrocytes in the course of pathogenesis, ongoing work is determining the full roles of other cell types (e.g. interneurons, microglia, oligodendrocytes, etc.).
Molecular Mechanisms of HD Pathogenesis

Molecular pathways that are disrupted in HD are numerous, as are the hypotheses regarding pathogenic mechanisms that they have generated. While roles are not completely resolved, it is apparent that some cell types contribute more to the pathology of HD than others. Striatal MSNs and cortical CPNs, the most affected in HD, see both cell-autonomous and non-cell-autonomous toxicity in HD, with the synergistic effects likely being the key factor in these cells’ vulnerability and eventual loss. The question of what molecular changes underlie both the mHTT induced cellular and molecular dysfunction, as well as the aberrant signaling that contributes to the cellular dysfunction and eventual death, becomes the second step of the two-step reductionist approach.

Levels of the Huntingtin protein themselves do not explain the higher vulnerability of cortex and striatum in HD, as these regions have lower expression compared to relatively spared areas such as hippocampus and cerebellum (Sharp et al., 1995), though it is possible that cis-acting modifications of Huntingtin in MSNs and CPNs could mediate enhanced neuronal vulnerability. Huntingtin undergoes numerous posttranslational modifications (PTMs), including phosphorylation, SUMOylation, ubiquitination, acetylation, palmitoylation, and cleavage into smaller fragments (reviewed in Ehrnhoefer et al., 2011). Of these PTMs, several have been shown to play a direct role in HD pathogenesis. Work in the Yang lab using BAC transgenic mice tested whether phosphomimetic mutation of serines 13 and 16 in the mHTT N-terminus 17 amino acid (N17) domain could alter disease course, finding disease symptoms abolished in mice transgenic for the phosphomimetic mHTT species. Notably, mice with the same construct but with either (1) serines 13 and 16 unaltered, or (2) mutated to phosphoresistant alanine, showed HD-like phenotypes (Gu et al., 2009). Likewise, phosphorylation of serine 421 has been shown
to be protective (Metzler et al., 2010; Warby et al., 2005). SUMOylation and ubiquitination have been likely play roles in proteasomal degradation of HTT. SUMOylation of HTT by SUMO-1 and PIAS in particular has been shown to regulate insoluble mHTT aggregates (O’Rourke et al., 2013). In addition, cleavage of full-length HTT into fragments may play a pathogenic role as well, with evidence that N-terminal mHTT fragments exhibit enhanced toxicity compared to full-length. There is evidence that cleavage of mHTT at the caspase 6 site at amino acid residue 586 generates an N-terminal fragment that accumulates in the nucleus (Warby et al., 2008), with mutation of the 586 site blocking toxicity in YAC mice (Graham et al., 2006), although further studies have shown that caspase 6 knockout mice have reduced HTT expression and aggregates, but still have cleavage at the 586 site (Gafni et al., 2012), while others have shown no effect of caspase 6 on mHTT fragments in Q150 knockin mice (Landles et al., 2012). Further, work that will be expanded upon in this thesis has shown that the N17 domain of HTT acts to keep relatively small N-terminal fragments out of the nucleus, where they appear to exert enhanced toxicity (Atwal et al., 2007; Gu et al., 2015).

Transcriptional profiling has shown that mHTT expression has wide-ranging effects on multiple cellular pathways and processes (reviewed in Cha, 2007). To study HD transcription, microarray and RNA-seqencing experiments have been undertaken in postmortem patient brain (Hodges et al., 2006), animal models (Becanovic et al., 2010; Strand et al., 2007), and cell models (Runne et al., 2008; Sipione et al., 2002). Huntingtin has been shown to play a direct role in altering transcription by differentially interacting with multiple transcription factors and transcription regulators (Sugars and Rubinsztein, 2003), many of which appear interesting in the study of HD pathogenesis (e.g. CBP, SP1, and NF-κB, NRSE). Transcription profiling in HD postmortem brains faces the complication of the change in tissue composition resulting from the
loss of MSNs and CPNs, however the mouse models seem to recapitulate the transcriptional landscape (Kuhn et al., 2007). Of the striatal transcription changes seen in profiling studies, a large number of down-regulated genes seem to be directly related to the function of the striatal MSNs (e.g. Drd1, Drd2, Gpr6, Darpp-32, Cnr1, etc.), while others have yet unknown function in these cells (e.g. Ddit4l, Wt1). Cellular signaling pathways that seem to be disrupted include Ca$^{2+}$ signaling, GPCRs, genes involved in mitochondrial function, and synaptic proteins and cytoskeletal proteins (e.g. Actn2). Additionally, cortical gene expression has implicated reduced BDNF transcription, and thus trophic support for striatum, possibly with impaired TrkB receptor function playing a role as well (Plotkin et al., 2014; Zuccato et al., 2001; 2005). With the relatively low levels of traditional differential gene expression sometimes seen in full-length human transgenic HD mouse models (Becanovic et al., 2010), work in this thesis will include the use of the highly sensitive Weighted Gene Coexpression Network Analysis method to group highly co-expressed genes regardless of their differential expression (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). Improvements to the methods for cDNA library construction from small amounts of RNA, combined with cell-type-specific expression of fluorescent proteins (Crook and Housman, 2012) or tagged ribosomal proteins (Shema et al., 2015), have driven the ability to perform transcriptional profiling in subsets of neurons that are thought to drive the pathogenesis of HD and other disorders. It will be fascinating, for example, to interrogate the molecular underpinnings of differential D1 vs. D2 MSN vulnerability in HD (Reiner et al., 1988). In addition to the use of transcriptional dysregulation for hypothesis generating work, the lowering of costs and multiple options for assaying transcription (e.g. microarray, NanoString, and RNA-seq) allow for the use of transcription signatures as a sensitive readout of future perturbation studies.
Aberrant cortico-striatal signaling likely contributes to the shift from vulnerable neuronal populations to the outright death of susceptible cells. Hypotheses extended for this include toxic glutamatergic signaling, morphological changes, withdrawal of trophic support, loss of cortico-striatal connectivity, and changes in MSN receptor localization or function (Cepeda et al., 2007; Raymond et al., 2011). Direct and indirect pathway MSNs, comprising 95% of the neurons of the striatum, were previously thought to be morphologically and electrophysiologically indistinguishable. Advances in the use of cell-type-specific promoters to identify genetically distinct cell populations has led to work that shows the D1-expressing and D2-expressing neurons are electrophysiologically different and receive different input from CPNs (Kreitzer and Malenka, 2008). D2-expressing MSNs appear to receive increased cortical input and be more excitable, with a lower threshold for action potential firing and evidence of a more compact dendritic arbor (Gertler et al., 2008). Current models suggest increased cortical activity, as seen early in the disease process, exerts preferential toxicity on D2-expressing MSNs in the striatum. Death of these MSNs would unbalance the cortico-striatal-thalamo-cortical (CTSC) signaling loop, with reduced signaling from D2-MSNs to globus pallidus externus reducing inhibition of thalamocortical projection neurons, leading to increased cortical activity, and promoting motor dysfunction (Chevalier and Deniau, 1990). Another hypothesis for the enhanced vulnerability of MSNs to cortical signaling is that MSN glutamate receptors undergo a shift from synaptic to extrasynaptic locations (Milnerwood and Raymond, 2010). Synaptic signaling by N-methyl-D-aspartate receptors (NMDARs) is thought to induce pro-survival signaling, while extrasynaptic NMDARs activate alternative pro-death signaling pathways. Interestingly, synaptosome preparations that allow separation of synaptic and extra-synaptic fractions shows that HD mice undergo a shift of NMARs from the pro-survival synaptic to the pro-death extra-synaptic fraction
(Milnerwood et al., 2010). Mechanisms underlying this receptor shift remain unclear, but treatments that specifically block extrasynaptic NMDAR signaling improved pro-survival CREB signaling and some behavioral deficits (*ibid*).

Figure 1.1. Schematic of the two-step reductionist approach and selected mechanisms implicated in HD pathology. First, critical cell types for pathogenesis are determined, here CPNs and MSNs. Second, molecular pathogenic mechanisms in those critical cell types are determined.
1.6 Conclusions

Huntington’s disease affects an estimated 30,000 patients in the United States and Europe, with another 150,000 at risk for developing the disease. Patients and their families are subjected to years of worsening symptoms and debilitation until the ultimate death of the patient. To say that the disease is brutal is an understatement, with patients facing relentless progression of cognitive dysfunction, loss of motor control, and psychiatric symptoms. Although the sheer number of affected individuals is lower than other neurodegenerative diseases, such as Parkinson’s and Alzheimer’s diseases, Huntington’s remains a particularly important target for neurodegenerative disease research because of its monogenetic etiology. However, and despite the relatively simple genetics that are at the root of the disease, HD pathogenesis remains complex.

HD preferentially kills neurons of the striatum and cortex, which seem to have some amount of intrinsic vulnerability as well as evidence of susceptibility to toxic cell-cell interactions. Within these cells, multiple pathways go awry in HD. Of particular challenge is how to determine which of these molecular pathways contributes to the disease and which are epiphenomena. The use of sophisticated tools and methods to determine the molecular pathways that are changed in the precise cells that are affected gives new insight into disease processes and has the potential to inform future studies to intervene in the processes in vivo. Neurodegenerative diseases likely share salient mechanisms, whether they are compensatory or pro-degenerative, and insight into HD molecular pathology has potential to inform other disease fields.

Finally, we are at a time when treatment of HD is looming near. The use of safe viral vectors for gene therapy means that long-lasting expression or repression of disease relevant
genes is no longer a pipe dream. Studies that identify the regional, cellular, and molecular
determinants of HD pathology are crucial in informing where to target these putative treatments.
New antisense oligonucleotide (ASO) therapies that look to reduce the level of disease-causing
mHTT are entering clinical trials this year, however it is unclear whether the ASOs will be able
to engage and lower HTT in human patients as well as whether lowering in the cells and brain
regions that are ASO-accessible will slow or prevent the onset of disease symptoms. These trials
represent a step towards developing the first drugs that truly treat the disease, instead of those
that reduce symptoms despite ongoing progression.
Chapter 2

Assessment of Cell Autonomous and Non-cell Autonomous Toxicity in HD Mice
2.1 Introduction

With the importance of cell-cell interactions in the manifestation of the full brunt of HD symptoms already implicated by previous studies (e.g. Bradford et al., 2009; 2010; Gu et al., 2005; 2007), work in the lab sought to further define the cell types necessary for HD pathogenesis by using conditional deletion of full length mHTT. This study is an excellent proof-of-concept for the use of the two-step reductionist approach to studying HD pathogenesis: first identifying the cellular determinants of disease, and then using molecular tools to assess the signaling pathways and molecules that are affected in those cells.

Previous work in the Yang lab led to the development and characterization of the BACHD mouse line, which expresses full-length human huntingtin from a BAC containing the entire 240kb human HTT locus (Gray et al., 2008). This mouse line exhibits adult-onset, progressive motor phenotypes (e.g. rotarod deficits at 2, 6, and 12 months of age), anxiety- and depressive-like symptoms (e.g. increased time spent in the dark in light-dark box testing and increased time spent immobile in the forced swim test), cortico-striatal electrophysiological dysfunction (e.g. reduced medium- and large amplitude spontaneous synaptic currents in MSNs at 6mo), evidence of transcriptional dysregulation and loss of cortical trophic support for striatum (e.g. reduced cortical BDNF transcription at 6mo), and neuropathology (e.g. cortical and striatal atrophy, striatal dark neuron degeneration, and the presence of mHTT aggregates at 12mo) (Gray et al., 2008; Menalled et al., 2009; Spampanato et al., 2008). Power analyses of the behavioral phenotypes suggest that group sizes of N=22 would be sufficient to provide 80% power of detecting a 30% improvement in these measures (e.g. rotarod), while only 5 mice would be necessary for the same power to detect a 30% improvement in striatal atrophy (Gray et al., 2008).
Of particular note, the BACHD transgene features exon1 of mHTT flanked by LoxP sites, allowing for the conditional deletion of exon1 and the translational start sequence in cells that express Cre recombinase (Fig. 2.1A) (Gong et al., 2007; Gray et al., 2008; Heintz, 2001). By genetically deleting or reducing the transgene copy number in genetically defined cell types (e.g. CPNs, MSNs, astrocytes, etc.), one can assay contribution of these cells to HD phenotypes. Importantly, previous work that used conditional genetics to “turn-off” expression of mHTT transgene in mice reversed neuropathology and ameliorated behavioral phenotypes (Yamamoto et al., 2000), suggesting that this approach has traction in our model to determine cell types and circuits in which mHTT is needed for disease. To begin this work, Cre expression was genetically targeted to reduce mHTT expression in CPNs, MSNs, or both CPNs and MSNs in the BACHD background, also comparing behavioral, neuropathological, and molecular phenotypes to wildtype (WT) littermate mice. The behavioral and neuropathological work described in this chapter is published in (Wang et al., 2014), however the characterization of the molecular signatures in BACHD mice and the related crosses was not included in the article.

2.2 Genetic Reduction of BACHD Transgene and mHTT Expression

To target Cre expression to CPNs and MSNs in BACHD mice, we used Emx1-Cre mice (Iwasato et al., 2000) and Rgs9-Cre mice (Dang et al., 2006), which were first crossed to generate Emx1-Cre x Rgs9-Cre double transgenic (or ER) mice in the inbred Fvb/NJ background. ER mice were crossed to Rosa-LacZ reporter mice, allowing the use of β-
Figure 2.1. Genetic reduction of mHTT transgene and expression. (a) Schematic of the BACHD transgene showing loxP sites flanking exon1 of 97Q HTT. Cre-mediated excision of exon1 eliminates the translational start codon and polyQ repeat. (b) Staining for β-galactosidase in LacZ reporter mice show the pattern of Cre-mediated LacZ expression due to Emx1-Cre, Rgs9-Cre, and Emx1-Cre x Rgs9-Cre mice. This pattern should match the deletion of mHTT transgene in BACHD mice crossed to these Cre lines. Images representative of three independent experiments. Scale bar = 2mm. (c,d) Cortical (a) and striatal (b) expression of Cre-dependent YFP in Ai3 reporter mice. YFP signal in green, NeuN staining for neurons in red, composite third image shows colocalization in yellow. Images representative of two independent experiments. Scale bar = 100 µm. (e) Western blot probed with 1C2 antibody against expanded polyQ shows reduction in specific tissues in Cre-expressing mouse lines. α-tubulin was used as loading control. Below, results of densitometry quantifying reduction of mHTT expression. N = 4 per genotype. * P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant; one-way ANOVA followed by LSD post hoc test. Values represent mean ± SEM. (f) In situ hybridization using BACHD exon1 as a probe reveals reduction of BACHD-expressed mRNA after Cre-mediated excision of the transgene in BE, BR, and BER mice. Images representative of two independent experiments. Scale bar = 1mm.
galactosidase to determine regions of Cre expression (Soriano, 1999), and a yellow fluorescent protein reporter line (Ai3; Madisen et al., 2009). Importantly, Emx1-Cre-mediated reporter expression was limited to just the cortex and hippocampus, while Rgs9-Cre mediated reporter expression in just the striatum (Fig. 2.1B). Further, the fluorescent reporter showed that Cre-mediated YFP expression is limited to NeuN co-staining neurons in the cortex in Emx1-Cre mice and in the striatum in Rgs9-Cre mice (Fig. 2.1C-D). Mice expressing double transgenic for Emx1-Cre and Rgs9-Cre showed additive expression patterns of the two single Cre transgenes. With the expression pattern of the Cre transgenes confirmed, the ER mice were then crossed with hemizygous BACHD mice, also in the FvB/NJ background, to produce the following genotypes: WT, BACHD, Emx1-Cre, Rgs9-Cre, Emx1-Cre x Rgs9-Cre, BACHD x Emx1-Cre (BE mice), BACHD x Rgs9-Cre (BR mice), BACHD x Emx1-Cre x Rgs9-Cre (BER mice). WT, BACHD, BE, BR, and BER mice were used to study the behavioral, molecular, and neuropathological consequences of removing full-length mHTT in genetically defined cell types (I will refer to the cohort of all genotypes as the BER cross). Western blotting for expanded polyQ proteins, such as mHTT, using the 1C2 antibody showed that protein levels are reduced in the cortex in BE (70% reduction) and BER (80% reduction) mice compared to BACHD, and in the striatum in BE (40% reduction), BR (44% reduction), and BER (80% reduction) mice compared to BACHD, but protein levels are not reduced in the cerebellum, which has not undergone Cre-induced transgene excision (Fig. 2.1e).

While it may be initially concerning that the cortical inactivation (BE) mice exhibit reduced mHTT levels in the striatum, where there should not be Cre-mediated transgene reduction, this result is explainable by the presence of mHTT in cortico-striatal axons that can not be separated from the striatal tissue during dissection. Evidence of this is the reduction of
mHTT levels in the striatum in the BER mice (80% reduction) compared to BR (44% reduction). To further satisfy that mHTT reduction is specific to the defined cell types in the cross, we performed in situ hybridization to show that mHTT transcript is reduced in the cortex in BE mice, in the striatum in BR mice, and in both cortex and striatum in BER mice (Fig. 2.1f). In summary, our mouse cross mediated efficient and specific reduction of BACHD transgene expression levels in mice crossed with cell type-specific Cre mice, allowing the genetic dissection of the role of CPNs and MSNs in BACHD phenotypes.

2.3 Amelioration of Behavioral and Neurodegenerative Phenotypes in BER Mice

To assess the fully-characterized behavioral phenotypes seen in the BACHD line, mice from the BER cross were assessed for motor phenotypes in the accelerating rotarod test at 2, 6, and 12mo of age, the open field at 6 and 12mo, the forced swim test at 12mo, and the light-dark box at 12mo. BACHD mice show decreased motor performance in the rotarod test beginning at 2mo (Gray et al., 2008; Menalled et al., 2009), worsening at 6mo and again at 12mo, findings that were recapitulated in mice from this cross (2mo data not shown). Interestingly, by 6mo, BE and BER mice show significant improvement over BACHD mice, with BER mice returning nearly to WT levels (Fig. 2.2a). The same pattern is seen in 12mo rotarod testing, with BACHD mice performing most poorly, followed by BR, and with BE and BER showing significant improvement compared to BACHD and BER being statistically indistinguishable from WT (Fig. 2.2b).

BACHD mice exhibit reduced spontaneous locomotor activity compared to WT littermates at 6 and 12mo of age in the open field test, however cortical and striatal inactivation (in the BER mice) rescues this phenotype at 6mo (Fig. 2.2c). At 12mo both BE and BER mice
Figure 2.2. Amelioration of behavioral and neuropathological deficits in BACHD mice crossed to Cre lines.  (a,b) 6mo (a) and 12mo (b) testing in the accelerating rotarod test showed significant amelioration of motor coordination deficits in BER mice compared to BACHD at both ages, while BE mice show significant improvement at 6mo. BR mice do not show significant improvement compared to BACHD mice at either age. (c,d) Distance traveled in the open field test at 6mo (c) shows significant improvement of BACHD-related hypoactivity in BER mice, while at 12mo (d) both BE and BER mice are improved in comparison to BACHD. (e,f) Forced swim testing (e) and light-dark box exploration (f) at 12mo revealed significant rescue of the BACHD psychiatric-like phenotypes (depressive-like and anxiety-like, respectively) in both BE and BER mice, which reached levels indistinguishable from WT. (g-i) Neuropathology studies at 12mo showed significant rescue of forebrain weight (g) in BER mice compared to BACHD, while BR mice showed no improvement. The stereological measure of cortical (h) and striatal (i) volume loss seen in BACHD mice is restored to WT levels in BER mice. * P < 0.05, ** P < 0.01, *** P < 0.001; one-way ANOVA followed by LSD post hoc test. Values represent mean ± SEM. N ≥ 13 for 6mo behavioral studies and N = 12 for 12mo behavioral studies. N = 8 for neuropathology studies.
show this amelioration of decreased locomotor activity (Fig. 2.2d). The forced swim test assays depressive-like behavior, which manifests as greater time spent immobile in the testing chamber. While BACHD and BR mice exhibit more time spent immobile in the forced swim test, both BE and BER mice exhibit amelioration of this phenotype to levels indistinguishable from WT littermates (Fig. 2.2e). In addition, BE and BER mice spend a similar amount of time in the dark chamber in the light-dark box test, compared to the greater amount of time spent in the dark by BACHD and BR mice, suggesting that BE and BER mice show reduced levels of anxiety-like behavior compared to BACHD and BR (Fig. 2.2f).

Neuropathologically, BACHD mice exhibit reduced forebrain weight, as well as reduced striatal and cortical volumes. Fitting with the degree of behavioral amelioration, BER mice exhibit a significant increase in forebrain weight compared to BACHD and BR mice, while BE mice remain a non-significant intermediate between the two genotypes (Fig. 2.2g). As a more sensitive measure of atrophy, unbiased stereological measurement of cortical and striatal volumes also shows this pattern, with reduced volume in BACHD compared to WT, rescue to WT levels in BER mice, and BR and BE mice showing a non-significant partial rescue (Fig. 2.2h-i).

In summary, longitudinal behavioral assessments and assays of neuropathology found a pattern of amelioration in the cohort of BACHD mice crossed with cell type-specific Cre mice (summarized in Table 2). First, compared to BACHD mice, striatal inactivation of the mHTT transgene in BR mice was not able to rescue any behavioral or neuropathological phenotypes. Second, cortical inactivation of mHTT transgene in the BE mice was sufficient to rescue a subset of motor dysfunction (i.e. 6mo rotarod and 12mo open field locomotion), and all measures of psychiatric-like deficits (i.e. light-dark box and forced swim), but not rescue any
Table 2. Summary of behavioral and neuropathological rescue in mice expressing Cre recombinase in defined neuronal populations.

<table>
<thead>
<tr>
<th>BACHD Phenotype</th>
<th>Rescue Effect (Percent Amelioration of BACHD)</th>
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<tbody>
<tr>
<td></td>
<td>BR</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td><strong>Motor Deficits</strong></td>
<td></td>
</tr>
<tr>
<td>Rotarod (6mo)</td>
<td>ns</td>
</tr>
<tr>
<td>Rotarod (12mo)</td>
<td>ns</td>
</tr>
<tr>
<td>Spontaneous locomotion (6mo)</td>
<td>ns</td>
</tr>
<tr>
<td>Spontaneous locomotion (12mo)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Psychiatric-like Behavior</strong></td>
<td></td>
</tr>
<tr>
<td>Anxiety (light-dark box)</td>
<td>ns</td>
</tr>
<tr>
<td>Depression (forced swim)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Neurodegeneration</strong></td>
<td></td>
</tr>
<tr>
<td>Forebrain weight</td>
<td>ns</td>
</tr>
<tr>
<td>Cortical volume</td>
<td>ns</td>
</tr>
<tr>
<td>Striatal volume</td>
<td>ns</td>
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</table>

measures of neuropathology. It is prudent to regard the lack of rescue in these tests as underpowered to definitely rule out any such effect, however, as these tests were designed with sufficient power to confirm rescue effects rather than to rule them out. Finally, genetic reduction of the mHTT transgene in cortex and striatum in the BER mice produced at least a partial, though significant, rescue in all tests, including behavior and neuropathology, with many BER phenotypes being statistically indistinguishable from WT. This pattern suggests that mutant huntingtin expression in cortex and striatum is sufficient to drive these major phenotypes in HD mice. Surprisingly, given the extreme vulnerability of striatal MSNs in HD, genetic reduction of mHTT expression in the striatum was not sufficient to rescue any behavioral or neuropathological readouts. This result, in combination with the result from the BER rescue, suggests that the role of the cortex in driving striatal degeneration may be even greater than suspected. To support this notion, cortical reduction of mHTT expression, even in the absence of
striatal mHTT reduction, still ameliorates some motor phenotypes and all of the psychiatric-like phenotypes tested, despite a lack of effect on neuropathology.

2.4 Transcriptional Profiling in Identifies Cell-autonomous and Non-cell-autonomous Transcriptional Dysregulation

Behavioral studies of the full cohort of BACHD mice with reduction of mutant huntingtin in genetically defined cell types confirmed that CPNs and MSNs play key roles in the development of HD-like phenotypes in mice. This represents the first step of the two-step reductionist approach to studying neurodegenerative disease pathology: identification of cells that contribute to disease pathogenesis. To undertake the second step of the two-step reductionist approach, we performed transcriptional profiling on mice derived from the same cross. Tissues from WT, BACHD, BE, BE, and BER mice were collected at three ages (2mo, 6mo, and 12mo), and from multiple brain (cortex, striatum, thalamus, and cerebellum) and peripheral tissues (liver, heart, skeletal muscle, and blood), in the interest of future studies assessing age-dependent mechanisms and central versus peripheral effects of reducing mHTT expression in the brain. In all, a total of 91 mice were dissected for the study, with one mouse being excluded due to a liver tumor found during the dissection.

Previous studies of select striatal genes in BACHD mice have claimed that there are few gene expression changes, even in comparison to other full-length transgenic models (i.e. YAC128; (Pouladi et al., 2012), so we first sought to assess whether we could find transcriptional dysregulation in the BACHD inactivation series at 12 months of age in the most affected tissues, cortex and striatum. We performed RNA-sequencing, achieving excellent read depth averaging roughly 82M reads / sample. Of these, an average of 70M reads per sample,
roughly 85%, were uniquely mapped to the mouse reference atlas MM10 using STAR. In the quality control and normalization process, one male WT striatal sample was identified as an outlier and removed from further analyses. The remaining samples were used for differential gene expression (DGE) using DESeq2 and WGCNA.

DGE analyses identified a number of genes up- and down-regulated in the cortex and striatum in 12mo BACHD mice. The majority of genes do not meet the strict multiple comparisons testing threshold cutoff of FDR < 0.05, however there are quite a few striatal genes that meet the FDR < 0.1 cutoff for suggestive significance (119 genes, 95 down-regulated and 24 up-regulated; Fig. 2.3a). At this same threshold, there are just 4 genes dysregulated in the cortex (Tacr3 and Tmem150c up-regulated, and Ttr and Prlr down-regulated). Due to the low number of genes differentially expressed in the cortex, I will focus the DGE analyses on the striatum, however later sections will show that there is additional information that can be gleaned from the cortical data using WGCNA. The genes differentially expressed in the striatum of BACHD mice at FDR < 0.1 are enriched for glycoproteins, calcium binding, and regulators of synaptic plasticity (DAVID gene ontology); canonical GPCR and axon guidance signaling, with upstream regulators Htt, Adrbk2, and CREB (Ingenuity Pathway Analysis); and markers of neurons, genes down in Alzheimer’s disease, human/chimp caudate markers, and postsynaptic density proteins (HDinHD.org anRicher, which utilizes the anRichment package in R). Many of these genes are classically dysregulated in HD mouse expression datasets (e.g. Pkp2, Pprk1, Actn2, Igfbp4, Clspn, Cnr1, Gpr6, and Adora2a), however DGE is only mildly preserved in human caudate and CAG150 datasets (Fig. 2.3b). Of interest in refuting the claim that BACHD mice exhibit fewer gene expression changes than YAC128 mice (Pouladi et al., 2012), we compared genes that are dysregulated at the FDR < 0.1 level in BACHD and published 12 and 24mo YAC128 data.
(Becanovic et al., 2010), as well as 6mo striatal Q175 RNA-sequencing data available at HDinHD.org. Surprisingly, at 12 and 24mo individually, no YAC128 genes passed the FDR < 0.1 threshold, while 119 BACHD and 6108 Q175 genes are differentially expressed (Fig. 2.3c; Langfelder et al., unpublished data). However, by combining the 12 and 12mo YAC128 datasets, 13 genes reach significance.

With establishment of transcriptional dysregulation in the striatum of BACHD mice at 12mo, next came assessing gene transcription changes correlating with the genetic reduction in the cortex, striatum, or both tissues. To accomplish this, genes that are transcriptionally dysregulated between BACHD and WT at FDR < 0.1 was searched for the subsets that were significantly (even if only partially) normalized in BE, BR, or BER vs. BACHD comparisons at the P < 0.05 significance threshold. These are the genes that can be considered to have some degree of significant “rescue” in the striatum compared to BACHD. If transcriptional dysregulation is a largely cell autonomous process caused by mHTT expression, we should see the largest number of rescued genes in BER and BR striatum, with cortical reduction of mHTT (in BE mice) not playing a large role in striatal transcriptionopathy. Instead, matching with the amelioration of behavioral phenotypes, the largest number of rescued genes are found in BER mice (72 out of 119 genes), followed by BE (32 of 119), and BR (18 out of 119) (Fig. 2.3d). IPA indicates that genes rescued in the BER striatum are enriched for canonical GPCR signaling, synaptic long term depression/potentiation, and calcium signaling, however there is minimal enrichment for the genes rescued in BE and BR striatum. Ingenuity upstream analysis also found that the striatal BER and BR rescue groups are enriched for transcripts regulated by HTT, suggesting a degree of cell-autonomous rescue that is not seen in the BE
Figure 2.3. BACHD mice exhibit mild HD-like transcriptional dysregulation at 12mo of age. (a) Numbers of genes down- (blue) and up-regulated (pink) in BACHD striatum (left) and cortex (right) at different statistical thresholds. (b) Correlation of BACHD differential expression with published human and mouse datasets, as well as with the BACHD-ΔN17 data presented in Chapter 2 and public 6mo Q175 data. BACHD striatal transcription is mildly correlated with human HD caudate datasets (Durrenberger et al., 2014; Hodges et al., 2006). Red indicates high correlation of two genesets, blue indicates opposite direction. Cells with numbers reach significance P < 0.05. (c-d) Numbers of genes down- and up-regulated reaching suggestive significance threshold of FDR < 0.1 in the striatum and the numbers of overlapping dysregulated genes in the striatum between 12mo BACHD, combined 12 and 24mo YAC128, and 6mo Q175.
rescue genes (Table S1). Together, these results point to a smaller cell autonomous transcriptional program that is altered in the striatum of BACHD mice that can be rescued by striatal mHTT activation, but also that cortical signaling drives significantly more transcriptionopathy, as revealed by the non-cell autonomous rescue of more than a quarter of the significant genes. It is also promising to confirm for future therapeutic trials that a majority of gene transcription changes seen in BACHD mice can be at least partially corrected by targeting the cortex and striatum.

While the DGE data yield insight into some of the most robust transcriptional changes in the BACHD mice, and the subsequent rescue in the BE, BR, and BER mice, we undertook Weighted Gene Coexpression Network Analysis (WGCNA; (Langfelder and Horvath, 2008; Zhang and Horvath, 2005) to glean more information on dysregulated transcriptional networks that may not meet the DGE threshold, but are still disrupted in HD mice. WGCNA utilizes correlation networks to reduce a cluster of similarly expressed genes to a “module,” whose average expression pattern can be then analyzed for disease relevance. Additionally, the genes that more conform to the average expression pattern of the module (summarized by the module eigengene, or ME) are hypothesized to be more important to these modules (Langfelder et al., 2013). By using modules instead of differential expression of the entire transcriptome, WGCNA largely avoids problems with multiple testing corrections. WGCNA has been applied to the study of multiple systems biology questions, yielding insight into co-expressed gene networks in the brain, periphery, cell culture, and even on proteomics and genomics data (Fuller et al., 2007; Kogelman et al., 2014; Miller et al., 2010; Oldham et al., 2006; Shirasaki et al., 2012; Tian et al., 2014).
WGCNA performed by Peter Langfelder resulted in 28 striatal and 33 cortical modules (Fig. 2.a-b), ranging from 33 to 2894 genes in the striatum and 36 to 2820 in the cortex. Plotting of ME vs. genotype allowed for the identification of modules that are dysregulated in BACHD.

**Figure 2.4. WGCNA coexpression dendrograms, module assignment, and differential expression.** (a,b) Striatal (a) and cortical (b) dendrograms indicating groups of genes that have similar expression patterns across conditions. These are genes are grouped into modules, which are assigned color and number labels. Below, gene expression heatmaps indicate differential expression of genes within the indicated modules (red, up-regulated; blue, down-regulated). For example, the striatal module M2, which is colored blue, contains genes that are predominantly up-regulated in BACHD vs. WT. These genes, however, are not predominantly up-regulated in BER vs. WT striatum.
compared to WT and are rescued in BER (see striatal modules M1, M19, M44, and cortical module M19). In addition to these criteria, some striatal modules were also rescued or partly rescued in BR (cell autonomous rescue, see striatal modules M38 and M49) or in BE (non-cell autonomous rescue, see striatal modules M2, M24, M41, and M45), and some cortical modules were at least partially rescued in BE (cell autonomous rescue, see cortical modules M4, M6, M43, and M49), however no cortical modules were restored in BR mice (See Tables 3-4 and S2). The two largest of the striatal modules, M1 and M19, have close to 3000 genes each and appear to be genes that are rescued in BER, but not BE or BR. M1 genes are down-regulated in BACHD, and are enriched for protein ubiquitination pathway, mitochondrial, and phagosome maturation pathway genes, as well as genes that have previously identified as being down-regulated in Alzheimer’s disease. M19 genes, which are up-regulated in BACHD, are enriched for axon guidance, acute myeloid leukemia, and RAR activation pathways, as well as for glutamatergic synapse, small GTPases, and nervous system development genes.

Striatal M2 is the major module of down-regulated genes that are rescued in a non-cell autonomous manner in BE mice. The 335 genes in this module are enriched for cAMP signaling, the superpathway of inositol phosphate compounds, striatal markers, neuronal markers, and genes localized to dendritic spines. It also contains some of the well-known genes from HD profiling, for example Actn2, Ddit4l, Gpr6, Cnr1, Oprk1, and Igfbp4, making this module of high interest. Striatal M45 is a small module (55 genes) shows the same pattern of down-regulated genes that are rescued in BE mice, but is enriched for oligodendrocytes genes. Striatal M42 genes show the opposite pattern: up-regulation in BACHD that is rescued in BE, as well as BER. This smaller module (87 genes) is enriched for cell adhesion and epoxysqualene biosynthesis, as well as choline degradation genes. Striatal modules M38 and M49 appear to be
Table 3. Striatal coexpression modules rescued with Cre-mediated deletion of mHTT.

<table>
<thead>
<tr>
<th>Module (Size)</th>
<th>ME vs. Genotype Plot</th>
<th>Rescue Type</th>
<th>Hub Genes</th>
<th>IPA Canonical Pathways</th>
<th>userListEnrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Str M1 2894</td>
<td>BACHD down-regulated genes rescued in BER only</td>
<td>Impact. Mf, Sec22a, Bp27, Serror1, Apb61a1, Phk3a, Act10, Canx, Arf1b</td>
<td>Protein ubiquitination</td>
<td>Mitochondrial dysfunction</td>
<td>Phagosome maturation</td>
</tr>
<tr>
<td>Str M2 335</td>
<td>Partial, non-cell autonomous, BE rescue in striatum, BER rescue</td>
<td>Itpr1, Pp1p1, Ppp2r3, Garn13, Mct1, Smad2, Acam1, Lmbr2, Opr155, Pgcmt11</td>
<td>cAMP signaling</td>
<td>Superpathway of inositol phosphate compounds</td>
<td>Striatum global marker</td>
</tr>
<tr>
<td>Str M19 2885</td>
<td>BACHD up-regulated genes rescued in BER only</td>
<td>Bar1, Spec, Scn1, Cacln, Hslr2, Rlr1, Fosq1, Zwint8, Vars, Ccl21a1</td>
<td>Axon guidance</td>
<td>Glutamatergic synapse</td>
<td>Ras / Rho guanyl-nucleotide exchange factor activity</td>
</tr>
<tr>
<td>Str M24 155</td>
<td>BACHD up-regulated genes rescued in BER, evidence of partial BE rescue</td>
<td>Sotr9a, Nkrd1, Mrosk1, Kir2, Cgmr, BC012158, Card10, Efr3, Cacl2, 2810300D17Fk</td>
<td>Methylopropionate biosynthesis</td>
<td>Triacylglycerol degradation</td>
<td>Nucleus accumbens markers</td>
</tr>
<tr>
<td>Str M34 365</td>
<td>BACHD up-regulated genes rescued in BER</td>
<td>Clec16a, Ipsec2, Wpt2, Bocor1, Gm12090, Ccl3, Kmt2a, Rpr, Rnf165, Dilgap2</td>
<td>Wnt / β-catenin signaling</td>
<td>Ephrin receptor signaling</td>
<td>Axin binding</td>
</tr>
<tr>
<td>Str M38 192</td>
<td>BACHD up-regulated genes rescued in BER, BER</td>
<td>Mdc21, Rap1gap, Lmp1, Tpm2, Shk5a, Itpr7, Acrod4, Sgam3, Nnfs, Narf</td>
<td>Cdk5 signaling</td>
<td>Salvage of pyrimidine ribonucleotides</td>
<td>Amyloid processing</td>
</tr>
<tr>
<td>Str M41 93</td>
<td>Partial, non-cell autonomous, BE rescue in striatum, BER rescue</td>
<td>Grik2, Kcna4, Csmi3, Cnn5, B2a, Rims2, Sgzc, Nos1, AW148154, 4rpr1p</td>
<td>NOS signaling in skeletal muscle</td>
<td>Neflin signaling</td>
<td></td>
</tr>
<tr>
<td>Str M42 87</td>
<td>Non-cell autonomous BE rescue in striatum, BER rescue</td>
<td>Aco3, Eda2b, Pdym, Pdzm4, Msat21, Cdk11a, Sfrp1, Pig5, Sus2, Ami2g2</td>
<td>Epoxypropane biosynthesis</td>
<td>Choline degradation</td>
<td></td>
</tr>
<tr>
<td>Str M44 68</td>
<td>BACHD up-regulated genes rescued in BER only</td>
<td>Prkd, Smag2, Diap1, Dskdl, Sgmer, Nhe2, Sd14a35, Tnnem109, Gatt2f, Arfla</td>
<td>Axon terminus</td>
<td>Response to histamine</td>
<td>Negative regulation of cAMP signaling</td>
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<tr>
<td>Str M45 55</td>
<td>Non-cell autonomous BE rescue in striatum, BER rescue</td>
<td>Tpat, R3, Pdx5, Car2, Pp1, Etv2, Gpase4, Ckn11, Serpinb1a, Aips</td>
<td>Granulocyte / agranulocyte adhesion and diapedesis</td>
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<td>Oligodendrocyte</td>
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<tr>
<td>Str M49 38</td>
<td>Cell autonomous BR rescue in striatum, BER rescue</td>
<td>Adm4, Zfp664, Sf1c39a, 493106c0270fik, Dwe51, Tpp4kat, Adck3, Zbtb33, Rspn1, Lirb</td>
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Table 4. Cortical coexpression modules rescued with Cre-mediated deletion of mHTT.

<table>
<thead>
<tr>
<th>Module (Size)</th>
<th>ME vs. Genotype Plot</th>
<th>Rescue Type</th>
<th>Hub Genes</th>
<th>IPA Canonical Pathways</th>
<th>userList/Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctx M4 72</td>
<td></td>
<td>Cell autonomous BE rescue in cortex, BER rescue</td>
<td>Stxbp6, Plen, Grp, Nrsgrf2, Mr7, Afpqap28, Isof1, 4933404D12Rq, Cep3t11, Slpr3</td>
<td>GPCR-signaling</td>
<td>Aging, Nucleus accumbens markers</td>
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<tr>
<td>Ctx M6 116</td>
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<td>Partial cell autonomous BE rescue in cortex, BER rescue</td>
<td>Adcy5, Asphb1, Cablax2, Tk1, Xnc3, Fam311a, Ccl1, Sugg1, Ilpr3, Acot1</td>
<td>Gustation pathway</td>
<td>Sensory perception of umami taste</td>
</tr>
<tr>
<td>Ctx M19 47</td>
<td></td>
<td>BER Rescue only</td>
<td>Csgt5, Argrf1, S1c1a3, Mic1, Adh1111, Ape1, Csp1, Scc4a1, S1pr1, Ptk1</td>
<td>Sphingosine-1-phosphate signaling, FXR/RXR activation, IL-12 signaling and production in macrophages</td>
<td>Validated astrocyte, Positive regulation of nitric oxide synthase</td>
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<tr>
<td>Ctx M43 81</td>
<td></td>
<td>Cell autonomous BE rescue in cortex, BER rescue</td>
<td>Gsl1, I33, Fam13a, Hcp14, Cmm110, Zfp946, Pdp111x, Ptp1, Post1, Man1</td>
<td>Glutamine biosynthesis</td>
<td>Putamen markers, Oligodendrocyte, Parahippocampal gyrus markers</td>
</tr>
<tr>
<td>Ctx M49 38</td>
<td></td>
<td>Cell autonomous BE rescue in cortex, BER rescue</td>
<td>Dcat6, Camklc, Cnmd2, Tf, Cyp7b1, Socs2c, Dgclp, Fox3, Spats2l, Gm1</td>
<td>Nrf2 oxidative stress response, Tetrahydrofolate salvage</td>
<td>Cortical neurons, Differentially expressed in autism</td>
</tr>
</tbody>
</table>

rescued in a cell autonomous fashion in the BR mice. M38 is up-regulated in BACHD, normalized in BR and BER, and contains genes enriched in Cdk5 signaling, amyloid processing, and microglia activation pathways. M49 has the opposite pattern of expression, and this very small module (just 38 genes) shows only mild enrichment for enhancer binding activity.

In contrast to the differential expression analysis of cortical transcription, WGCNA identified several modules of co-expressed genes that are associated with BACHD. The six cortical modules that show normalization of BACHD expression in at least the BER mice are, on the whole, smaller than the striatal modules (max size of 116 genes). Cortical M19 is the slightly down-regulated module that appears to be normalized in just BER and is enriched in sphingosine-1-phosphate, FXR/RXR activation, IL-12 production, and is very specific to astrocytes. The other cortical modules exhibit at least a partial cell autonomous rescue in BE as well as BER. M4 and M49 are up-regulated in BACHD, with M4 showing enrichment in GPCR
signaling, genes associated with aging and the nucleus accumbens, and M49 genes being enriched for Nrf2-mediated oxidative stress, cortical neurons, and genes differentially expressed in autism. M6, which is down-regulated in BACHD and partially rescued in BE and BER, is enriched for gustation pathway, PKA, and GPCR nutrient sensing signaling, as well as the sensory perception of umami flavor. M43, the last of the down-regulated cortical modules that is rescued in BE and BER is enriched for glutamine biosynthesis and markers for several types of cells and regions (putamen, oligodendrocytes, and hippocampus).

2.5 Amelioration of Synaptic and Pathology in BER Mice

Since cortico-striatal signaling is altered in HD mice, including BACHD, we sought to assess whether genetic reduction of mHTT expression in our rescue mice could ameliorate this aberrant signaling. Actn2 is a basal ganglia-enriched gene that shows reduced expression in HD patient brains and multiple mouse models, including BACHD. Actn2 binds to both actin and N-Methyl-D-aspartate (NMDA) receptor NR1 and NR2B subunits, which are highly expressed in MSNs, and is thought to play a role in anchoring the receptors at the post synaptic density (PSD; reviewed in (Otey and Carpen, 2004). This function is Ca\(^{2+}\)/calmodulin dependent, with calmodulin directly competing for Actn2 NMDA receptor binding (Wyszynski et al., 1997). Since altered NMDA receptor-dependent Ca\(^{2+}\) signaling and localization of the receptors have both been proposed as potential toxic mechanisms in HD (Fan et al., 2007; Milnerwood and Raymond, 2010; Raymond et al., 2011), Actn2 is an interesting target for further research to dissect the mechanisms by which altered cortico-striatal signaling may contribute to HD pathogenesis. We undertook staining of Actn2, as well as another PSD-95, another postsynaptic
marker protein that does not show evidence of down-regulation in our transcription data but has been linked to NMDAR dysfunction in HD (Fan et al., 2009), in addition to synaptophysin, a

Fig. 2.5. Rescue of synaptic pathology in mice with genetic reduction of mHTT transgene. (a-d) Immunostaining (a) of presynaptic marker protein synaptophysin (top), and postsynaptic proteins Actn2 (middle) and PSD-95 (lower) show loss of these markers in BACHD compared to WT that is restored in the Cre-expressing mice. Scale bar = 100µm. (b) Quantitation of synaptophysin immunofluorescence across genotypes. (c) Quantitation of Actn2 immunofluorescence. (d) Quantitation of PSD-95 immunofluorescence. * P < 0.05, ** P < 0.01, *** P < 0.001; one-way ANOVA followed by LSD post hoc test. Values represent mean ± SEM. N ≥ 7 for all.
presynaptic marker protein. Staining levels of all three synaptic proteins were reduced in the striatum of BACHD mice at 12 months of age (Fig. 2.5a-d). BER mice exhibited normalization of Actn2 and synaptophysin levels to WT levels. BE mice, however, partially restored expression of both these synaptic proteins, suggesting that mHTT can exert both cell autonomous effects on synaptophysin as well as non-cell autonomous effects on Actn2. In contrast, BR mice did not show a restoration of the presynaptic marker synaptophysin but did exhibit a partial, significant rescue of Actn2 staining in the striatum, suggesting there is also influence of cell autonomous mHTT in changes in these marker proteins. PSD-95 levels were restored in the striatum with genetic reduction of mHTT in cortex (BE), striatum (BR), and both (BER). In summary, staining of synaptic marker proteins adds further evidence that mHTT exerts cell autonomous pathogenic effects on the striatum, and that mHTT expression in the cortex contributes non-cell autonomously to striatal dysfunction.

2.6  Transcriptionopathy in Cortico-striatal Projection Neurons Identifies Npas4 Loss as a Potential Pathogenic Mechanism in HD Mice

To further interrogate the role of aberrant cortico-striatal dysfunction in striatal pathogenesis in HD, we sought to understand the role that transcriptional dysregulation may play in this specific neurocircuit. Since previous studies of HD transcriptionopathy have been performed in HD patients of animal models have used intact tissue, these result in a complete picture of HD transcription changes in an intact animal, however tissue is a heterogenous mixture of neuronal and non-neuronal cells, some of which may not have an etiological role in HD pathogenesis, and thus present average expression across that tissue. Cell models, while they are more “pure” from a composition standpoint, lack the full disease context to assess non-
cell-autonomous toxicity. To utilize the full disease context, with a far less complex cellular composition than dissected tissue, we performed unilateral striatal FluoroGold (FG) injections to retrograde label cortico-striatal projection CSPNs, used fluorescent-activated cell sorting (FACS) to purify the subset of FG+ CSPNs from the contralateral cortex (to avoid FG+ cells near the injection tract), and ultimately perform RNA-seq in HD vs. WT mice. This subset of CSPNs, called intratelencephalically-projecting (IT) CSPNs, are found in layer five of the cortex, send projections through the corpus callosum to the contralateral striatum, and are thought to play a role in movement planning and reward prediction (Reiner et al., 2010). FluoroGold is an extremely efficient retrograde dye (Zingg et al., 2014), and IT CSPNs were readily purified from mouse cortex 3 weeks post injection (Fig. 2.6a-e). Sorted neurons were enriched for CSPN markers Satb2 and BDNF, and not for markers of other cell types (Darpp-32 for MSNs, GFAP for astrocytes, Calb for a subset of interneurons, Mbp for oligodendrocytes), with the exception of Cd68, indicating that there may be some microglia sorted with the retrograde-labeled CSPNs (Fig. 2.6f-h). We performed these experiments at 2mo in knock-in Q175+/Q mice (referred to here as Q175) and WT littermates, as these mice exhibit robust behavioral phenotypes beginning around 2mo (Menalled et al., 2012; Smith et al., 2014). 2 male and two female mice were used per genotype, with the mice generating ~16,700 retrograde-labeled, FACS-collected cells per contralateral cortex in WT and ~17,000 cells in Q175. Since the RNA yield from this low number of cells was small (in the several nanogram range), we utilized the NuGen Ovation Ultra-low Mass linear amplification library prep kit on one nanogram of total RNA, and sequencing was performed as before at a depth of approximately 75M reads per sample (of which ~58M, or ~77%, uniquely mapped to the mouse genome). We used our earlier criteria of genes significant at the FDR < 0.1 level and found 23 transcripts that show differential
Figure 2.6. Sorting of retrograde-labeled cortical FluoroGold cells suggests enrichment in markers for cortical pyramidal neurons. (a) Schematic brain section showing dorsal striatum FluoroGold injection site. (b) Bilateral FluoroGold immunofluorescence three weeks after striatal injection shows intense FluoroGold signal in the ipsilateral striatum and cortex, with lower, primarily cellular signal in the contralateral cortex. (c) FluoroGold positive cells in the contralateral cortical deep layers have morphology suggesting they are CPNs. (d,e) FACS sorting scatterplot of FluoroGold (Indo1 channel) versus dead cell marker 7-AAD (PerCP channel) shows no FluoroGold positive cells in the negative control (d), while the FluoroGold retrograde labeled sample (e) shows FG⁺ cells. Both are representative traces of 10,000 FACS events. (f-h) Quantitative PCR for makers of CPNs (f), MSNs and a subclass of interneuron (g), and glia (h) show that the sorted CSPNs express cortical neuronal markers and not markers of other cells, with the exception of possible microglia expression (seen via Cd68 expression).
expression in our HD IT CSPNs (see Table 5), including some activity dependent genes, like the transcription factor Npas4, that the lab had identified as interesting in work analyzing the ΔN17 microarray data discussed in later chapters. Other transcripts of interest include structural proteins such as Bfsp2, Prph, and Cdhl8, trafficking-related proteins Sorcs3 and Lamp5, and calcium-related proteins Ryr2 and Syt6 that could play a role in pathogenic cortico-striatal signaling at the synapse.

Npas4, is an immediate early gene that has been identified in activity-dependent protective signaling (Zhang et al., 2009) and plays roles in regulating domain-specific inhibition in excitatory neurons (Bloodgood et al., 2013) as well as inhibitory-excitatory balance (Spiegel et al., 2014). Of further interest, Npas4 regulates BDNF transcription, which is down-regulated

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in the cortex of HD patients and mice, and loss of which is thought to contribute to striatal pathogenesis via loss of trophic support (Gauthier et al., 2004; Strand et al., 2007; Zuccato et al., 2001; 2005). To confirm the reduction of Npas4 in cortical neurons in Q175 mice, we co-stained 2mo WT and Q175 mice with Npas4 and NeuN to identify neurons. Deep layer neurons consistent with CPNs had a roughly 30% reduction in Npas4 staining in 2mo Q175 (Fig. 2.7a-b). To further generalize this reduction, we extended the staining to 12mo old mice from the WT, BACHD, BE, BR, BER cross. Npas4 staining was significantly reduced by roughly 45% in BACHD cortex (Fig. 2.7c-d). BE mice showed a similar, more variable reduction that did not reach statistical significance, as did the 40% reduction in BR mice. The 20% reduction in BER cortex compared to WT was also not significant, although the rescue of approximately 25% compared to BACHD was significant. In summary, Npas4 levels were found to be reduced in sorted, retrograde-labeled IT CSPNs in 2mo Q175 mice compared to WT. We confirmed the reduction in cortical neurons in Q175 mice with staining for Npas4, and then extended the finding to the BACHD mice with genetic reduction of mHTT. While Npas4 levels are significantly rescued in BER mice compared to BACHD, they are still reduced in comparison to WT. Lastly, there is not evidence of cell-autonomous or non-cell autonomous rescue of Npas4 levels in the BE or BR mice, indicating that Npas4 could be a more generalized marker of cortico-striatal dysfunction.

2.7 Conclusions

Huntington’s disease is an excellent proof of concept for the use of the two-step reductionist approach to studying the pathogenesis of neurodegenerative disorders. Using this approach, with insight gleaned from previous studies, we showed that genetic
Figure 2.7. Immunofluorescent staining confirms reduction of Npas4 expression in cortical neurons. (a,b) Staining (a) with quantitation (b) of Npas4 and NeuN in the deep cortical layers of 2mo WT and Q175 mice shows reduction in Npas4 signal that colocalizes with NeuN⁺ neurons. (c,d) Npas4 staining (c) is also reduced in 12mo BACHD mice, and partly normalized in BER mice (d). Images representative of three experiments. * P < 0.05, Student’s t-test.
reduction of mHTT expression in the cortex and striatum is sufficient to ameliorate behavioral deficits and neuropathology. Further, we interrogated the molecular changes underlying disease pathogenesis in these critical regions using genome-wide transcriptional profiling. Insights into these molecular pathogenic changes were confirmed by staining for proteins at the cortico-striatal synapse, which were altered in BACHD and reduced to varying degrees in our mHTT lowering mice. The approach was taken one step further by using retrograde dye to label CSPNs for FACS, followed by transcription profiling, identifying more genes that may be linked to cortico-striatal dysfunction in HD.

In the first step of the two-step approach, we used Cre-loxP conditional genetics, first confirming that Emx1-Cre and Rgs9-Cre is specific to the cortex (with some hippocampal expression) and striatum, respectively. Using this Cre-loxP system crossed into the BACHD background, we showed reduction of both mHTT transcript and protein, following our cohort of mice with genetic reduction of mHTT in the cortex, striatum, or both cortex and striatum, with mHTT expression unperturbed in the rest of the body through 12mo of age. Longitudinal behavioral phenotyping identified that BER mice, with genetic reduction in cortex and striatum, saw amelioration of HD-like behavioral deficits to near WT levels; BE mice, with genetic reduction of mHTT expression in the cortex, saw partial improvement in some motor tests, such as 6mo rotarod, and 12mo open field, but near complete restoration of psychiatric-like deficits (i.e. light-dark box and forced swim); in contrast, BR mice, with mHTT reduction in the striatum did not exhibit any significant rescue of behavioral deficits compared to BACHD mice. This confirmed that we had identified a crucial set of neurons for the pathogenesis of HD: the Emx1 and Rgs9 positive cells of the cortex and striatum. Additionally, these results added evidence that, while cell autonomous toxicity underlies some of the vulnerability of striatal MSNs in HD,
the full brunt of the disease is only brought on in a non-cell autonomous manner with expression of mHTT in the cortical pyramidal neurons.

In the second step of the two-step approach our analyses of the cohort of WT, BACHD, BE, BR, and BER mice lead to some interesting insights into the transcriptional dysregulation underlying normalization of behavioral deficits in these mice. RNA-seq and standard differential expression analyses identified 119 genes that are differentially expressed in the striatum of BACHD mice, with 72 of these genes rescued when mHTT is genetically reduced in the cortex and striatum of BER mice. Further, a subset (31) of these 119 differentially expressed striatal genes are rescued in the striatum in a non-cell autonomous fashion in the BE mice, and 19 genes were rescued in a cell autonomous fashion in the striatum of BR mice. We utilized WGCNA to obtain more insight into gene expression network changes in these genotypes, and found that multiple modules associated with the BACHD genotype are rescued with our inactivation lines. While the largest striatal modules (M1, M19) rescue BACHD in just the BER mice, multiple other modules highlight gene networks that are normalized in a non-cell autonomous fashion in the striatum of BE mice (e.g. M2, M42, M45). The cortical modules are interesting in that they have just one module that is restored in BER, but not BE (M19); the rest of the BER-rescued modules are all at least partially rescued in BE mice (M4, M6, M43, and M49). This seems to suggest that gene network changes correlated with disease symptoms are more cell autonomously regulated in BACHD cortex, unlike in the striatum, which has multiple networks affected in a non-cell autonomous fashion by mutant huntingtin deletion in the cortex.

Finally, we attempted to delve into the molecular changes underlying HD pathogenesis in IT type cortico-striatal projection neurons. These neurons or the pyramidal tract neurons that do not project contralaterally, provide the cortical input to the striatal MSNs, and hence molecular
pathogenesis in these cells could provide insight into aberrant cortico-striatal signaling that creates a second hit to vulnerable MSNs. Transcriptionally altered genes in these cells, even at two months of age in Q175 mice, include structural proteins that could be involved in the morphology or function of cortico-striatal synapses, trafficking-related proteins that could be involved in altered neurotransmitter release, calcium-related proteins that could affect synaptic release, and Npas4, an activity-dependent transcription factor that is responsible for activity-induced neuroprotective transcriptional programs, domain specific inhibition, excitatory-inhibitory balance, and BDNF transcription (ibid). Npas4 reduction in cortical neurons was confirmed with staining of 2mo Q175 mice and then in the 12mo BACHD mice. Npas4 was found to be partially, though significantly rescued in the cortical neurons of BER mice, but not BE or BR.

The narrative written by this study of the cells and molecular substrates of HD-like pathology in mouse models of HD is fairly consistent: cells vulnerable to HD undergo cell autonomous molecular perturbation caused by mHTT expression, however there are synergistic effects of mHTT beyond these vulnerable cells that lead non-cell autonomously to the full scope of HD-like pathology.

2.8 Materials and Methods

Generation and breeding of conditional HD mice. BACHD mice were generated in the FvB/NJ background as described previously (Gray et al., 2008). Emx1-Cre (E) (Iwasato et al., 2000) and Rgs9-Cre (R) (Dang et al., 2006) mice were backcrossed into the FvB/NJ background >10 generations, then crossed to each other, resulting in 25% Emx1-Cre x Rgs9-Cre (ER) double transgenic mice. ER mice were crossed to BACHD mice, resulting in the following genotypes:
WT, BACHD, E, R, ER, BE, BR, BER. WT, BACHD, BE, BR, and BER mice were used for experiments. Mice were bred and housed under standard conditions, consistent with NIH guidelines and approved by the UCLA Chancellor’s Animal Research Committee. Mice were genotyped using the following primers. BACHD: 5’-gagccatgattgtgctatcg-3’ and 5’-agctacgtgctcagaaaa-3’. Emx1-Cre: 5’-gcgttccccagagccccgctacctcact-3’ and 5’-ggatccgccccgctcataacccagtg-3’. Rgs9-Cre: 5’-aggatgatcggacgaagage-3’ and 5’-tcgccccacaaactgaacaga-3’.

**Cre Reporter Assays.** Double transgenic Cre mice (ER) were crossed with lacZ reporter mice (Gt(ROSA)26Sor<sup>tm1Sor</sup>) from the Jackson Laboratory (JAX003309). Mice with Cre transgenes and lacZ transgene were used for β-galactosidase staining using our previously published protocol (Yang et al., 1997). ER mice were also crossed with Ai3 YFP reporter mice (Gt(ROSA)26Sor<sup>tm3(CAG-EYFP)HZE</sup>) from the Jackson Laboratory (JAX007903) (Madisen et al., 2009). 3mo old mice were perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline, brains were post-fixed overnight in 4% PFA and then in 30% sucrose at 4°C. 20µm brain sections were cut using a Leica 1800 cryostat and stained for using anti-NeuN (1:1000, Millipore MAB377) followed by anti-mouse Alexa Fluor 594 (1:300, Life Technologies A11005) as in (Gu et al., 2005).

**Western blotting.** Proteins lysates and Western blots were performed as in our previous studies (Gray et al., 2008). Blots were probed using 1C2 (antibody specific for expanded polyQ, 1:3000, Chemicon MAB1574) and anti-α-tubulin (1:3000, Sigma T9026). Two male and two female mice per genotype at 3mo were used for quantification of Western blots using densitometry.

**In situ hybridization.** A probe consisting of the mHTT fragment between the two loxP sites in the BACHD transgene was used as cDNA template, and <sup>35</sup>S-UTP radiolabeled RNA was
transcribed using the RNAMaxx High Yield Transcription Kit (Agilent). Hybridization was performed as in (Lobo et al., 2006) on one sections from male and one female mouse per genotype.

**Behavioral Studies.** All behavioral studies used n = 12-15 per genotype.  
*Accelerating rotarod:* Mice were trained on an Ugo Basile 7650 accelerating rotarod (4-40 rpm) for two days with three trials per day before testing. Mice were trained to walk on the accelerating rod and replaced on the rod if they fell during the first 20 seconds of a trial. After training, the same cohort of mice was repeatedly tested for rotarod performance for three trials per day over three days at 2mo, 6mo, and 12mo of age.  
*Light-dark box:* The Light-Dark box is a behavioral assay that consists of a polypropylene box (45 x 21 x 21 cm) unequally divided into 1/3 dark and 2/3 light. Separating the dark and light side is a wall with an opening (8.5 x 5 cm) to allow mice to move free between the chambers. Each test is started by placing the mouse in the lit chamber at the opposite end facing the opening to the dark chamber. Each test trial lasted for exactly 10 min. Behavior for the LD assay was scored by examining the latency to enter the light box and the number of light-dark transitions. To score a transition from one area to the other, all four paws had to cross each region. Time spent in each area was calculated from the time of first entrance into the light box.  
*Open field:* Automated open field analysis was used to assess locomotor activity. Mice were tested in the automated open field (Coburn) at 2, 6, and 12mo. Sessions lasted 15 minutes.  
*Forced swim:* Mice were allowed to acclimate to experimental room at least one hour before testing period. Mice were placed in transparent plastic cylinders 15 cm in diameter X 25 cm high containing water that is 25°C. Animals placed in the water were recorded with a video camera for 6 minutes. Swimming behavior was assessed for the last 4 minutes of the test, and immobility duration scored as the time spent immobile (floating without
excess movement), excluding movement necessary to keep the animals’ heads above water. All testing except open field was performed during the light phase of the light/dark cycle. Behavioral testing was performed by researchers who were blind to mouse genotype.

**Neuropathology.** Brain weight and stereological measurement of cortical and striatal volume was performed as described (Gray et al., 2008; Gu et al., 2009) using n = 8 mice per genotype at 12mo.

**BER Tissue RNA extraction, library prep, and sequencing.** Tissue was dissected from mice in the BER cross at 2mo, 6mo, and 12mo. Brain (cortex, striatum, thalamus, cerebellum) and peripheral tissue (blood, heart, liver, skeletal muscle) were dissected simultaneously and immediately flash-frozen on dry ice. Once all tissue was dissected, samples were arranged into randomized batches and cortical and striatal RNA was prepared using a miRNA permissive kit (mirVana, Life Technologies). All 12mo cortical and striatal samples were sent for Bioanalyzer quantitation of RNA quality and concentration. The highest quality RNA samples were selected for each genotype, with balanced sex ratio (2 male and 2 female per condition), and showed excellent quality for library preparation (Avg. RIN = 8.54, low = 7.3, high = 10). These 40 samples (two tissues, five genotypes, and four biological replicates) were again randomized and sent for library preparation using Illumina Ribo-Zero ribosomal RNA depletion and sequencing using 2x69bp rapid runs at the UCLA Neuroscience Genomics Core facility. Sequencing was performed using 2x69bp Rapid runs.

**FluoroGold retrograde labeling, fluorescence-activated cell sorting, RNA extraction, library preparation, and sequencing.** 4% FluoroGold (FG; Santa Cruz, SC-358883) was prepared by dilution in embryonic transfer buffer (10mM Tris-Cl, 0.1mM EDTA, 100mMNaCl, pH 7.5) and filtered using 0.22μm syringe filter before use. 1μL FG solution was injected unilaterally into
the striatum of WT and Q175 mice at 2 months of age using a stereotaxic injection frame (coordinates -1.55, 0.75, -3.00) at 0.2µL/minute infusion rate. After three weeks, contralateral cortices were dissected and underwent papain/DNase dissociation as in (Crook and Housman, 2012). FACS was performed at the UCLA Johnson Comprehensive Cancer Center Flow Cytometry Core. Cells were collected directly into RNA extraction buffer and immediately processed following the manufacturer’s protocol (PicoPure RNA Kit, Life Technologies). RNA quality and concentration was assessed using Bioanalyzer and 1ng of high quality total RNA was sent for library preparation using the NuGen Ovation Ultra Low Mass library kit at the UCLA Neuroscience Genomics Core. Two male and two female mice were used per genotype. Sequencing was performed as above.

**BER Differential Gene Expression and WGCNA.** Analyses were performed by Peter Langfelder in Steve Horvath’s group. In the BER quality control and quantile normalization process, one male WT striatal sample was identified as an outlier and removed from further analyses. No samples were excluded in the quality control and normalization process for the sorted CSPNs. For the BER study, differential gene expression (DGE) was performed using DESeq2 and WGCNA in R (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). For sorted CSPNs, DGE was performed by the UCLA Informatics Center for Neurogenetics and Neurogenomics (ICNN) using edgeR in R.

**Immunofluorescent Staining.** 12mo mice from the BER cross were used for synaptic pathology studies (N = 7-10 per genotype). For Npas4 studies, 2mo Q175 and WT mice and 12mo BER cross mice were used (N = 3 per genotype). All mice were transcardially perfused using 0.1M PBS and 4% PFA and post-fixed as above. Mice for the synaptic pathology studies were cut thin (5µm), while those for Npas4 staining were cut at 40µm. Immunofluorescent staining was
performed using the following antibodies. Anti-Synaptophysin (Millipore MAB5258A4, 1:200), anti-Actn2 (Epitomics EP2529Y, 1:500), anti-PSD-95 (UC Davis/NIH NeuroMab 75-028, 1:500), anti-Npas4 (Abcam ab110855, 1:500), and anti-NeuN (Millipore MAB377, 1:1,000). These were followed by the compatible Alexa Fluor conjugated secondary antibodies (Life technologies goat anti-rabbit and goat anti-mouse Alexa Fluor-594 A11037 and A11005, goat anti-rabbit Alexa Fluor-488 A11008, 1:300). Sections were imaged using a Zeiss LSM510 confocal laser scanning microscope and immunofluorescent intensity measured using ImageJ (NIH). All images for each study were taken under identical conditions and quantitated blind to genotype.
Chapter 3

Accelerated HD-like Phenotypes, Enhanced Nuclear Toxicity, and Transcriptional Dysregulation in a Transgenic Mouse Model Lacking the N17 Domain of Mutant Huntingtin
3.1 Introduction

Post-translational modification of the mutant huntingtin protein can play a large role in its propensity to cause disease. Amongst the numerous modifications huntingtin can undergo (reviewed in Ehrnhoefer et al., 2011), our lab has previously studied the role of the 17 amino acid domain at the N-terminus of huntingtin (N17). This evolutionarily conserved domain (Tartari et al., 2008) undergoes numerous post-translational modifications, including phosphorylation, ubiquitination, and sumoylation, with these modifications altering aggregation and toxicity (Steffan, 2004; Thompson et al., 2009). Our lab found that mimicking phosphorylation of serines 13 and 16 of the N17 domain (SD mutations) led to the complete abolishment of behavioral and neuropathological phenotypes in BACHD-SD mice, while mice with the same amino acid residues mutated to phosphoresistant alanine (SA mutations) exhibited similar behavioral phenotypes as the BACHD mice they were derived from (Gu et al., 2009).

Additional work on understanding the role of the N17 domain HD pathogenesis identified that it plays a role in cytoplasmic retention of huntingtin, and deletion or mutation of the domain can lead to greatly accelerated accumulation of nuclear huntingtin in cultured cells (Atwal et al., 2007; Rockabrand et al., 2006).

Cellular localization of expanded polyQ proteins has been found to play large roles in disease processes. In spinocerebellar ataxia type 1 (SCA1) and spinal and bulbar muscular atrophy (SBMA), both polyQ diseases, nuclear localization of the mutant proteins is critical for the development of disease pathology (Katsuno et al., 2002; Klement et al., 1998). Although huntingtin is largely localized to the cytoplasm, it can enter the nucleus (Atwal et al., 2011), mHTT undergoes differential interaction with transcription factors compared to HTT (Sugars and Rubinsztein, 2003), and is thought to exert toxicity in both locations (Ross and Tabrizi,
Further, targeting mHTT fragments to the nucleus using nuclear localization signals has been found to accelerate disease pathogenesis in mice (Benn et al., 2005). To further understand the role of the N17 domain in mHTT-induced pathogenesis, specifically its roles in aggregation and nucleocytoplasmic localization, our lab developed transgenic mice based on the BACHD line, but with deletion of amino acids 2-16 of the N17 domain (BACHD-ΔN17 mice). This chapter will discuss the behavioral, neuropathological, and transcriptional characterization of this mouse line, which was recently published in Neuron (Gu et al., 2015).

### 3.2 Generation of BACHD-ΔN17 Mice

BACHD-ΔN17 mice were made by modifying the BAC originally used to make the BACHD line (Gray et al., 2008), with deletion of the codons encoding amino acids 2-16 in exon 1 (Fig. 3.1a). Control mice were also made that contracted the polyQ domain from the 97Q found in the BACHD to unpathogenic 31Q (these mice are termed BAC-WT-ΔN17). Transgenes were microinjected into fertilized FvB/NJ embryo pronuclei to generate founders, of three lines of BACHD-ΔN17, N, L, and A lines, (N line, being the more comprehensively studied is referred to throughout this work as BACHD-ΔN17) and one line of BAC-WT-ΔN17. BACHD-ΔN17 express mHTT at roughly a quarter the level seen in BACHD mice (Fig. 3.1b). BACHD-ΔN17 and BAC-WT-ΔN17 mice crossed onto the murine Htt null background were found to rescue Htt knockout embryonic lethality at a Mendelian ratio, indicating that the ΔN17 forms of human huntingtin maintain essential huntingtin functions in development (Fig. 3.1c-d).
Figure 3.1. Transgene design, expression, and rescue of murine Htt null lethality. (a) Schematic depicting transgene constructs used to generate BACHD-ΔN17 and BAC-WT-ΔN17 mouse lines. (b) Western blot of brain lysate probed with antibody against expanded polyQ (1C2) shows relative expression of mHTT between BACHD and BACHD-ΔN17 lines. α-tubulin loading control is shown below. (c) Both transgenic ΔN17 mouse lines rescue Htt null embryonic lethality. (d) Western blot probed for expanded polyQ (1C2) and N-terminal HTT/Htt (PW0595A) shows the lack of WT Htt in brain lysates from Htt null BACHD-ΔN17 transgenic mice. All blots representative of three independent experiments.
3.2 Longitudinal Phenotyping of BACHD-ΔN17 Mice

BACHD-ΔN17 mice and WT littermates were evaluated for behavioral phenotypes longitudinally up to 11mo. Mice expressing human full length huntingtin tend to be larger than WT mice (Menalled et al., 2009), which may be due to altered IGF signaling (Pouladi et al., 2010), and both male and female BACHD-ΔN17 are heavier than WT littermates by 2mo of age. Unlike the BACHD line, which maintains heavier body weight through life, BACHD-ΔN17 mice are roughly the same weight as WT by 7mo, and continue to lose weight until they are significantly lighter by 10-11mo (Fig. 3.2a). The transgenic mice also exhibit early mortality, with supportive care being necessary for mice aged 10-11mo.

Motor phenotypes are often the most sensitive assays to detect early HD-like behavior, and the BACHD mouse line exhibits progressive rotarod deficits beginning at 2mo (Gray et al., 2008; Menalled et al., 2009; Wang et al., 2014). BACHD-ΔN17 mice exhibit normal performance in the rotarod test at 1mo, but exhibit progressively decreased performance in the test beginning at 2mo and continuing through 6mo. Despite BACHD mice being able to run rotarod, albeit with reduced performance, beyond 12 months of age, by 8mo, BACHD-ΔN17 mice are no longer able to perform the accelerating rotarod test (Fig 3.2b). The transgenic mice also undergo progressive loss of motor coordination in gate testing, showing normal gate at 2mo and largely normal although worsening gate at 6mo, but significant deficits in stride length for all limbs, limb overlap, and both front and rear base length (Fig. 3.2c). Observing the mice in the home cage, we noticed that they tended to wobble and would spontaneously fall. Falling is a significant concern for HD patients, and the most common reason for the patients to end up in long-term care (Busse et al., 2009; Grimbergen et al., 2008; Wheelock et al., 2003). We quantitated falls in 10mo mice, and compared to WT mice, which do not exhibit spontaneous
Figure 3.2. Age-dependent motor deficits and evidence of neurological decline in BACHD-ΔN17 mice.  
(a) Body weight of male (M) and female (F) mice. Both male and female BACHD-ΔN17 mice initially gain weight compared to their WT littermates, however they weigh similar amounts by 7mo and begin to lose weight at 10-11mo.  
(b) Accelerating rotarod shows similar motor coordination at 1mo, however BACHD-ΔN17 mice exhibit impairment by 2mo. Impairment is progressive, and by 8mo the mice are unable to perform the task.  
(c) Gait analyses show no deficits at 2mo, and only a single effect on stride overlap at 6mo. At 8mo, however, gait is severely impaired, with deficits in stride length for all limbs, stride overlap, and front and rear base lengths.  
(d) Unlike WT mice, BACHD-ΔN17 mice exhibit spontaneous falls at 10mo of age.  
(e) Photograph showing unkempt fur due to reduced grooming in BACHD-ΔN17 mice at 10mo and a WT littermate of the same age.  
(f) Photograph showing 10mo old BACHD-ΔN17 and WT mice. At this age, the transgenic mice are often found on their backs in the homecage and make little effort to right themselves. This phenotype is not observed in WT littermates. * P < 0.05, ** P < 0.01, *** P < 0.001; Student’s T-test. Values represent mean ± SEM.
falls, BACHD-ΔN17 fell at an average close to once every 90 seconds (Fig 3.2d). Also from observing mice in the home cage, we noticed that aged BACHD-ΔN17 mice presented ruffled, ungroomed, dirty coats starting around 10mo (Fig. 3.2e). These mice also spend large amounts of time inverted, on their backs in the home cage, often below the food and water sources, and toward end-stage exhibit urine scalding (Fig. 3.2f).

We tested BACHD-ΔN17 mice using the open field paradigm at 2, 4, and 8mo, before the mice became sedentary. At 2mo, the transgenic mice exhibited behavior indistinguishable from WT, including normal distance traveled, velocity while moving, and rearing behavior (Fig. 3.3a-c). By 4mo, the BACHD-ΔN17 mice showed significant deficits in two of these behaviors, exhibiting reduced distance traveled and velocity while moving, however rearing behavior was still normal. By 8mo, these deficits were more pronounced, and rearing behavior was almost completely abolished. Further, 5mo BACHD-ΔN17 mice in the forced swim test show dramatically more time spent immobile (roughly three times the immobility time as WT mice), indicating these mice experience depressive-like behaviors (Fig. 3.3d).

One of the striking clinical features of Huntington’s disease is the typically adult onset of progressive chorea, a phenotype that has not been recapitulated in previous mouse models of the disease (Crook and Housman, 2011). Due to this, perhaps the most interesting behavioral phenotype exhibited by the BACHD-ΔN17 line is that transgenic mice begin showing abnormal head and upper body movements beginning between 6-7mo. These movements include smooth swinging of the head and retroflex of the neck lasting several seconds (Fig. 3.4a), and are reminiscent of choreiform or dystonic movement (and hence referred to as chorea/dystonia-like movements, or CDLs). The CDLs are progressive, increasing in frequency roughly ten fold between 7 and 10 months (Fig. 3.4b). Importantly, local field potential recording of the mice
Figure 3.3. Open field and forced swim testing reveal further behavioral deficits in BACHD-ΔN17 mice. (a-c) Open field testing shows normal behavior at 2mo of age. By 4mo, the transgenic mice exhibit hypoactivity in measures of total distance (a) and velocity (b). These phenotypes are worse by 8mo, when the mice exhibit severe deficits in vertical plane entries (c), a measure of rearing behavior. (d) Mice were tested in the forced swim apparatus at 5mo, before the onset of severe motor deficits. BACHD-ΔN17 mice spent more time immobile in this test, indicating depressive-like behavior. * P < 0.05, ** P < 0.01, *** P < 0.001; Student’s T-test. Values represent mean ± SEM.
Figure 3.4. Chorea/dystonia-like behavior in BACHD-ΔN17 mice is progressive and associated with aberrant striatal activity. (a) Composite photograph showing abnormal head movement in CDL behavior. (b) Quantitation of CDL behavior in BACHD-Δ17 mice and WT littermates shows onset at 6-7mo and progression until end stage at 10mo. Data shown as mean ± SEM. Two-way ANOVA determined significant genotype-time interaction (P < 0.0001). (c) Cortical field recordings with simultaneous video taping show no epileptiform activity associated with CDL behavior in 8mo mice. (d,e) Striatal gamma activity in WT (d) and 8mo BACHD-ΔN17 mice (e) highlighting the presence of abnormal gamma-frequency activity in the transgenic mice. Expanded traces below (e) show large-amplitude gamma events. (f,g) Aberrant gamma frequency events are progressive, with cumulative probability plot of the inter-event interval in younger (f) and older mice (g). Thin traces are individual mice and thicker trace is average probability. Red = BACHD-ΔN17, black = WT.
with simultaneous video recording ruled out the possibility of seizure inducing these movements (Fig. 3.4c). Instead, BACHD-ΔN17 mice exhibit large, low gamma-frequency events in the striatum that are associated with the CDLs, with WT mice not exhibiting such gamma-frequency activity (Fig. 3.4d-e). Interestingly, these gamma-frequency events are progressive, increasing with age between 4-5mo and 8-10mo of age (Fig 3.4f-g).

Based on these phenotypes, we concluded that BACHD-ΔN17 mice exhibit accelerated disease-related motor, gait, and psychiatric-like phenotypes compared to BACHD mice. Further, the mice exhibit behavioral deficits (i.e. CDLs) that are novel to this disease model. These CDLs are not due to cortical seizure activity, but instead are associated with abnormal activity in the striatum.

3.3 Neuropathology in BACHD-ΔN17 Mice

Existing HD mouse models often recapitulate selective brain atrophy, but none exhibit the profound and selective loss of striatal MSNs (Crook and Housman, 2011; Vonsattel, 2008). We sought to assess the degree of neuropathology in BACHD-ΔN17 mice using multiple methods. First, BACHD-ΔN17 mouse forebrains and cerebella were weighed, finding that although 2mo transgenic forebrains weighed the same as WT, by 6mo the HD mice exhibited significant loss. This deficit was progressive at 8mo and finally at 10mo, when the BACHD-ΔN17 mice exhibited a striking 34% loss of forebrain weight (Fig 3.5a). Cerebellar weight was unaffected in the HD mice until 10mo of age, when the transgenic mice exhibit a slight, though significant decrease in weight, consistent with the mild atrophy seen in HD patient cerebellum at
Figure 3.5. Progressive neuropathology in BACHD-ΔN17 mice. (a) Forebrain weight is normal at 2mo in BACHD-ΔN17 mice, but significantly decreased at 6mo, 8mo, and 10mo compared to WT. Two-way ANOVA shows significant effects of genotype and age (P < 0.0001). In contrast, cerebellum is relatively spared and shows only mild loss of mass by 10mo. (b,c) Unbiased stereological measurement of cortical and striatal area (b) shows decrease in both in BACHD-ΔN17 mice at 10mo, while the number of Darrp-32+ neurons in the striatum (c) is also reduced. * P < 0.05, ** P < 0.01, *** P < 0.001; Student’s T-test. Values represent mean ± SEM. (d,e) Immunohistochemistry for astrocytes with antibody against GFAP shows increased astrogliosis in striatum and deep layers of cortex (d), with the astrocytes increasing in density and changing morphology (e), indicating reactive state.
end-stage (Vonsattel, 2008). We undertook unbiased stereology to assess whether loss of cortical and striatal volume could account for the reduced brain weight in BACHD-ΔN17 mice. At 10mo of age, the HD mice exhibited a roughly 35% reduction in cortical and striatal volume (Fig. 3.5b). Further, the number of DARPP-32+ MSNs was reduced by roughly 40% in the striatum compared to WT littermates (Fig. 3.5c).

HD patients exhibit increased gliosis, with pronounced astrogliosis, microgliosis, and oligodendrogliosis in affected brain regions at end stage (Waldvogel et al., 2014). To assess gliosis in BACHD-ΔN17 mice, we undertook staining for astrocytes using antibody against astrocytic marker glial fibrillary acidic protein (GFAP). GFAP immunohistochemistry identified marked increases in reactive astrocytes in the striatum and deep layers of cortex in the HD mice relative to WT controls (Fig. 3.5d-g).

3.4 Characterization of mHTT Subcellular Location and Species

A striking finding in HD patients, first discovered in transgenic mice, is the presence of aggregated mHTT species, and in particular inclusion bodies, in the brain (DiFiglia et al., 1997). Much research has been performed on these aggregates, which were initially thought to be the source of mHTT toxicity, although later studies have found them to be protective (Arrasate et al., 2004). The emerging picture is one in which mHTT inclusion bodies are formed as part of the cellular response to mHTT, acting with autophagy and proteasomal degradation pathways to try to sequester and degrade the protein (Arrasate and Finkbeiner, 2012). Importantly, cellular localization of mHTT also plays a role in toxicity, with nuclear mHTT exerting considerable toxicity (Benn et al., 2005). To assess huntingtin aggregation in BACHD-ΔN17 mice, we undertook immunohistochemical staining of HTT aggregates using the EM48 and S830
Figure 3.6. Accelerated accumulation of nuclear mHTT aggregates in BACHD-ΔN17 mice. (a,b) Immunohistochemistry using EM48 (a) and S830 (b) antibodies against aggregated mHTT shows progressive increase in nuclear huntingtin aggregates, starting at 4mo in the cortex and present in both cortex and striatum at 6mo. (c) Immunofluorescent staining shows S830+ aggregates are predominantly in neuronal nuclei, co-labeling with NeuN. (d) S830+ mHTT nuclear inclusions also co-label with antibody against ubiquitin, as is seen in postmortem HD patient neuropathology.
antibodies against aggregated huntingtin (Gutekunst et al., 1999; Sathasivam et al., 2001).

Unlike BACHD mice, which exhibit predominantly neuropil aggregates only late (i.e. 12mo), BACHD-ΔN17 mice exhibit diffuse mHTT nuclear staining and small nuclear inclusions in the cortex by 4mo. Further, by 6mo, diffuse nuclear staining and larger nuclear inclusions are found in both cortex and striatum (Fig. 3.6a-b). Immunofluorescent staining confirmed that BACHD-ΔN17 mHTT aggregation is specific to NeuN+ neuronal nuclei (Fig. 3.6c), and also found that the mHTT nuclear inclusions co-stain with ubiquitin (Fig. 3.6d), similar to HD patients (DiFiglia et al., 1997). In comparison to the BACHD line, BACHD-ΔN17 mice exhibit greatly accelerated mHTT aggregation and nuclear localization instead of neuropil.

We sought to further assess the role of the N17 domain in the cellular localization of mHTT. First, we undertook subcellular fractionation and Western blotting in BACHD and BACHD-ΔN17 mice at 1mo and 10mo of age. Despite previous work indicating that N17 is required for cytoplasmic retention in N-terminal fragments, we found both full-length mHTT and ΔN17-mHTT in the cytosolic fraction at 1mo and 10mo in the BACHD and BACHD-ΔN17, respectively (Fig. 3.7a). In the nuclear fraction, however, we were only able to detect mHTT in 10mo BACHD-ΔN17 mice, and then only as a high molecular weight species consistent with mHTT aggregates. These high molecular weight species in the brains of BACHD-ΔN17 mice stain with antibodies directed against the N-terminus of huntingtin (S830), but not the C-terminus (7667), suggesting that they may be aggregated N-terminal fragments (Fig. 3.7b). Further, dissociation of the high molecular weight species using formic acid treatment resolved the putative aggregates into two small polyQ+ positive bands (Fig. 3.7c). This suggests that the huntingtin N17 domain is critical for the cytoplasmic localization of smaller N-terminus
fragments of expanded polyQ huntingtin. To follow-up on this idea, we tested different mHTT N-terminal fragment lengths to see whether N17 deletion would impact subcellular localization.

Figure 3.7. N17 domain mediates nuclear accumulation of small N-terminal mHTT fragments. (a,b) BACHD and BACHD-ΔN17 mice show differential accumulation of nuclear mHTT aggregates as mice age. (a) Probing of the nuclear fraction with S830 antibody against mHTT exon1 aggregates shows the presence of aggregated mHTT in the nucleus only in aged BACHD-ΔN17 mice, while probing with 7667 antibody against the HTT C-terminus does not show the presence of mHTT. Lamin B1 and α-tubulin loading controls were used for nuclear and cytosolic fractions, respectively. (c) Western blot following formic acid treatment to dissolve mHTT aggregates shows the presence of small N-terminal mHTT fragments in the nucleus of BACHD-ΔN17 mice but not the cytosol. Blot was probed with 1c2 antibody against expanded polyQ. (d,e) Cell culture experiments using N-terminal fragments of 46Q mHTT with- and without the N17 domain (d) show that the N17 domain is critical for cytoplasmic localization of smaller fragments (i.e. exon1 and CpA/N114), with N17 deletion shifting nuclear cytoplasmic ratio towards the nucleus (e). N17 deletion does not change the nuclear-cytosolic distribution of the caspase 6 (N586) fragment.
We tested three fragments: the caspase 6 586 amino acid cleavage fragment (Graham et al., 2006), the CpA 114 amino acid cleavage fragment (Lunkes et al., 2002), and an exon 1 fragment (Sathasivam et al., 2013). These were modified to have 46 polyglutamine repeats and versions were created with and without amino acids 2-16 of the N17 domain. These constructs were transfected into HEK293 cells and cytoplasmic versus nuclear huntingtin was quantitated via immunofluorescent staining. Interestingly, while the N17 domain acted to prevent nuclear localization of the smaller exon 1 and CpA fragments, the absence of N17 did not affect the localization of the longer caspase 6 fragment, which remained cytoplasmic even with N17 deleted (Fig. 3.7d-e). Taken together, our in vivo and cell culture results suggest that the N17 domain is critical for the cytoplasmic localization of small mHTT fragments that are likely formed in the aging BACHD-ΔN17 mice.

3.5 Characterization of BACHD-ΔN17 and BAC-WT-ΔN17 Control Lines

An important consideration when generating transgenic mice is the potential for insertion effects. To ensure that BACHD-ΔN17 behavioral and neuropathological phenotypes are the result of the HTT transgene and not due to disruption of any endogenous genes, we sought to confirm key phenotypes in two additional lines of BACHD-ΔN17 mice (A line and L line). L line mice express equivalent amounts of ΔN17-mHTT compared to the N line described above, however these mice exhibit even earlier onset of disease, dying around 5mo (Fig. 3.8a). These mice also exhibit CDL behavior earlier than the N line, with typical onset at 4-5mo (Fig. 3.8b). At end stage, L line mice also exhibit significant forebrain atrophy and mHTT nuclear aggregate staining. The BACHD-ΔN17 A line expresses a far lower amount of mHTT, with undetectable
levels in the brain lysate soluble fractions, and just a faint band detectable in the insoluble fraction (Fig. 3.8c). A line mice appear normal up to 18mo, when they begin to lose weight and

Figure 3.8. Confirmation of key phenotypes in additional BACHD-ΔN17 mouse lines. (a-e) BACHD-ΔN17-L line mice exhibit earlier disease phenotypes, with body weight loss at 5mo (a), premature lethality (b), onset of CDLs at 4mo (c), reduced forebrain and cerebellar weight at 5mo (d), and nuclear mHTT aggregates in both the cortex and striatum (e). (f-j) BACHD-ΔN17-A line mice express faintly detectable amounts of mHTT (f), but still exhibit the late onset of body weight loss by 24mo (g), progressive CDLs beginning at 18mo (h), reduced forebrain and cerebellar weight (i), and cortical aggregation of nuclear mHTT, but fewer nuclear inclusions (j). * P < 0.05, ** P < 0.01, *** P < 0.001; Student’s T-test. Values represent mean ± SEM.
exhibit CDLs (Fig. 3.8d). This line also shows late neurodegenerative pathology, with decreased forebrain weight at 24mo and mHTT nuclear aggregates appearing at 18mo and progressing to larger inclusions by 24mo (Fig. 3.8e). These phenotypes confirmed that the profound effects found in BACHD-ΔN17 mice are ΔN17-mHTT dependent and not caused by insertional effects.

A further consideration is whether the behavioral phenotypes in BACHD-ΔN17 mice are elicited in an expanded polyQ-dependent matter. To assess this, we also surveyed behavior and neuropathology in BAC-WT-ΔN17 mice, which have a non-pathogenic 31Q polyglutamine repeat. If the phenotypes seen in BACHD-ΔN17 mice are recapitulated in BAC-WT-ΔN17, it would argue for toxicity caused by a more general N17-dependent mechanism. This was not the case, however, and BAC-WT-ΔN17 mice showed normal life span, body weight, no evidence of forebrain or cerebellar atrophy, and no rotarod deficits through 12mo of age (Fig. 3.9a-d). These mice also showed no evidence of mHTT aggregation (Fig 3.9e-f).

### 3.6 Progressive Transcriptionopathy in BACHD-ΔN17 Mice

To assess the consequences of accelerated nuclear mHTT accumulation on gene transcription, we performed longitudinal microarray profiling on BACHD-ΔN17 and WT mice at 2mo (onset of motor phenotypes), 7mo (age of CDL onset), and 11mo (end-stage). Longitudinal profiling at these phenotypically-selected timepoints was performed to allow for the assessment of progressive transcription disruption. Cortex and striatum were collected from four mice per genotype and age (two males and two females each), for a total of 48 samples. Expression profiling was performed using Illumina Mouse Ref-8 v2.0 bead-based arrays, with >25,000 probes for >19,000 unique transcripts.
Figure 3.9. BAC-WT-ΔN17 mice do not show behavioral or neuropathological phenotypes. (a) Unlike BACHD-ΔN17, BAC-WT-ΔN17 mice do not exhibit body weight loss at 12mo. Brain weight is not affected through 19mo (b), and there is no evidence of atrophy of brain or other tissues in these mice at 8mo (c). Further, the BAC-WT-ΔN17 mice perform as well as WT mice in the accelerating rotarod at all ages tested, up to 12mo. (e,f) Immunohistochemistry with S830 shows no mHTT aggregation in cortex (e) or striatum (f). Student’s T-test and 2-way ANOVA were used to look for differences between genotypes and genotype and age as appropriate. Values represent mean ± SEM.
Differential expression analysis of WT vs. BACHD-ΔN17 mice showed little evidence of transcriptional dysregulation at 2mo, with only one gene from striatum or cortex reaching our multiple comparisons corrected threshold of FDR < 0.1. (Acy3, which is up-regulated in the striatum) (Table S3). At 7mo, however, 395 cortical genes meet this significance threshold (top five down-regulated: Ddit4l, Pdyn, Nptx2, Wfs1, Tnnc1; top five up-regulated: En2, Pou4f1, Twist1, Onecut2, Bex4), as do a striking 2063 striatal genes (top five down-regulated: Ddit4l, Dmkn, Ryr1, Inhba, and B230373P09Rik; top five up-regulated: Spp1, Lyz, Lyzs, Gfap, and Il). At this age, the striatal down-regulated gene list is noticeably populated with striatal marker genes that are classically down-regulated in HD striatum (e.g. Actn2, Drd1a, Drd2, Pde10a, and Cnr1) (Cha, 2007; Mazarei et al., 2010). At 11mo, the number of cortical genes with significant dysregulation increases to 3435 (top five down-regulated: Ddit4l, Egr2, Slc23a3, Nptx2, and Lrrn6a; top five up-regulated: En2, Twist1, Pou4f1, Barx1, and Hoxc6) and striatal genes increase to 3129 (top five down-regulated: Ddit4l, Scn4b, Egr2, Ryr1, and Dmkn; top five up-regulated: Lyz, Nrn1, Lyzs, S3-12, and Spp1). Impressively, gene transcription changes in the striatum of our BACHD-ΔN17 mouse model progressively overlap with transcriptionopathy observed in HD patient brain, with no significant overlap at 2mo, but significant overlap of both up- and down-regulated genes at 7 and 11mo (Fig. 3.10a-b). By focusing on DGE at the 7mo timepoint, we can avoid potential confounds from cellular composition changes (e.g. caused by loss of striatal MSNs at the 11mo timepoint). DAVID gene ontology found the up-regulated striatal genes at 7mo enriched for lysosomal genes, tetraspanins, and immune functions, while the down-regulated genes were enriched for neuronal projections/dendrites, palmitiate and lipoproteins, and synaptic genes (Table S4). The up-regulated cortical genes are enriched for
Figure 3.10. Progressive HD-like transcriptionopathy in BACHD-ΔN17 striatum. (a,b) Heatmap (a) of up-regulated (red) and down-regulated genes and Venn diagrams (b) showing genes in common between HD patient arrays and BACHD-ΔN17 mice highlights the progressive overlap of these genes. $P =$ hypergeometric test. (c) Cluster dendrogram based on hierarchical clustering of probes based on topological overlap indicates probes that are highly correlated. Dynamic tree cutting led to assignment of seven coexpression modules. (d,e) Heatmap and module eigengene expression in Turquoise module (d) and Blue module (e) identify age- and genotype-dependent down- and up-regulated coexpression networks, respectively. (f,g) Enrichment analysis using anRicher. (h,i) Network visualization of Turquoise (h) and Blue (j) modules showing top hub genes (inner circle), with genes previously identified in HD expression papers colored purple. Genes previously identified to be involved in other neurodegenerative diseases, such as frontotemporal dementia and Alzheimer’s disease are colored red.
homeobox-related genes, while the down-regulated genes are enriched for calmodulin binding, pleckstrin homology, and ion channel activity.

Instead of static pictures of gene transcription at the three ages, we sought to understand the progressive nature of transcriptionopathy in our new HD mice. To do so, we applied WGCNA to our dataset, splitting the analysis by tissue and focusing on the striatum. WGCNA identified seven modules, with the two largest modules of co-expressed genes showing strong patterns of gene expression correlating with disease state in the HD mice (Fig. 3.10c-e, Table S5). The largest of these modules, the striatal Turquoise module, contains 3247 genes. Plotting the Turquoise module eigengene expression against the microarray samples shows a striking pattern of expression, with genes in this module being down-regulated over time, but only in the BACHD-ΔN17 samples (Fig. 3.10c). The top ten Turquoise hub genes based on module membership are Actn2, Dnah1, Dfy, Rpel1, Cacng4, Dacmkl1, Lin7b, Rhobtb2, Inf, and Ddit4l (the most down-regulated gene by differential expression analysis) (Fig. 3.10h). Other notable genes with high membership this module include Rgs9, Gpr6, Ntrk3, Pde10a, Gabrd, Ryr1, Igfbp4, Drd2, Drd1a, and Cnr1. Dysregulation and dysfunction of many of these striatal marker genes has been previously implicated in striatal pathogenesis (Cha, 2007). IPA found the turquoise module to be enriched for genes involved in cAMP and G protein-coupled receptor (GPCR) signaling, Wnt/β catenin signaling, axon guidance, synaptic long-term potentiation / depression, and Huntington’s disease-related genes (Table S6). Further enrichment analysis, using the geneset enrichment R anRicher package (Miller et al., 2011), showed that the Turquoise module is enriched for genes expressed in neurons, glutamatergic cortical neurons, genes dysregulated in HD striatum in previous studies, genes related to schizophrenia, glutamatergic synapses, and human / chimp caudate (Fig. 3.10f). The other disease-related
module, termed the striatal Blue module, contains 2141 genes that display the opposite
eexpression pattern to the Turquoise module: they are progressively up-regulated over time and
only in BACHD-ΔN17 samples (Fig. 3.10e). The top ten Blue hub genes based on module
membership are Copg, Lys, Thbs2, Lys, Copg, A2ld1, 1500012F01Rik, C4b, Gfap, and Ctse
(Fig. 3.10i). Other notable genes with high module membership include Lgals3 (Mac2), Grn,
Lamp2, Thr2, En2, Wt1, Abca7, Tspo, Thr2, Myd88, Cdh86, Cx3cr1 and numerous genes in the
complement cascade, such as C1qa, C1qb, C3, Tyrobp, Cfd33. IPA found enrichment of Blue
module genes in canonical pathways related to Type 1 diabetes mellitus, TREM1 signaling,
sphingolipid metabolism, the role of pattern recognition receptors in recognition of bacteria and
viruses, and apoptosis signaling. We again used anRicher to determine enrichment in datasets
from previous studies, and found that the Blue module is enriched for genes expressed in glia;
astrocytes most strongly, followed by microglia and then oligodendrocytes (Fig. 3.10g).

We sought to validate a subset of these transcription changes using quantitative PCR
(qPCR), as well as looking at immunohistochimical staining of several of the more interesting
genes. Using qPCR, we found that expression of six striatal module genes (Actn2, Drd2,
Cyp46a1, Gpr6, Drd1a, and Sortl) matched the down-regulation seen in the 11mo striatal
microarray data, while ten Blue module genes (Abca7, Clu, Apoe, Hdad1, Casp1, Copg, Thr7,
Apoc1, and Lys) matched the genes up-regulated (Fig. 3.11a). In addition,
immunohistochemical staining also confirmed that levels of dopamine receptors D1 and D2 are
reduced at 10mo in the HD mice (Fig. 3.11b-c). Immunostaining also suggests against potential
for tissue composition changes observed in older BACHD-ΔN17 striatum causing apparent
down-regulation of these striatal marker genes, as the remaining MSNs show decreased levels of
D1 and D2 receptors. We had previously stained for GFAP to assess the levels of astrocytosis in
Figure 3.11. Validation of selected striatal network hub genes. (a) Quantitative PCR of Turquoise module members (green bars) and Blue module members (blue bars) compared to WT in 10-11mo striatum. * P < 0.05, Student’s T-test. (b-d) Immunohistochemical staining of dopamine receptor D2 (b) and D1 (c) in the striatum shows decreased signal 10mo BACHD-ΔN17 mice. Quantified in (d). (E-K) While WT mice show no reactive microglia staining with Mac2 at 11mo (E), BACHD-ΔN17 mice show Mac2⁺ microglia staining progressively increasing at 8mo (G) and 11mo (H). Mac2⁺ reactive microglia (J) completely colocalize with Iba1⁺ microglia (K).
aged BACHD-ΔN17 brain, finding increased reactive astrocytes in the striatum and deep cortical layers at 10mo that corresponds to the increased Gfap transcription observed in the microarray data (Fig. 3.5d-g). To further assess the evidence for reactive gliosis in our aged HD mice, we stained for a marker of reactive microglia, Lgals3/Mac2, which is a hub gene in our Blue module and up-regulated in aged BACHD-ΔN17 striatum. Mac2+ cell staining is not seen 11mo WT mice, however we see an age-dependent increase in the number of Mac2+ cells that colocalize with Iba1, a marker of resting as well as reactive microglia, beginning at 6mo and progressing at 8 and 11mo in BACHD-ΔN17 striatum.

In summary of our longitudinal studies of transcription dysregulation in the BACHD-ΔN17 brain has identified both novel and known transcription abnormalities. First, we have described progressive down-regulation of striatal marker genes that are known to be down-regulated in HD. Some of these genes, such as Pde10a, Ntrk3, Gabrd, Ryr1, Gpr6 and Actn2, have evidence that they may have causal involvement in cell autonomous and non-cell autonomous pathogenic processes in HD. We have also described evidence for novel neuroinflammatory processes and gliosis in our HD mice, with our Blue module showing enrichment for astrocytes (e.g. Gfap, S100a6) and microglia (e.g. Lgals3/Mac2, Tspo, Cd68). This Blue module also has enrichment for genes in previous studies of Alzheimer’s disease (AD) transcription and contains genes that have been identified in genome-wide association studies of AD (e.g. Apoe, Trem2, Abca7, Ms4a6d, and Grn) (Karch et al., 2014), as well as immune-related genes recently implicated in the disease (e.g. C1qa, C1qb, C3, Tyrobp, Cd33) (Zhang et al., 2013), and genes involved in apoptosis. With such neuroinflammation not before observed in HD mouse models, but a key feature of postmortem HD patient pathology, this is a critical
finding of face validity that may make BACHD-ΔN17 mice a better model for testing therapeutics targeting inflammatory processes in HD and other neurodegenerative diseases.

3.7 Conclusions

To assess the impact of the huntingtin N17 domain in HD pathogenesis in vivo, we developed transgenic mice based on the BACHD line, but lacking amino acids 2-16 of the N17 domain. Similar experiments, using small N-terminal fragments of HTT and performed in cell culture, identified the N17 domain as a cytoplasmic retention signal and showed a role in nuclear export (Atwal et al., 2007; Maiuri et al., 2013; Rockabrand et al., 2006; Zheng et al., 2013). This study is the first, however, to test the role of N17 in disease pathogenesis using nearly full-length mHTT (i.e. missing just amino acids 2-16). The ΔN17 form of mHTT maintains essential HTT functions in development and rescued Htt null embryonic lethality at a Mendelian ratio. Despite a lower transgene expression level than the BACHD line, BACHD-ΔN17 mice exhibited greatly accelerated HD-like behavioral deficits, neurological decline, neurodegeneration, nuclear accumulation of mHTT aggregates, and robust HD-like transcriptional dysregulation.

We performed a comprehensive survey of HD-relevant behaviors and phenotypes and found robust deficits. BACHD-ΔN17 mice exhibit onset of rotarod deficits at the same age as BACHD mice (Menalled et al., 2009; Wang et al., 2014), however the progression of these symptoms is greatly accelerated, with the new line unable to perform the rotarod test by 8mo of age. The mice also lose weight, exhibit progressive gait abnormalities, rearing deficits, and hypoactivity. Of the behavioral phenotypes, the CDL behaviors are of particular interest, as no previous HD mouse models have exhibited such overt HD-like movement. These movements are progressive, with onset at 6-7mo, and it is important to note that care was taken to exclude
seizure activity as a cause for the abnormal head movement, as seizures have been found to cause abnormal head movement in other mice (Noebels et al., 1990). These key behavioral phenotypes were confirmed in two additional BACHD-ΔN17 mouse lines, including one with very low transgene expression, suggesting that the toxic effects of ΔN17-mHTT expression require very little of the protein over time to cause disease.

Neuropathologically, the BACHD-ΔN17 mice exhibit profound forebrain atrophy and mild cortical atrophy. Concomitant with this atrophy, both cortex and striatum show volume loss, and striatum shows reduction of MSNs. Staining for mHTT aggregates shows a progressive increase in nuclear staining, starting at 4mo with diffuse nuclear staining in the cortex, and by 6mo showing both diffuse staining and neuronal inclusions in both the cortex and striatum. In addition to the neuronal loss and atrophy, these mice exhibit robust gliosis, with increased reactive astrocytes and activated microglia, similar to end stage HD patients.

Our cell culture experiments shed some light on the role of the N17 domain in regulating mHTT cellular localization. In mHTT N-terminal fragments, removing N17 shifts the localization of small (exon1 and CpA) fragments to the nucleus, while it does not affect the localization of the longer caspase 6 fragment. Since nuclear mutant huntingtin has well-established toxicity, and the cleavage of full-length mHTT into N-terminal fragments has a long standing as a proposed pathogenic mechanism in HD, it would make sense that the absence of N17 would cause accelerated mHTT accumulation in the nucleus where it exerts its toxicity.

BACHD-ΔN17 mice also exhibit progressive transcriptionopathy, with HD-like reductions in striatal marker genes and increases in genes involved in apoptosis signaling. WGCNA identified two modules of coexpressed genes that are progressively up- and down-regulated only in the transgenic mice. The down-regulated genes are enriched for cAMP/GPCR
signaling, calcium, and synaptic health. Loss of these genes may contribute to the functional impairment of the cortico-striatal circuit and thus the overall health of the MSNs. Further, the up-regulated genes show evidence of a robust induction of gliosis, with markers of reactive astrocytes and activated microglia. Although neuroinflammation is seen in HD and other neurodegenerative diseases, this signature is unique to this HD mouse model and may provide a novel avenue to test therapeutics designed to block runaway inflammation in the brain.

All of this work points to a two-hit model where full-length mHTT is first post-translationally cleaved, aberrantly spliced (Sathasivam et al., 2013), or undergoes alternative translation (i.e. repeat-associated non-ATG, or RAN translation; Pearson, 2011) to generate a small, toxic fragment that is still excluded from the nucleus and can largely only exert cytoplasmic toxicity. A second hit, which would occur when N17 is either functionally or physically impaired, would then end the cytoplasmic retention or impair nuclear export, causing the mHTT fragment to accumulate in the nucleus where it exerts nuclear toxicity through, amongst other mechanisms disrupting normal transcription.

Lastly, an analysis of this mouse line as a general mouse model for HD studies. The face validity, as shown by the behavioral deficits and CDLs, neuropathology including overt MSN loss, and transcriptional dysregulation that includes neuroinflammation make the BACHD-ΔN17 line an attractive model for studying the pathogenesis of HD and other neurodegenerative diseases where neuroinflammation may play a pathogenic role. In addition, the early onset and accelerated progression of phenotypes, compared to other full-length mouse models could make this line ideal for testing candidate therapeutics that do not rely on an N17-dependent mechanism. This is a key point: that the BACHD-ΔN17 line does not exhibit construct validity matching even BACHD mice (thanks to the 15 amino acid N17 deletion), which have lower
construct validity compared to the knock-in models. To this point, these mice have been a successful tool to understand the role of the N17 domain and nuclear mHTT in vivo. Ultimately, the question will need to be answered of whether these mice show predictive validity, however that will require successful therapeutics for the HD patients.

3.8 Materials and Methods

Generation and breeding of BACHD-ΔN17 and BAC-WT-ΔN17 mouse lines. BAC modification was performed as previously described (Gray et al., 2008; Gu et al., 2009). Modified BAC constructs were sequenced prior to injection into fertilized FvB/NJ oocyte pronuclei at the UCLA Transgenic Core. Mice were genotyped using the following primers: 5’-gagccatgattgtgctatcg-3’ and 5’-agctacgctgctcacagaaa-3’ or 5’-gcaacgtgctgctctc-3’ and 5’-gagcagcttggaaca-3’. Transgenic mice were bred and maintained under standard housing conditions consistent with NIH guidelines, and all procedures were approved by the UCLA Institutional Animal Care and Use Committee.

Quantitation of Transgene Expression. Brain extracts were taken from the transgenic mouse lines and quantitated by Western blot with mHTT specific antibody against the human polyproline domain (MAB5492, Millipore), 1C2, and α-tubulin as previously described (Gu et al., 2009). Three mice were used per genotype at 6 weeks of age.

Behavioral Testing. All tests used at least ten mice per genotype and timepoint. Investigators were blinded to genotype during experiments and analyses. Quantitation of CDL behavior and spontaneous falls was manually performed in the last three minutes of a five minute video recording of individual mice. Mice (at least ten per genotype and age) were placed in a 30cm x 30cm x 30cm clear Plexiglass container and videotaped. CDL behavior was defined as the
mouse having all four paws on the ground, or the hind paws on the ground while the fore paws touching the wall of the text box, while the head extends 80° beyond the body plane. One bout of CDL behavior was considered one episode of the mouse raising its head past 80° and then lowering it to the body plane. Spontaneous falls were defined as the mouse’s body touching the ground with all four paws leaving the ground while moving or rearing. Rotarod performance was performed as described (Gray et al., 2008; Gu et al., 2009; Wang et al., 2014). Rotarod testing was performed at 1, 2, 4, 6, and 8mo of age. Testing in the open field was performed at 2, 4, and 8mo as described (Wang et al., 2014). Gait abnormalities were assessed using a 25cm x 45cm x 80cm walking path linked to the home cage. Mice were encouraged to walk towards the home cage by an overhead light and with gentle touch, if necessary, during training. During testing, mice had their paws painted with non-toxic paint and the floor lined with paper. Stride length (distance between consecutive footprints of the same paw), base length (distance hindpaw-to-hindpaw and forepaw-to-forepaw), and overlap (distance superimposed between hind- and forepaw) were measured. Mice were tested in the forced swim paradigm at 5mo age, as mice were not severely motor impaired at this timepoint. Forced swim testing was performed as described in (Wang et al., 2014).

**Electroencephalography.** **Surgeries.** Surgeries were performed under aseptic conditions on mice weighing 25–35g, at 3-4 and 7-9 months of age, according to a protocol approved by the University of California Los Angeles Chancellor’s Animal Research Committee. Under isoflurane anesthesia (2–2.5% in O₂), animals were mounted into a standard Stoelting stereotaxic frame with blunt ear bars. Body temperature was maintained at 37°C using a rectal probe and a water circulating heating pad. The cranium was exposed through a small midline scalp incision and 2 small (~ 0.5 mm diameter) holes were drilled in the cranium. With the aid of a
micromanipulator, two sterilized stainless steel recording electrodes (PlasticsOne, Roanoke, VA) were lowered into the cortex (from the rostral confluence of the nasal sinus, anteroposterior (AP), -3.0 mm; mediolateral (ML), -1.7 mm; dorsoventral (DV), -0.5 mm and into the dorsal striatum (AP: -3.0 mm; ML: -1.7 mm; DV: -2.0 mm) through one of the holes. The second hole was drilled above the cerebellum to insert the ground electrode. The bone was dried and cranioplastic cement (Lang Dental, Wheeling, IL) was used to anchor electrodes to the skull. During the recovery period we administered 0.01-0.02 ml/g warm saline solution subcutaneously twice a day to prevent dehydration. After surgery, the mouse was monitored continuously until recovery, as demonstrated by its ability to maintain sternal decumbency and to exhibit purposeful movement. Data Acquisition. Synchronous video-EEG recordings were carried out at least 7 days post surgery in freely moving mice in an 8-inch diameter recording chamber. Electrodes were connected to a custom made buffer amplifier to eliminate cable movement artifacts. All field signals were referenced to the implanted ground electrode. Field potential recordings were band-pass filtered and amplified (0.3 Hz - 1 kHz, gain: 1000, Model 3500, A-M Systems, Carlsborg, WA), digitized on-line at a rate of 2048 s⁻¹ (PCI-MIO16E-4 data acquisition board National Instruments, Austin, TX), and acquired continuously (SignalExpress, National Instruments, Austin, TX). Data Analysis. All data analysis was performed using IGOR Pro v6.22A (Lake Oswego, OR). Morlet wavelet analysis was performed for time-frequency analysis of local field potential recordings. Gamma events were detected in 1000s long segments of the dorsal striatal recordings chosen 2 hours after starting the video-EEG monitoring. The recording segment was band-pass filtered in the lower gamma band (25-55 Hz), the absolute values of the oscillations were smoothed with the Savitzky-Golay method. Gamma events were detected using a threshold 7 times larger than the baseline standard deviation. Cumulative
probability plots of inter-event intervals were constructed by pooling equal numbers of gamma events from each animal.

**Neuropathology.** Immunostaining for mHTT aggregates was performed using S830 (1:30,000 dilution, gift from Dr. Gillian Bates) and EM48 (1:50 dilution, Thermo) antibodies using published methods (Gu et al., 2009). Other antibodies used were anti-GFAP (1:20,000, Dako Z0334), anti-Mac2 (1:500, Santa Cruz h-160), anti-NeuN (1:1,000, Millipore MAB377), anti-ubiquitin (1:1,000, Dako Z0458), anti-D2 (1:1,000, Sigma WH0001813M1), anti-D1 (1:1,000, Sigma HPA013393), and anti-Darpp-32 (1:1,000, Santa Cruz sc-11365). ImageJ software was used to compare relative amounts of expression as described (Wang et al., 2014). Weighing and stereological measurements of brain were performed as described (Gu et al., 2009).

**Cell culture experiments.** HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum (Gibco) and passaged every three days. Cells were transfected with Lipofectamine 2000 (Invitrogen) or Fugene HD (Promega) according to manufacture instructions. DNA constructs of mHTT exon 1 (46Q) fragments were generated from mHTT-CFP by PCR and fused with an HA tag (mHTT-46Q-Exon1). The CpA and caspase 6 fragments were generated by inserting additional sequences to mHTT-46Q-Exon1 using the Gibson assembly kit (New England Biolabs) according to manufacture instructions. The deletion of N17 from aforementioned DNA fragments was generated by mutagenesis using QuickChange II XL kit (Agilent). All constructs were cloned into pcDNA3.1 for mammalian expression. Plasmid DNAs were titrated to produce similar level of transgene expression. HEK293 cells were plated on coverslips in 24-well plates and transfected with one of these plasmids. 48 hours after transfection, cells were fixed with 4% paraformaldehyde followed by the treatment of PBS containing 0.25% Triton X-100 (PBST). Cells were then probed with an anti-HA antibody (Cell
Signaling) in PBST containing 10% normal goat serum, followed by incubation of an Alexa 488-conjugated secondary antibody (Life Technologies). Nuclei were visualized with Hoechst 33258 staining (Sigma). Confocal images were taken using a Leica TCS SP2 confocal microscope and analyzed by ImageJ (NIH). In brief, the fluorescence intensity of the nuclear and cytosolic mHTT was quantified and the ratio of the mean intensity of nuclear/cytosolic signal was calculated.

**Longitudinal Transcription Profiling. RNA extraction.** Four mice, two male and two female, from HD-ΔN17 and WT littermates at ages 2, 7 and 10-11 months were used for microarray experiments. Animals were deeply anesthetized with pentobarbital prior to dissection. Brains were dissected in ice cold 0.01M phosphate buffered saline, made with DEPC-treated water, and flash-frozen on dry ice. Tissue was stored at -80°C until all tissue was ready for RNA preparation. Tissues were then assigned into randomized batches for RNA extraction. Tissue was disrupted and homogenized in Trizol (Invitrogen) before RNA was extracted using Qiagen RNeasy kit with QIAshredder columns (Qiagen), per the manufacturers’ recommendations, including on-column DNase digestion. Total RNA was stored at -80°C until use, avoiding freeze-thaw cycles. **Microarray.** Microarray processing was performed by the UCLA Neuroscience Genomics Core (UNGC, www.semel.ucla.edu/ungc). Briefly, whole genome gene expression profiling protocols began with 100ng total RNA isolated from cortical and striatal tissue processed as described above. Samples were quantitated using Ribogreen fluorescent assay and normalized to 10ng/ul prior to amplification. Amplified and labeled cRNA was produced using the Illumina specific Ambion TotalPrep kit. 1st and 2nd strand cDNA was produced using the Ambion kit and purified using a robotic assisted magnetic capture step. Biotinylated cRNA was produced from the cDNA template in a reverse transcription reaction.
After a second Ribogreen quant and normalization step, amplified and labeled cRNA was hybridized overnight at 58°C to Illumina MouseRef-8 v2.0 expression arrays. Hybridization was followed by washing, blocking, staining and drying on a Little Dipper processor. Array chips were scanned using an iScan reader, and expression data were extracted and compiled using BeadStudio software (Illumina). Raw data were analyzed by Fuying Gao in Giovanni Coppola’s lab using Bioconductor packages as previously described (Coppola et al., 2011), and both raw and normalized data are available under GEO accession number GSE64386. Quality assessment was performed by examining the interarray Pearson correlation, and clustering based on the top variant genes was assessed. Contrast analysis of differential expression was performed by using the LIMMA package (Smyth, 2004). After linear model fitting, a Bayesian estimate of differential expression was calculated, and significance threshold was set at p<0.005. WGCNA. Weighted gene co-expression network analysis was performed using methods detailed in (Oldham et al., 2006; 2008). Briefly, after selecting the 8,000 most variable probes, the absolute Pearson correlation coefficients between one gene and every other screened gene were computed, weighted and used to determine the topological overlap, a measure of connection strength, or ‘neighborhood sharing’ in the network. A pair of nodes in a network is said to have high topological overlap if they are both strongly connected to the same group of nodes. In gene networks, genes with high topological overlap have been found to have an increased chance of being part of the same tissue, cell type or biological pathway. Probes were clustered based on topological overlap, and a dynamic tree-cutting algorithm was used to “cut” 7 branches (modules) off the cluster dendrogram (Langfelder and Horvath, 2008). Resulting modules were characterized using a gene set enrichment analysis (collapseRows function in the R WGCNA package, Miller et al., 2011) and plotted using the software VisANT 3.5 (Hu et al., 2009).
Quantitative PCR. Total RNA was used to generate cDNA using the QuantiTech Reverse Transcription Kit (Qiagen) according to the manufacturer’s recommendations. KAPA SYBR Fast SYBR Green master mix (KAPA Biosystems) was used with 20ng cDNA per reaction. Quantitative PCR (qPCR) was performed using a Roche LightCycler 480 thermocycler with the manufacturer-supplied software calculating each reaction’s second derivative max value (Roche Diagnostics; Indianapolis, IN). Primers (Valuegene) from were checked for specificity to target transcripts using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and, when possible, were selected to span exon-exon junctions. Actb was used as a reference gene. Standard curves were used to determine amplification efficiency for each primer set and were determined based on serial dilutions of wildtype mouse brain cDNA. Relative quantification was performed using the ΔΔCt method (Livak, 2001), with at least three biological replicate samples, and with each reaction performed in triplicate. Primers used for qPCR can be found in Table A1.

Statistical Analyses. Western blot quantification was determined by one-way ANOVA followed by Tukey’s multiple comparisons post hoc test. Electroencephalography data used the Kolmogorov-Smirnov test. Statistical significance was assessed as reaching P < 0.05. Two-way ANOVA was used to assess effects of time and genotype, followed by Bonferroni post hoc testing. Student’s t-test was also used when appropriate. Overlap between genesets was performed using the hypergeometric test in R (phyper function) using the number of shared gene symbols between gene sets as the background. All data are expressed as mean ± standard error of the mean. Graphing and statistics were also performed using Prism software (Graphpad).
Chapter 4

Conclusions and Future Directions
4.1 The Use of Animal Models to Understand Pathogenic Mechanisms in HD

HD, as a monogenetic disease, is an ideal case for the use of genetically engineered animal models to study disease mechanisms. While non-invasive measures of HD progression have improved (especially with the TRACK-HD and PREDICT-HD studies (Paulsen et al., 2008; Tabrizi et al., 2009), we are learning more about the state of the brain as premanifest mutant HTT gene carriers age, phenoconvert, and develop HD symptoms. There are, of course, experiments cannot be performed in human patients, such as longitudinal transcription studies of the vulnerable brain regions, conditional genetics, perturbation with viral vectors, genetic crosses, or preclinical trials, all of which are important methods for interrogating disease mechanisms. HD animal models, ranging from flies to mice, to sheep, pig, and non-human primate, have all provided utility in answering specific questions about the disease (Li et al., 2015; Pouladi et al., 2013). This thesis seeks to unravel the molecular bases underlying two distinct aspects of Huntington’s disease using mouse models: first, what gene expression changes could underlie cell autonomous and non-cell autonomous mHTT toxicity in the cortex and striatum, and second how loss of the mHTT N17 domain causes exacerbated behavior, neuropathology, and strikingly HD-like transcriptionopathy.

4.2 Cortico-striatal Interaction and Cell Autonomous vs. Non-cell Autonomous mHTT toxicity

Using Cre-LoxP conditional transgenic mHTT mice, we undertook a two-step approach to the issue of cortical and striatal vulnerability in HD. By genetically reducing the mHTT transgene in the most vulnerable regions to HD neurodegeneration, we were able to assess the contributions of striatal cells, cortical cells, and the cortico-striatal circuit to disease symptoms.
Lowering mHTT in both cortical and striatal cells achieved amelioration of all tested behavioral phenotypes and rescued neuropathology compared to the BACHD mice, while reducing mHTT in the cortex rescued psychiatric-like phenotypes and partially ameliorated some motor symptoms without having an effect on neurodegeneration. Somewhat surprisingly, reduction of mHTT expression in the striatum did not rescue any measured HD-relevant phenotypes. These studies largely confirmed using genetics what has been studied for years—that MSNs are somehow intrinsically vulnerable to disease, but that mHTT expression causes aberrant cortico-striatal interaction (be it synaptic signaling, loss of trophic support, or other mechanisms) drives the full brunt of disease symptoms.

After genetic determination that dual cortical and striatal mHTT reduction, and to a lesser extent cortical reduction, ameliorates disease symptoms in the conditional transgenic mice, we sought to understand the molecular underpinnings of the behavioral and neuropathological rescue. RNA-sequencing is a powerful tool to assess transcriptome-wide alterations in molecular pathways, and it provided keen insight into the pathways perturbed by mHTT. The BACHD mice, despite a previous study claiming otherwise, do exhibit mild HD-like transcriptional dysregulation in the striatum by 12mo. Gene ontology and pathway analyses identified that these genes are involved in canonical GPCR and axon guidance signaling, and that a significant number have previously been found to have expression altered by Htt. Of the 119 genes deemed dysregulated by fold change, over half (72) are rescued in the striatum with the genetic reduction of mHTT expression. Additionally, more than a quarter of striatal dysregulated genes (31) are significantly ameliorated with reduction of mHTT expression in the cortex, suggesting that this subset of genes may be non-cell autonomously mis-regulated in response to aberrant cortico-striatal signaling caused by the cortical expression of mHTT. Further analyses using Weighted
Gene Coexpression Network Analysis identified genes that, although they have subtle transcriptional fold changes between conditions, have robust coexpression patterns in response to genotype. These modules are of great interest for future work, as they contain genes that are dysregulated in a manner unresponsive to mHTT reduction in striatum or cortex alone (e.g. striatal module M2, with genes related to the ubiquitin-proteasome system and mitochondria), are dysregulated in response to mHTT, but rescued with cortical and cortical plus striatal mHTT reduction (e.g. striatal M2, non-cell autonomous gene changes related to cAMP signaling and inositol phosphate compounds), and genes rescued with striatal reduction and cortical plus striatal reduction (e.g. striatal M38, with cell autonomous gene changes related to Cdk5 signaling and amyloid processing).

To further look into possible mechanisms underlying aberrant cortico-striatal signaling, we assessed synaptic marker staining that shed light on the loss of pre-synaptic (i.e. synaptophysin) and post-synaptic markers (i.e. Actn2 and PSD-95). Actn2, a scaffold protein that links NMDA receptors to the postsynaptic density (Wyszynski et al., 1997), is both cell-autonomously (partially restored in BR striatum) and non-cell autonomously (further restored in BE striatum), with levels indistinguishable from WT in BER striatum. Further, we performed RNA-sequencing on a sorted subset (IT type) of cortico-striatal projection neurons, finding amongst the few changes that several genes involved in calcium or neurotransmitter release (i.e. Ryr2, Syt6), and the activity-dependent neuroprotective transcription factor Npas4 all undergo altered transcription in HD mice at an early age that precedes motor deficits. Npas4 was also found to be dysregulated in the cortex of BACHD mice, with levels restored by genetic reduction of mHTT.
Npas4 is an attractive candidate to test therapeutic intervention in our mouse models. First, Npas4 is primarily expressed in neurons, where it functions as an immediate early gene governing activity-dependent neuroprotective (it is one of Hilmar Bading’s so-called “activity-regulated inhibitor of cell death” or AID genes) and plasticity transcriptional programs, with Npas4−/− mice exhibiting neuronal death (Bloodgood et al., 2013; Ooe et al., 2009; Zhang et al., 2009). Npas4 transcription can be down-regulated by stress signaling (Furukawa-Hibi et al., 2012) and REST (Bersten et al., 2014), which has been previously implicated in HD transcriptionopathy (Buckley et al., 2010). Further, Npas4 has a role in regulation excitatory versus inhibitory input and regulates BDNF transcription, suggesting that its misregulation could play a causal role in altering multiple aspects of cortico-striatal signaling (Bloodgood et al., 2013). We have recently developed viral constructs to drive Npas4 expression or further reduce it in our mice. Preliminary results suggest that these constructs can elevate and lower Npas4 expression, respectively, however much work remains to be done to characterize the role of Npas4 in HD.

Finally, we have saved RNA and tissue from multiple brain and peripheral tissues from the BER mouse cross. We have the opportunity to perform sequencing or other analyses on any of these tissues, with several questions arising that would yield interest. First, what are the progressive transcription changes in the BACHD cortex and striatum that are normalized? Tissue was collected and processed for RNA from all mice at 2, 6, and 12mo, so longitudinal transcriptional study of these brain regions would be relatively easy. Also collected, though not processed yet for RNA, are other tissues: thalamus, cerebellum and more interestingly peripheral tissues such as skeletal muscle, heart, blood, and liver. These tissues could be used to address the question of what affect central mHTT reduction has on the periphery. In addition, addressing
this question could reveal peripheral markers that marker progression of the disease that are restored with central mHTT reduction, that is to say candidate biomarkers for mHTT reduction studies that don’t require invasive collection methods.

4.3 **N17 Deletion Accelerates and Exacerbates HD-like Behavioral, Neuropathological, and Transcriptional Phenotypes**

To assess the role of the huntingtin N17 domain in regulating mHTT localization *in vivo*, we engineered mice based on the well-characterized BACHD mouse line, but lacking amino acids 2-16 in the N17 domain. The resulting ΔN17 mHTT species lacks a domain that previous work has shown plays critical roles in HD pathogenesis (Gu et al., 2009; Thompson et al., 2009) and cell culture studies had found to be critical for cytoplasmic retention, membrane localization, and nuclear exclusion (Atwal et al., 2007). Somewhat surprisingly, BACHD-ΔN17 mice exhibit greatly accelerated disease course, with onset of rotarod symptoms at a similar age to BACHD mice that still have the intact N17 domain (Gray et al., 2008; Menalled et al., 2009) but progression that leaves the mice unable to perform the rotarod task by 8mo. In comparison, BACHD mice complete the rotarod task, albeit at a reduced level well past 12mo. Concomitant with reduced motor performance, BACHD-ΔN17 mice exhibit phenotypes not seen in previous HD mouse models: chorea/dystonia-like head movements starting between 6-7mo, neurological decline including falls, loss of grooming, and urine scalding, and the overt loss of striatal MSNs.

Nuclear mHTT accumulation, as can be seen with immunohistochemical staining and by Western blot, confirmed that deletion of the N17 domain leads to the accumulation of mHTT species in the nucleus *in vivo*, with the particular species appearing to be small N-terminal fragments. Further work in cells points to the likelihood that N27 mediates the nuclear exclusion
of specific sized mHTT N-terminal fragments, with exon1 and CpA fragments requiring N17 for exclusion, while the longer caspase 6 fragment does not. This suggests that mHTT post-translational cleavage into small N-terminal fragments, a long-studied mechanism in HD pathogenesis, followed by impairment of the N17 domain either by further cleavage, conformation change, or by binding of function-disrupting interactors, could drive these fragments into the nucleus to obtain the accelerated toxicity seen in the mice.

Transcriptional effects of nuclear mHTT are robust and age-dependent. Although there is only one significant gene between cortex and striatum of BACHD-ΔN17 versus WT mice at 2mo (Ay3, which is up-regulated in the striatum), by 7mo there are 2063 genes changed in the striatum and another 395 in cortex. By 11mo, when there are severe measures of atrophy, neurodegeneration, and even cell loss in the striatum, 3129 genes are dysregulated in striatum and 3426 in cortex. We again used WGCNA to make sense of the transcriptionopathy, finding that two large modules were associated with gene expression changes over age in the transgenic mice and not in the wildtype. The major down-regulated striatal module, the Turquoise module, is enriched for cAMP and GPCR signaling, axon guidance, and HD-related signaling, with particular enrichment for neuronal genes at the postsynaptic density. Interestingly, the major up-regulated module, the Blue module, is enriched for immune response, apoptosis signaling, and sphingolipid metabolism, and particularly enriched for genes found in astrocytes, microglia, and oligodendrocytes. This is particularly novel, as staining confirmed the presence of reactive gliosis in our aged BACHD-ΔN17 mice, a phenotype that is not seen in the other full-length mouse models but is very relevant to HD and other neurodegenerative disease patients.

Lastly, our discussion of progressive BACHD-ΔN17 transcriptional deficits is limited just to the striatum. Despite generating the raw transcriptional data, we have yet to thoroughly
assess the gene transcription changes in the cortex in this new mouse model. With the emphasis that we have put on cortico-striatal dysfunction being critical for disease pathogenesis in HD, this aspect of BACHD-ΔN17 transcriptional deficits remains an important vein that must be tapped to gain understanding of this model.

4.4 Applying Mouse Studies to Human Patients

The ultimate measure of these studies is whether they get the field closer to meaningful disease modifying or quality of life improving treatments for Huntington’s. Here, we have undertaken studies designed to find the cellular and molecular drivers of disease pathogenesis, with the intention of finding ways to intervene in the process. With the large number of pathways disrupted by mHTT expression, it is likely that therapies that proximal to the disease-causing mutation are most likely to affective.

Our study of cortico-striatal interaction driving disease pathogenesis raises interesting questions for future trials that look to reduce HTT expression, either through delivery of antisense oligonucleotides (ASOs, Kordasiewicz et al., 2012) or by gene editing or transcriptional repression with zinc finger nucleases (ZNFs, Garriga-Canut and Agustín-Pavón, 2012), which appear to be the closest leads to therapeutic trials. The greatest questions for these HTT lowering therapies are when and where to apply these therapies, as the delivery methods can be invasive and spreading limited (Lu and Yang, 2012). To this end, we think that our study shines light on the question of where to treat. Surprisingly, reduction of mHTT expression in the striatum alone, which was a likely target for some therapies, did not rescue disease symptoms in our model mice. Cortical reduction of mHTT expression ameliorated some phenotypes, but did not improve neuropathological readouts. We found maximal behavioral rescue, with mice
statistically indistinguishable from WT, in BACHD mice crossed with Emx1-Cre and Rgs9-Cre, suggesting that treatment of both the cortex and striatum will attain the greatest chance of ameliorating disease symptoms. Our study, and its conclusions with regard to HTT lowering therapies, are not without caveats, though. Emx1 is expressed in embryonic stages and Rgs9 expression begins perinatally, leading to excision of mHTT exon1 early. Thus, our study does not address the effects of mHTT lowering therapies begun after development. Although knockout of mHTT in the adult mice has been shown to improve behavioral phenotypes (Yamamoto et al., 2000), the study did not attempt the dissection of critical cell types as we have done here.

Our study of the HTT N17 domain as well has shed light onto possible mHTT proximal therapeutics. We found that N17 is critical for the nuclear exclusion of small N-terminal fragments that are known to be pathogenic in HD. We proposed a model for the enhanced nuclear toxicity that involves both the cleavage of mHTT into the small N-terminal fragments, and a second step that impairs the N17 domain and leads to nuclear accumulation of the toxic species. Whether the impairment of N17 is functional or physical, disruption of this domain’s function could lead to loss of the ability to exclude these small fragments from the nucleus, where they exert tremendous toxicity. Proteomics studies aimed at determining the interactors involved in these putative trafficking, cleavage, or domain-blocking events would likely lead to insight on how to modulate these events to reduce mHTT translocation. What is not yet known is whether the ΔN17-mHTT is a physiologically relevant fragment.

Although likely not a proximal mechanism, our transcriptional studies of BACHD-ΔN17 mice identified the striking presence of HD- and AD-like neuroinflammation in our aged HD mice. This phenotype has not been seen in other full-length models. It is tempting to speculate
about the role of inflammation in the neurodegenerative disease process—that is say simply whether the inflammation is a pathogenic mechanism, protective mechanism, or a response to the overt neurodegeneration, however current studies have not addressed this. A recent study looking at transcription profiles in multiple neurodegenerative and psychiatric diseases identified common genes that are activated or repressed in AD, ALS, HD, MS, PD, and schizophrenia, suggesting that at least some features of neuroinflammation are shared (Durrenberger et al., 2014). If these inflammatory mechanisms are indeed pathogenic, lessons learned from our BACHD-ΔN17 may also inform the biology and treatment of other CNS diseases.
Appendix

Table A1. From Chapter 3, primers used in qPCR validation of BACHD-ΔN17 modules.

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