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GABAergic Dysfunction in Huntington Disease

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ABSTRACT

GABAergic neurons are responsible for regulation of neuronal signaling through inhibition. Alterations in the delicate balance of excitation and inhibition have been observed in many neurological disorders, including neurodegenerative diseases. Huntington disease (HD) is an autosomal dominant neurodegenerative disorder that is characterized by motor, psychiatric, and cognitive disturbances. As the hallmark movement pathology of HD is hyperkinesis it is intriguing to postulate that alterations in inhibition, and by extension within GABAergic cell networks, could be part of the pathogenesis of this disorder. In order to elucidate the role of GABAergic cell populations in HD we have used behavioral paradigms, molecular techniques including immunostaining and real-time PCR, and electrophysiology to assess the consequence of mutant huntingtin (mthtt) expression on cellular functioning and viability. Using immunostaining, we observed both aggregated and soluble expression patterns throughout the brain of multiple mouse models. Investigation of transcript levels revealed alterations in cell markers and essential proteins in a variety of brain regions. Finally, electrophysiological investigations revealed pathological alterations in neuronal function concurrent with expression of mthtt. The work presented here holds implications for the understanding of the underlying disease mechanisms of HD.

Keywords: GABA, interneuron, huntingtin, parvalbumin, PGC-1α

DEDICATION

As part of this thesis work, I was given the honor to shadow in the UAB HD clinic and met some of these patients first hand. I would like to first and foremost dedicate this work to them and their families- this is for those people who have been rooting for me, and all other researchers, in hopes that we will one day provide them with the answers and the aid that they so greatly deserve.

For my family. You have been there for me from the beginning, every step of the way. Thank you for the biggest things, the love, the support, the laughs. I love you.

For my friends. You have made Alabama like home to me and in the process become my second family. Thank you for the intangible everyday things that put every stress in prospective. I love you.

For my fiancé. You have worked as hard as I have for this. Thank you for keeping me grounded, always. I love you.

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LIST OF ABBREVIATIONS

CalB	Calbindin
GAD67	Glutamic Acid Decarboxylase
HD	Huntington Disease
HTT	huntingtin
IPSC	inhibitory postsynaptic current
MTHTT	mutant huntingtin
PC	purkinje cell
PN	pyramidal neuron
PV	Parvalbumin
Q-RT-PCR	Quantitative Real Time Polymerase Chain Reaction
TTX	Tetrodotoxin
APV	(2*R*)-amino-5-phosphonopentanoate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione

CHAPTER I

INTRODUCTION

Huntington Disease

Huntington disease (HD) is a debilitating neurological disorder characterized by progressive deterioration of cognitive, motor, and psychiatric function. HD is caused by an aberrant expansion of the CAG repeat (encoding glutamine) in exon 1 of the huntingtin (htt) gene, *IT15 (Group 1993).* The disease is genetically inherited in an autosomal dominant fashion. Age of onset is inversely correlated with the length of the CAG repeat (Brandt, Bylsma et al. 1996); while disease severity is directed correlated with repeat length. In patients, a repeat length shorter than 35 CAGs will result in an HD asymptomatic individual (Group 1993). With a repeat length between 36 and 39 there is incomplete penetrance. Once the repeat has extended beyond 39, HD will present with symptoms around midlife with a disease progression of about 15 years until death. Extreme CAG repeat length can occur and results in an early onset form of the disease known as juvenile onset HD (JOHD) (Nance and Myers 2001).

HD was first described by Dr George Huntington in 1872 in a paper entitled “On Chorea” (Brody and Wilkins 1967). He characterized the disease as what is now termed

autosomal dominant and described its hallmark motor phenotype. Clinically, HD presents most often with the motor dysfunction termed chorea. These involuntary dancelike movements are hallmark to the disease and are the most visually obvious to the observer. HD symptomology also includes lessening of fine motor control, abnormal gait, increased occurrence of depression (concurrent with a high rate of suicide attempts; ~12% increase (Schoenfeld, Myers et al. 1984)) and cognitive disturbances. Behavioral symptoms include hallucinations, irritability, paranoia, and psychosis (Paulsen, Ready et al. 2001). It is thought that some of these non-motor phenotypes may arise prior to the motor abnormalities in the pathological timeline, though this tends to be patient-specific (Kirkwood, Su et al. 2001).

Presently, there are no disease-onset or progression-altering treatments. Commonly, HD patients are prescribed antidepressants and anti- anxiolytics (reviewed in (Adam and Jankovic 2008; Mestre, Ferreira et al. 2009). Also the drug, tetrabenazine has been successful in lessening the chorea burden on these patients. This treatment works as a vesicular monoamine transporter inhibitor. It treats hyperkinetic disorders through the enhancement of metabolic degradation of monoamines (Singer 2012). In 2008 this drug became the first Food and Drug Administration-approved treatment of the chorea associated with HD (Yero and Rey 2008). This therapy has become a valuable tool for neurologists in management of the movement component of the disorder, though over time, patients often must increase dosage which still fails to combat the progressive deterioration brought on by HD.

Regional Specificity

HD variably affects distinct brain regions and cell populations. The region primarily affected in HD is the striatum, comprised of the caudate and putamen (Vonsattel, Myers et al. 1985; Reiner, Albin et al. 1988; Aylward, Sparks et al. 2004). Within this region there is severe atrophy of the projection neuron population (medium spiny neurons, MSN). MSNs make up 95% of the neuronal population of the striatum and so loss of this population results in a dramatic thinning of the entire region. MSNs are so named as they display extensive spines beginning about 20 microns from the soma and continuing throughout the dendritic arborization (Wilson and Groves 1980). Functionally, the MSNs of the striatum comprise an integral part of the basal ganglia system of motor control (Wilson and Kawaguchi 1996; Grillner, Hellgren et al. 2005). Within other brain regions there is variable atrophy; another common finding post mortem is the thinning of the cerebral cortex (Halliday, McRitchie et al. 1998; Rosas, Liu et al. 2002; Rosas, Koroshetz et al. 2003). Studies have shown, with varying severity, involvement of other regions as well including the cerebellum (Rodda 1981; Fennema-Notestine, Archibald et al. 2004).

Mouse Models

As HD is a genetic disorder with a known cause animal modeling has become a powerful tool for exploration of the disease course and associated pathologies. Multiple mouse lines have been made in attempts to recapitulate some or all of the features of the human disease. This includes pharmacological, transgenic, knock-in, and conditional models. The models express a variety of CAG repeats within either the full-length htt protein or the shortened exon one segment (Heng, Detloff et al. 2008).

Pharmacological models initially allowed investigators to assess pathological changes in mice with overt HD-like symptomology. Some of the earliest work was done with quinolinic acid (Beal, Kowall et al. 1986; Sanberg, Calderon et al. 1989; Beal, Ferrante et al. 1991), kainic acid (Mason and Fibiger 1978), and 3-nitro-proprionic acid (Borlongan, Koutouzis et al. 1995; Borlongan, Koutouzis et al. 1997; Borlongan, Koutouzis et al. 1997). Valuable insights were gained into the pathological timeline that resulted from neuronal assault with these toxins; however, the discovery of the htt gene allowed for genetic models to come to the forefront of research, with the vast majority of work since being performed in a genetic model.

The first genetic mouse model was the R6 line created in 1996 in the laboratory of Dr. Gillian Bates (Mangiarini, Sathasivam et al. 1996). The R6/2 mouse line is one of the most widely characterized mouse models of HD. This mouse line and its relative R6/1 line were created using exon 1 of the human htt gene under control of the human htt promoter (Mangiarini, Sathasivam et al. 1996; Hansson, Petersen et al. 1999; Li, Popovic et al. 2005). The R6/2 model expresses exon one of the mthtt gene with approximately 150-170 CAG repeats (Mangiarini, Sathasivam et al. 1996). The R6/2 line has a very severe phenotype with an abbreviated lifespan of only 13-15 weeks for animals with approximately 150 CAG repeats (Carter, Lione et al. 1999; Stack, Kubilus et al. 2005), early onset of mthtt+ inclusions (Meade, Deng et al. 2002), overt motor abnormalities by 6 weeks of age (Stack, Kubilus et al. 2005), and neurophysiological signaling abnormalities in a variety of brain regions beginning at 4 weeks of age (Klapstein, Fisher et al. 2001). By end stage, the brains of R6/2 mice weigh an average of 20% less than wildtype littermates (Stack, Kubilus et al. 2005).

While the R6/2 rapid disease progression and severe symptomology have provided valuable insights into the etiology of HD, many speculate that the severity of the disease more closely models the most extreme patient cases, the juvenile onset form of the disease (or even just a generic poly-glutamine disorder), and therefore fails to offer complete insights into adult onset HD, the more common form of the disease (Cepeda-Prado, Popp et al. 2012). Other transgenic models include the N171-82Q (Schilling, Becher et al. 1999; Luthi-Carter, Strand et al. 2000; Klivenyi, Bende et al. 2006) , YAC (Hodgson, Agopyan et al. 1999; Zeron, Fernandes et al. 2004; Zhang, Li et al. 2008; Joshi, Wu et al. 2009), BADHD (Gray, Shirasaki et al. 2008), and transgenic rat model (Kantor, Temel et al. 2006; Nguyen, Kobbe et al. 2006) whose phenotypes are described in table 1. Further investigation of the role of the cerebellum in the disease progression in the R6/2 mice can be found in **chapter II** of this dissertation.

In order to address the issue of physiological relevancy, many groups have attempted to more closely model the adult form of the disease. One avenue to this end is the use of knock-in models. These models are produced through insertion of the elongated mthtt into the endogenous locus for the gene; a gene fragment containing the CAG repeat was utilized. These models have displayed varying phenotypes and HD-like pathologies. One of the more commonly used knock-in models is the HdhQ line originally produced in the laboratory of Dr. Peter Detloff. The HdhQ lines were produced by a double insertion technique that removed the endogenous *htt* gene and effectively replaced it with the mutant form (Lin, Tallaksen-Greene et al. 2001). This lab has gone on to make models with a variety of CAG repeat lengths ranging from 50-350 CAGs. The initial report on this line was published in 2001 on the HdhQ150 line (Lin,

Tallaksen-Greene et al. 2001). These mice have a normal life expectancy with the onset of motor symptoms around 40 weeks of age. The HdhQ150 mice display striatal nuclear mthtt immunoreactivity and neuronal intranuclear inclusions by 37 weeks of age. This is followed by motor abnormalities and weight loss by 70 weeks of age (Heng, Detloff et al. 2008). The HdhQ200 variant of this mouse line presents with a similar phenotype though accelerated phenotype progression, with initial motor abnormalities presenting around 50 weeks of age (Heng, Duong et al. 2010). Further investigation of the role of the cerebellum in the disease progression in these mice can be found in **chapter II** of this dissertation. Other knock-in models include the HdhQ92 (Bayram-Weston, Jones et al. 2012; Brooks, Higgs et al. 2012) , HdhQ111 (Wheeler, White et al. 2000), and CAG 140 (Cummings, Andre et al. 2009; Hickey, Zhu et al. 2012) whose phenotypes are describes in table 1.

One of the hallmark physiological features of HD is regional and cellular specificity. In order to assess cell specific roles for mthtt and further, the roles of cell populations and regions in the disease pathology, several groups have utilized conditional mouse models. Restricted expression of the mutant protein can be established through use of the cre recombinase enzyme as part of the cre/lox system (Utomo, Nikitin et al. 1999). Essentially, the expression pattern of the enzyme can be restricted by placement of a cell specific promoter just prior to the enzyme gene that would then drive enzyme transcription only in cells that drive the promoter. This enzyme works to excise any piece of DNA that is surrounded by molecular tags termed loxP sites. In order to induce expression of a protein in specific locations, the gene of interest (mthtt in our case) is preceded by a stop codon that is floxed by loxP sites (Gu, Li et al. 2005). Upon co-

expression of the cre recombinase enzyme with the floxed gene, mice have the stop codon removed and therefore expression of the desired protein. This technique can also be used to conditionally remove a gene, in which case, the entire gene of interest would be floxed with loxP sites. We utilize the cre/lox system of conditional gene expression to investigate the role of mthtt in a subset of interneurons; this work is described in **chapter III**.

Circuitry & Regional Connectivity

Circuit connectivity through the brain is essential in the establishment of higher level processing. The brain areas thought to be involved in HD, the striatum, cortex, and cerebellum in particular, are connected through complex circuitry and essential projections interweaving the distinct regions and providing a basis for the interplay between regional dysfunction. Region specific susceptibility is seen in HD (Rosas, Liu et al. 2002; Hodges, Strand et al. 2006). The striatum receives direct input from the cerebral cortex in the form of excitatory transmission from the projecting cortical pyramidal neurons. Alterations in excitation from the cortex to the striatum have previously been implicated in Parkinson's Disease (Mallet, Ballion et al. 2006). The cerebellum also has lobule-specific connections to the cortex (Kelly and Strick 2003) and basal ganglia (Hoshi, Tremblay et al. 2005) and from the basal ganglia to the cerebellum (Bostan, Dum et al. 2010; Bostan and Strick 2010). Beyond neuronal synaptic connections the cortex also provides neurotropic support to the striatum in the form of brain derived neurotropic factor (BDNF) (Zuccato, Ciammola et al. 2001). In addition to the loss of actual connectivity, it has been suggested that this support network is compromised in a number of neurological disorders including HD.

Cellular Selectivity

Another intriguing finding in HD is that though the mutant protein is ubiquitously expressed, cell populations have exhibited variable vulnerability to pathology. Multiple reports have suggested that certain cell populations are largely spared or overtly at risk. (Aronin, Chase et al. 1995; Hodgson, Agopyan et al. 1999; Kosinski, Cha et al. 1999). Initial studies implicated dysfunction in and atrophy of the MSNs of the striatum as the predominate cause of the disease pathology seen in HD (Reiner, Albin et al. 1988; Ferrante, Kowall et al. 1991). Though it stands that MSNs are severely affected in the disease, additional cell populations have been implicated as having a role in the disease progression including the pyramidal neurons of the cortex (Cudkowicz and Kowall 1990), the interneurons of the cortex (Gu, Li et al. 2005) and the purkinje cells of the cerebellum (Jeste, Barban et al. 1984). In the work presented here we focus on investigation of GABAergic subsets of the neuronal populations in HD including purkinje cells of the cerebellum (**chapter II**) and parvalbumin positive interneurons of the cortex (**chapter III**).

Excitatory/Inhibitory Balance

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter utilized throughout the brain. It functions in inhibitory cell populations, termed GABAergic neurons, to regulate the signal transmission of other target neuronal populations (Cobb, Buhl et al. 1995; Wang and Buzsaki 1996; Bartos, Vida et al. 2002; Mann and Paulsen 2007). The role of these GABAergic cell populations will be investigated at length in this dissertation with regard to disease processes, molecular

Model/Line	Basic Information			Phenotype			References
	Host Species	Gene	Repeats	Motor	Psychiatric	Cognitive	
Pharmacological							
Quinolinic Acid	variable	--	--	hyperactivity	visuospatial deficits	procedural memory deficits, recall deficits	(Beal, Kowall et al. 1986; Sanberg, Calderon et al. 1989; Beal, Ferrante et al. 1991)
3-Nitro-proprionic Acid	variable	--	--	hyperactivity	deficits in habituation to open field	deficits on radial arm water maze	(Borlongan, Koutouzis et al. 1995; Borlongan, Koutouzis et al. 1997; Borlongan, Koutouzis et al. 1997)
Transgenic							
R6/1	mouse	human exon 1	116	gait abnormalities	decreased anxiety		(Hansson, Petersen et al. 1999; Li, Popovic et al. 2005)
R6/2	mouse	human exon 1	144	resting tremor, hypoactive, clasping, rotorod deficits	altered exploratory behavior	deficits morris water maze, T-maze	(Mangiarini, Sathasivam et al. 1996; Carter, Lione et al. 1999)
N171-82Q	mouse	human exon 1	82	rotorod deficits, clasping	minimal increased anxiety	deficits on radial arm water maze	(Schilling, Becher et al. 1999; Luthi-Carter, Strand et al. 2000; Kliveryi, Bende et al. 2006)
YAC	mouse	human full	72,128	gait abnormalities, hyperactive, clasping, ataxia	variable	deficits T-maze	(Hodgson, Agopyan et al. 1999; Zeron, Fernandes et al. 2004; Zhang, Li et al. 2008; Joshi, Wu et al. 2009)
Transgenic Rat	rat	human repeats	51	gait abnormalities	decreased anxiety	deficits radial arm maze, elevated plus maze	(Kantor, Temel et al. 2006; Nguyen, Kobbe et al. 2006)
Knock-In							
HdhQ92	mouse	human	90	none	altered prepulse inhibition	none	(Bayram-Weston, Jones et al. 2012; Brooks, Higgs et al. 2012)
HdhQ111	mouse	mouse	109	gait abnormalities		none	(Wheeler, White et al. 2000; Choo, Johnson et al. 2004)
CAG140	mouse	mouse	140	early increase in locomotion followed by later reductions	increased anxiety	none	(Cummings, Andre et al. 2009; Hickey, Zhu et al. 2012)
CAG150	mouse	mouse	150	hypoactivity, rotorod deficits, clasping	normal exploratory behavior	none	(Lin, Tallaksen-Greene et al. 2001; Heng, Tallaksen-Greene et al. 2007)
Conditional							
Cre/lox	mouse	mouse	variable	variable	variable	variable	(Diaz-Hernandez, Torres-Peraza et al. 2005;

Table 1.1: Commonly Used Huntington Disease Models.

In order to assess the physiological consequence of mthtt expression, a multitude of mouse models have been produced. Here we summarize some of the most commonly used varieties. This list is not exhaustive but includes several of models used for experiments in this thesis. Model type is separated by mode of production i.e.- pharmacological, transgene expression, knock-in, and conditional.

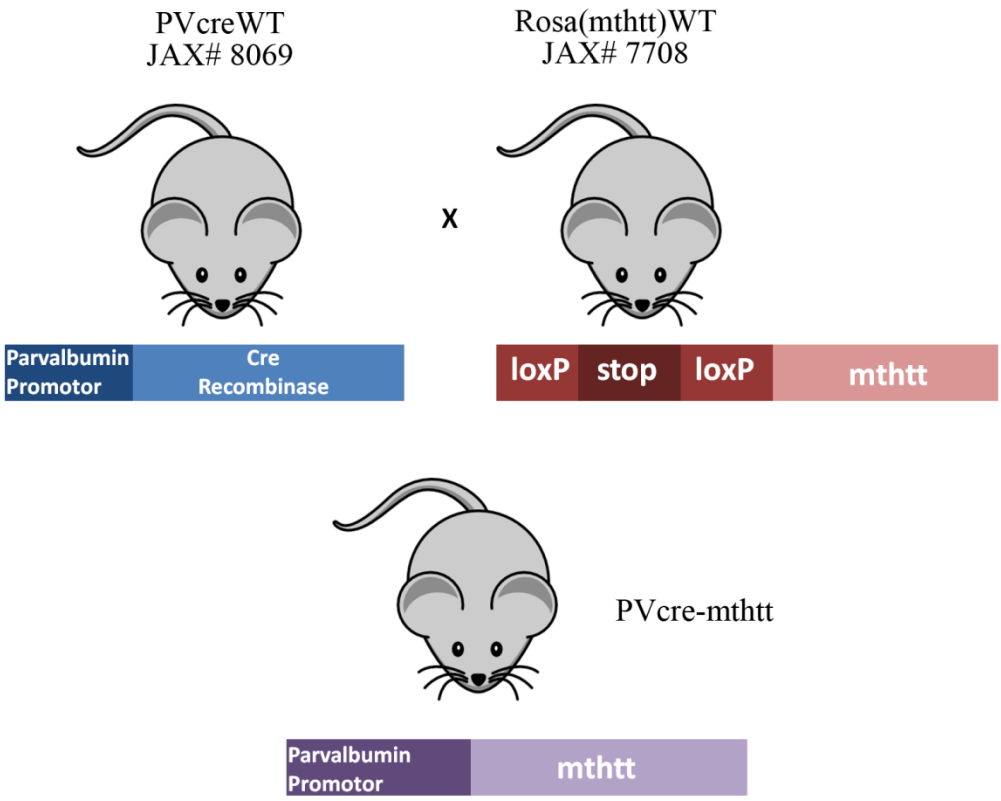

Figure 1.1: Breeding Scheme for Conditional Expression of mthtt.

To accomplish restricted expression of mthtt only in PV+ cell populations, we utilized the cre/lox system of gene expression. The original parent lines were purchased from Jackson Laboratories and then mated as illustrated to produce F1 offspring with exon 1 of the mthtt with approximately 103 CAG repeats with expression under control of the PV promoter.

alterations, and regional neuronal functioning.

Coordinated neuronal signaling is established through complex networks of inhibition and excitation working in tandem to properly encode information. The maintenance of the delicate balance between these two forms of signaling is imperative to the healthy functioning of the brain circuit as a whole. Alterations in either form of transmission can cause a wide range of neuropathologies. GABAergic cell populations can be divided into subtypes based on morphology, localization, or molecular content. Throughout these studies the molecular profile of these neurons will be used to identify key populations.

The cerebellum is the most posterior part of the human brain and commonly known to play a role in movement. This brain region has been shown to control coordination and precise movements while also playing a role in cognitive function (Rapoport, van Reekum et al. 2000). The cerebellar cortex is uniformly arranged into layers including the molecular layer, the PC layer and the granule cell layer (Kwong, Chan et al. 2000). The GABAergic interneurons of this region are mostly found in the molecular layer. The sole output neuron of this region is the GABAergic PC. PCs work to integrate incoming signals from the other regional neurons and accurately communicate these on to the deep cerebellar nuclei and then in to further downstream targets. PCs are pacemaking cells that fire in fine tuned rhythms and allow for the encoding of complex signals from this solitary output. As HD is a neurological disorder with a strong motor component it is intriguing to postulate that the cerebellum could be contributing to the lack of coordination and alteration in motor control observed in the disease. Further as HD is also a poly-glutamine disorder and most poly-glutamine disorders included

involvement of the cerebellum it follows that HD could display the same regional disruption. Antemortem brain scan studies have shown that thinning of the cerebellar cortex is present in some HD patients (Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004), including but not limited to the most severe cases of the disease. Loss of PCs was found to occur in adult HD patients through blind postmortem quantification (Jeste, Barban et al. 1984). Cerebellar atrophy has also been observed in multiple mouse models of HD (Turmaine, Raza et al. 2000). Further, primate studies have revealed that the mutant protein can be found throughout the cerebellum, though in the highest concentration within the PC layer (Gutekunst, Levey et al. 1995). **Chapter II** of this dissertation investigates the timeline of pathological occurrences in the cerebellum.

GABAergic interneurons of the cortex play in integral role in the regulation of network firing (Huang, Di Cristo et al. 2007). Interneurons are densely interconnected with the projection neurons, pyramidal cells, allowing for the precise entrainment of regional oscillations (Packer and Yuste 2011). PV+ interneurons are the most common type of interneuron in the cortex (Markram, Toledo-Rodriguez et al. 2004). These neurons entrain the network at the gamma frequency (Traub, Whittington et al. 1996; Wang and Buzsaki 1996). PV+ interneurons are uniquely primed to regulate network activity due to their intrinsic properties. PV+ interneurons express the potassium channel Kv3.1 which allow for the fast spiking nature of these interneurons (Perney, Marshall et al. 1992; Du, Zhang et al. 1996; Yeung, Thompson et al. 2005). Further, the calcium binding kinetics of PV also allow PV+ interneurons to fire in a non-adapting, non-

accommodating pattern (Kawaguchi 1993; Kawaguchi and Kondo 2002; Tepper and Bolam 2004).

The vulnerability of interneurons to a mthtt-mediated assault was long a point of contention, with early results suggesting a relative sparing of these neuronal populations, including a widely cited abstract (Harrington & Kowall, 1991). Since the initial reports many studies have disputed those claims and suggested the interneurons may play an integral role in the etiology of HD and the progression of the disease (Fusco, Chen et al. 1999; Kosinski, Cha et al. 1999; Meade, Deng et al. 2002; Giampa, Middei et al. 2009). In 2005, and elegant study came out from the laboratory of Dr. William Yang at UCLA (Gu, Li et al. 2005). In this report, through the use of the cre/lox system, conditional expression of mthtt in all neurons (nestin cre) was compared to restricted expression of the mutant protein just to pyramidal projection neurons in the cortex (emx cre). The results showed that projection neuron only expression was insufficient to recapitulate the phenotypes observed in the pan-neuronal model. The conclusion of this work was that since the resultant phenotypes were not identical that the interneurons must be playing a role in the pan-neuronal model; however as interneurons are a heterogeneous group, further characterization through subtype specific investigation will shed light on the roles of specific classes of interneuron populations in HD.

Normal huntingtin & Mutant huntingtin

As the normal function of the htt protein is unknown, it is unsurprising that contention arises upon the classification of HD as a pathology of loss-of-function or toxic gain-of-function. Studies have shown that genetic ablation of the gene results in

embryonic lethality (Duyao, Auerbach et al. 1995; Zeitlin, Liu et al. 1995). This finding suggests that the protein has some essential, as yet undetermined, function in development. Through mostly immunoprecipitation binding assays, normal htt has been found to interact with numerous other proteins with roles in diverse processes as transcription, transport, and cell signaling (Cattaneo, Rigamonti et al. 2001; Gauthier, Charrin et al. 2004; Landles and Bates 2004; Li and Li 2004; Caviston and Holzbaur 2009); though often the consequence of these interactions is unknown.

There is abundant evidence to suggest that toxicity in HD is mediated by a toxic gain-of-function mechanism. Multiple reports have suggested novel aberrant roles for the mutant protein in a wide range of processes including: disruption of calcium signaling (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), interruption of cellular trafficking and firing (Klapstein, Fisher et al. 2001; Morton, Faull et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009), and transcriptional dysregulation (Cha, Kosinski et al. 1998; Dunah, Jeong et al. 2002; Luthi-Carter, Hanson et al. 2002; Sugars and Rubinsztein 2003; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009; Zuccato, Valenza et al. 2010). It is likely this multifaceted assault that has complicated the pursuit of the underlying root cause of the disease. These roles for the mutant protein are also likely compounded by its propensity to form aggregates which may be contributing to the pathology of HD (DiFiglia, Sapp et al. 1997; Saudou, Finkbeiner et al. 1998).

Studies have shown that concurrent loss of htt and overexpression of mthtt can have synergistic negative effects in mouse models (Van Raamsdonk, Pearson et al. 2005; Leavitt, van Raamsdonk et al. 2006). Additionally, several groups have attempted to

rescue mthtt-associated pathologies with expression of htt to varying degrees of success (Leavitt, van Raamsdonk et al. 2006). The caveat appears to be that while the dual hit is most damaging, reconstitution of the normal protein does not fully rescue the pathologies caused by mthtt.

Transcription & Regulation of Parvalbumin

Peroxisome proliferator activated receptor gamma coactivator one alpha (PGC-1α) is a transcriptional coactivator that works through the binding of transcription factors to upregulate expression of its downstream targets (Finck and Kelly 2006; Cowell, Blake et al. 2007; Cowell, Talati et al. 2009). PGC-1α is highly concentrated in GABAergic cell populations (Cowell, Blake et al. 2007). PGC-1α has been thoroughly investigated in peripheral tissues throughout the body and due to its role in energy homeostasis has been termed the master regulator of metabolism (Handschin and Spiegelman 2006). Early work from this lab was aimed at identification of the gene programs regulated by PGC-1α in the central nervous system (Lucas, Markwardt et al. 2010).

In Lucas et al 2010, our lab demonstrated that PGC-1α is required for the proper expression of PV; PGC-1α was shown to directly regulate PV through induction of PV expression in a neuroblastoma cell line with treatment of PGC-1α. There was also loss of the calcium binding protein in the PGC-1α -/- animals. This finding was essential as it was some of the earliest evidence of PGC-1α's regulation of a non-metabolism-related gene program. Investigation into the consequences of loss of PGC-1α on cortical physiology is addressed in **chapter IV** of this document.

Previous reports have implicated PGC-1α as playing an important role in a number of aberrant processes in HD, reviewed in (Cui, Jeong et al. 2006; Weydt, Pineda et al. 2006; Tsunemi and La Spada 2012). In order to characterize the meaningful contribution of PGC-1α in HD, it is essential to first elucidate its role in normal physiological circumstances. Our lab has demonstrated that PGC-1α drives the expression of a variety of transcripts including PV and the calcium sensor synaptotagmin 2 (SYT2; unpublished observation). Ablation of PV (Schwaller, Dick et al. 1999) and SYT2 (Pang, Melicoff et al. 2006; Pang, Sun et al. 2006; Sun, Pang et al. 2007) through the use of knock-out animals causes asynchronous neurotransmitter release phenotypes that mirror electrophysiological deficits seen in HD models. Synchrony in neuronal firing in the motor cortex occurs during executive voluntary movements (Donoghue, Sanes et al. 1998; Hatsopoulos, Ojakangas et al. 1998; Riehle, Grammont et al. 2000) and could contribute to motor dysfunction in a variety of neurological motor disorders. It has been hypothesized that such synchrony is established through the control of pyramidal neurons by PV+ inhibitory interneurons synapsing on or near the soma and axon initial segment (Wang and Buzsaki 1996; Vreugdenhil, Jefferys et al. 2003). As PV and SYT2 are decreased in PGC-1α -/- mice, we postulate that there will be a loss of synchronous release of neurotransmitter and synaptic activity, as is observed in PV -/- (Vreugdenhil, Jefferys et al. 2003; Korotkova, Fuchs et al. 2010) and SYT2 -/- (Sun, Pang et al. 2007) animals. Such asynchrony could contribute to the motor symptoms observed in these

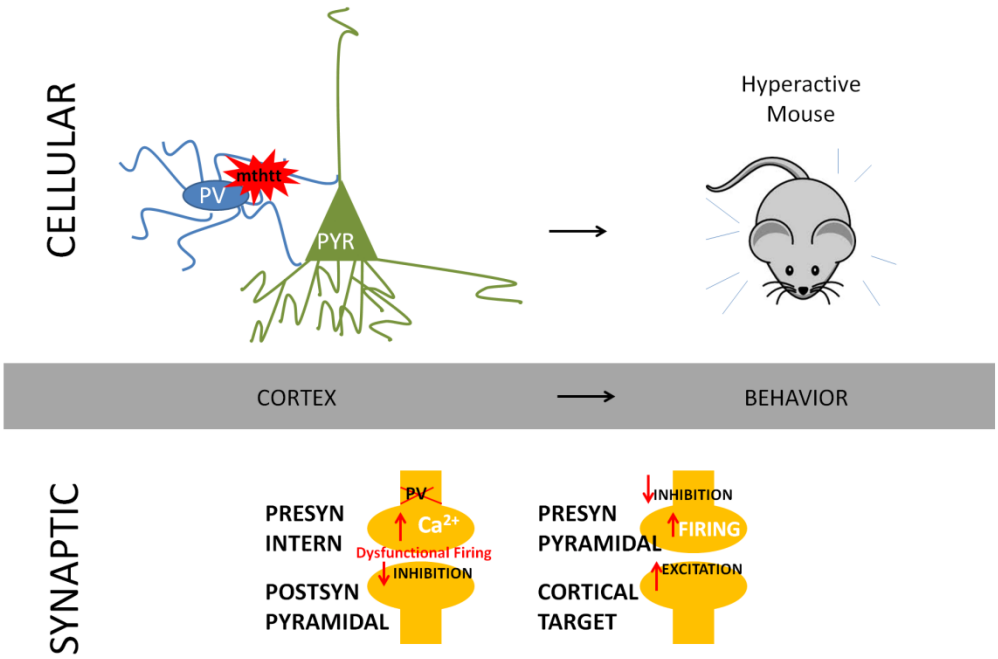

Figure 1. 2. Proposed Model for mthtt induced hyperactivity.

With restricted expression of mthtt only in PV+ cell populations, our initial assumption in this model is that the mutant protein will be acting in a cell-autonomous fashion in those cell populations. We hypothesize that mthtt could act at the level of the synapse reducing inhibitory output from the interneurons resulting in a hyperactive cortical network which could then contribute to a behavioral phenotype, including hyperactivity in a mouse model. Mthtt could be directly affecting levels of PV through disruption of transcription but also could be performing aberrant protein-protein interactions with synaptic machinery within the PV+ cells to result in dysfunctional firing independent of PV protein concentration.

mice, especially when occurring in the primary motor cortex. Experiments in Aim 1 are designed to elucidate the contribution of PV+ neuron dysfunction to the abnormalities in GABAergic signaling which we have observed in PGC-1α -/- mice.

Considering the importance of PV+ neurons in regulating cortical synchrony and the proposed interference of mthtt with normal synaptic activity, it is possible that mthtt disrupts synchronous release from these neurons, giving rise to hyperactivity. To investigate the involvement of PV+ interneurons in HD, we will use a transgenic approach to over-express mthtt specifically in PV+ neurons and will then evaluate motor behavior, changes in interneuron- specific transcripts, and inhibitory transmission at interneuron-pyramidal neuron synapses.

Thesis Focus

In order to address the overarching goal of elucidating the role of GABAergic cell populations in HD this thesis has taken on three aims. These aims will be addressed in three chapters each with separate specific hypotheses addressed with similar methods. The data presented in **chapter II** has been accepted as two manuscripts both at the journal of *Experimental Neurology*, the data from chapter 3 will form one manuscript which is in submission, and the data from chapter 4 represents one manuscript which is currently in preparation and one set of data that will supplement an ongoing submission.

The focus of **chapter II** is on the role of the cerebellum and its output neuron, the purkinje cell, in HD. Previous reports have been conflicting as to the extent of involvement of this brain region in the disease (Rodda 1981; Rosas, Koroshetz et al.

2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006; Van Raamsdonk, Warby et al. 2007; Carroll, Lerch et al. 2011). As HD is often thought of as a disease of the striatum and secondarily the cortex, the cerebellum is often overlooked. We have further investigated the potential role of the cerebellum in HD in two manuscripts entitled, "Disruption of Purkinje cell function prior to huntingtin accumulation and cell loss in an animal model of Huntington disease" and "Purkinje cell dysfunction and loss in a knock-in mouse model of Huntington Disease." In these works, highlighted in **chapter II**, we have assessed the transcriptional, protein, and firing abnormalities that occur within purkinje cells throughout the disease progression of HD. This work stems from an initial finding of a loss of parvalbumin expression in the cerebellum from the R6/2 mice. The main questions to be addressed in this section are: Where is the mutant protein expressed in the Purkinje cells? Along what timeline are inclusions present? Are cells lost throughout the disease progression? Do the output neurons continue firing as normal? These experiments were performed in transgenic mice as well as knock-in models and illuminated the pathological occurrences throughout the cerebellum with additional focus on the firing dysfunction of the purkinje cells.

Chapter III of this thesis focuses on the role of the parvalbumin subpopulation of cortical interneurons and the role that those neurons play in the etiology of HD. Through the use of a conditional mouse model of HD we investigate the motor phenotype, protein level expression of mthtt, and physiological consequences through the lifespan of these animals. The main questions to be addressed as part of this work are: Could expression of exon 1 of mthtt only in PV+ cells result in an HD-like motor phenotype? Does expression of mthtt in PV+ cells alone result in an altered transcriptional profile? Can these deficits

be rescued in acute slices with treatment of PV-like calcium buffers or in animals with PGC-1α itself? These experiments are in submission in the manuscript entitled, "Restricted expression of mutant huntington to parvalbumin-positive cells causes hyperactivity and altered synaptic function in the motor cortex". This work centers on the hypothesis that: *PV interneuron dysfunction will contribute to the motor and regional physiology alterations seen in HD and that expression of the mutant protein in that cell population will recapitulate some aspects of the HD-associated pathology.* The results provided evidence that PV+ cell population- specific expression of exon one of the mthtt gene with 103 CAG repeats is sufficient to cause hyperactivity at midlife and concurrent reductions in inhibitory transmission in the cortex.

Chapter IV investigates the physiological consequences of disrupting the expression of the mthtt-interacting protein PGC-1α. Our hypothesis was that *as PGC-1α is concentrated in GABAergic cell populations, loss of this coactivator, and by extension its downstream targets, will cause dysfunction in inhibitory signaling in the cortex*. The main questions to be addressed in this section are: Does loss of PGC-1α result in an electrophysiological phenotype similar to that seen with ablation of PV or SYT2? Are changes in the cortex similar to the alterations that have been shown in the hippocampus by Lucas et al 2010? In order to test this hypothesis we performed electrophysiological characterizations of both basal and evoked GABA release in the motor cortex. Further, these experiments utilized two animal models, one with global ablation of PGC-1α and one with conditional loss only from PV+ cell populations. These experiments allowed us to not only describe the consequence of loss of PGC-1α but to take this a step further into loss of the coactivator from a cell population in which it is known to regulate genes key

to inhibitory function.

Research Objectives

The main goal of this thesis research was to elucidate the role of GABAergic cell populations in the etiology of HD.

The Cerebellum. Early studies in the HD field implicated a role of the cerebellum (Jeste, Barban et al. 1984). Cerebellar, and often by extension PC, atrophy has been observed in both patients and animal models of HD (Kageyama, Yamamoto et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). Much of the recent research has neglected alterations in the region, focusing more on the striatum and cortex as the cardinal regions affected through the disease time course. In **chapter II** we used quantitative Real-Time PCR, immunohistochemistry, stereology and loose patch electrophysiology to describe and quantify transcript, protein, and physiological changes in Purkinje cells of two different HD mouse models.

PV Interneurons. Interneurons synchronize regional activity through vast connectivity with projection neurons. Within the cortex, inhibitory PV interneurons entrain the network at the gamma frequency and allow for local and temporal regulation of firing resulting in fine tuned propagation of neuronal signals throughout the region and its targets (Traub, Whittington et al. 1996; Wang and Buzsaki 1996; Packer and Yuste 2011). Network signaling dysfunction has been observed in neurodegenerative disorders

including HD (Palop, Chin et al. 2006). In **chapter III** we used quantitative Real-Time PCR, immunohistochemistry, and whole cell patch clamp electrophysiology to investigate the consequence of restricted expression of mthtt to PV+ cell populations.

PGC-1α & PV Interneurons. In order to fully understand the role of PV interneurons in HD we further assessed alterations in inhibitory transmission in a mouse model deficient in PGC-1α which is a transcriptional coactivator that directly regulates level of PV. In **chapter IV** we used whole cell patch clamp electrophysiology to assess inhibitory transmission within the motor cortex of mice with reduced PGC-1α and, as a consequence, reduced PV.

Specific Aims

General Hypothesis: GABAergic cell populations, especially PV+ subtypes, play an integral role in the pathogenesis of HD.

Specific Hypothesis:

I. We hypothesize that as part of the etiology of HD there will be transcript and protein level changes in purkinje cells in the cerebellum which will contribute to the motor phenotype observed in this disorder.

AIM: To evaluate and compare the transcriptional and motor phenotypes in mice with global expression of mthtt.

A. To assess the motor phenotype in the mouse models of HD.

B. To evaluate the transcriptional alterations in the cerebellum brought on by global expression of mthtt.

C. To evaluate the expression of mthtt protein throughout the cerebellum of these mice with double-labeling immunofluorescence.

AIM: To evaluate alterations in purkinje cell viability and spontaneous firing in the cerebellum of mice with global expression of mthtt.

A. To evaluate the population density of purkinje cells in the cerebellum of HD mouse models over the disease progression.

B. To evaluate the spike frequency of spontaneous activity from the purkinje cells in the cerebellum brought on by global expression of mthtt.

II. We hypothesize that PV+ interneurons will be vulnerable to a mthtt-associated pathology and that expression of exon 1 of mthtt in these neuronal groups will be sufficient to cause a motor phenotype.

AIM: To evaluate and compare the transcriptional and motor phenotypes in mice with PV+ cell specific expression of mthtt.

A. To assess the motor phenotype brought on by conditional expression of mthtt only in these populations

B. To evaluate the transcriptional alterations in the cerebral cortex, striatum, and cerebellum brought on by PV+ cell specific expression of mthtt.

C. To evaluate the expression of mthtt protein throughout the brain of these mice with double-labeling immunofluorescence to determine which neuronal populations are contributing to the phenotype.

AIM: To evaluate alterations in inhibitory neurotransmission in the cortex of mice with PV+ cell specific expression of mthtt.

A. To assess the frequency and overall properties of spontaneous inhibitory post synaptic currents (sIPSC) including: amplitude and frequency in the presence of glutamate receptor antagonists in order to evaluate basal GABA release.

B. To evaluate changes in spontaneous activity in the presence of Na+ channel antagonist TTX to block action potential activity in order to elucidate the role of mini IPSCs (mIPSCs) in any altered firing patterns.

C. To assess the alterations in short-term plasticity using the paired pulse recording paradigm with interevent intervals of 20, 30, and 100ms.

D. To evaluate levels of evoked GABA release as brought on by gamma train stimulation.

III. We hypothesize that as loss of PGC-1α results in a loss of PV, we will see alterations in spontaneous activity and evoked responses mirroring those changes seen in PV -/- and potential PV+ cell specific HD animals.

AIM: To evaluate the electrophysiological consequences of the loss of PGC-1α in the pyramidal cells of the motor cortex in PGC-1α -/- animals.

A. To assess the frequency and overall properties of sIPSC including: amplitude and rise and decay kinetics in the presence of glutamate receptor antagonists in order to evaluate basal GABA release in PGC-1α -/- animals.

B. To evaluate changes in spontaneous activity in the presence of Na+ channel antagonist TTX to block action potential activity in order to elucidate the role of mIPSCs in any altered firing patterns.

C. To assess the alterations in short-term plasticity using the paired pulse recording paradigm with interevent intervals of 20, 30, and 100ms.

D. To evaluate levels of evoked GABA release as brought on by gamma train stimulation.

Experimental Approaches

The experimental protocols used to address these specific aims focused on quantitative techniques to assess protein and transcript levels changes, forming a molecular basis for electrophysiological investigation. Qualitative immunostaining techniques were then used to localize the observed changes in molecular content and synaptic function. Detailed experimental protocols are included in the chapters.

Figure 1.3: Targeted Whole Cell Patch Clamp Electrophysiology.

In order to assess the consequence of mthtt expression in PV+ cell populations and in attempts to extend these findings to mice lacking PGC-1α and its targets, we performed visualized patch electrophysiology. Precision in the region used for recording was essential. Our efforts focused on the area highlighted above, the motor cortex anterior to and slightly post-formation of the hippocampus. (Position shown in coronally sectioned WT mice stained for PV, 30μm thickness).

CHAPTER II: A

DISRUPTION OF PURKINJE CELL FUNCTION PRIOR TO HUNTINGTIN ACCUMULATION AND CELL LOSS IN AN ANIMAL MODEL OF HUNTINGTON DISEASE

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Experimental Neurology and Dougherty, S. E., J. L. Reeves, et al. (2012). "Disruption of Purkinje cell function prior to huntingtin accumulation and cell loss in an animal model of Huntington disease." Exp Neurol **236**(1): 171-178.

Introduction

Huntington Disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive deterioration of cognitive, motor, and psychiatric function. HD is caused by an elongation of the CAG repeat in exon 1 of the huntingtin gene, with an inverse correlation between age of onset and repeat length (Group 1993). Expression of mutant huntingtin (mthtt) is ubiquitous throughout the brain and is thought to be involved in a number of pathological interactions. These functions include transcriptional dysregulation (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009), interruption of calcium signaling (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), and disruption of normal synaptic physiology (Klapstein, Fisher et al. 2001; Morton, Faull et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009).

While the striatum is the primarily affected brain region in HD, it is possible that other components of the motor circuit, such as the cerebellum, are involved in motor symptom development. Conflicting reports have been made on the pathology of the cerebellum in HD which range from the region being considered unaffected (Van

Raamsdonk, Warby et al. 2007; Carroll, Lerch et al. 2011) to the cerebellum being an important part of the disease progression (Rodda 1981; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006). The cerebellum appears to be more commonly affected in juvenile HD as exhibited by a loss in overall cerebellar volume (Kageyama, Yamamoto et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). It is imperative to explore cerebellar pathology in animal models of HD to determine the involvement of this brain region in the pathogenesis of HD.

The output neurons of the cerebellar cortex are the GABAergic Purkinje cells (PCs). Intranuclear inclusions have been found in PCs in postmortem tissue from HD patients and in knock-in animal models of HD (Adachi, Kume et al. 2001). Additionally PCs are vulnerable in both patients and animals models of other CAG repeat disorders (Koeppen 1991; Vig, Fratkin et al. 1996; Vig, Subramony et al. 1998; Garden, Libby et al. 2002; Koeppen 2005; Schulz, Borkert et al. 2010). Investigators have often considered the cerebellum to be unaffected in HD and used the cerebellum as a control region. Nonetheless, there is evidence to suggest a reduced density of PCs in HD patients (Jeste, Barban et al. 1984; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004) raising the possibility that PCs play a meaningful role in HD.

Little is known about PC function or survival in animal models of HD. To investigate the involvement of PC dysfunction in HD, we utilized the R6/2 model. The R6/2 mouse model of HD carries transgenic expression of exon 1 of the human huntingtin gene with an expanded CAG repeat (Mangiarini, Sathasivam et al. 1996). This

model has been shown to be a severe model of HD with an abbreviated lifespan, early symptom onset and enhanced disease severity. This increased severity might suggest a more global neurological assault (Ruocco, Bonilha et al. 2008); as such, it follows that investigation of cerebellar changes in the R6/2 mouse could provide insights into the involvement of this brain region in early onset HD.

In these experiments we explored the changes that occur in PCs of R6/2 mice using qRT-PCR, immunohistochemical staining, and electrophysiology. Here we report decreases in GABAergic cell markers and calcium binding proteins in the cerebellum, specifically the PC layer. These changes were accompanied by huntingtin accumulation and PC loss in symptomatic mice but preceded by alterations in cellular physiology. These studies suggest that PCs are vulnerable in R6/2 mice and may contribute to the development of motor symptoms in HD.

Methods

Animals.

The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all experimental protocols. The R6/2 mouse line was obtained from Jackson Laboratories and maintained through breeding male F1 generation offspring to WT hybrid (B6CBA) females (B6CBA-Tg (HDexon1)62Gpb/3J). The mice were obtained from Jackson Laboratories by way of an ovarian transplant female with ovaries from a sexually unviable R6/2 +/- female. All experiments were conducted with both male and female animals at ages 2, 4, 7, and 12 weeks. R6/2 mice carried a repeat

length of 167 as genotyped by Laragen, Inc (Culver City, CA). These animals were housed in groups of up to 5 animals per cage with food and water *ad libitum.*

Behavioral Assessment.

Behavioral analyses were conducted on littermates at four, six or twelve weeks of age during the lights on period (6am – 6pm). All experiments were conducted blind to the genotype of the animals.

Rotarod.

The rotarod apparatus (MedAssociates, St. Albins, VT) consisted of a 5-station treadmill with a computer-controlled stepper motor-driven rod with constant speed or accelerating speed modes of operation. Animals were trained on the rotarod for four days. On the first day of the training period, animals were placed on the treadmill at an accelerating speed of 2-24 rotations per minute (rpm) for a maximum of 60 seconds for a total of 5 trials. On the second through fourth days, the animals were placed on the treadmill at a constant speed of 16 rpm for a maximum of 60 seconds for a total of 5 trials per day. On the fifth day, animals underwent two trials each at rotating speeds 16, 20, 24, 28, and 32 fixed rpm. Each trial lasted for a maximum of 60 seconds, during which latencies to fall were recorded. Mice were allowed to rest for at least five minutes between each trial.

Open field.

Animals were placed in a square apparatus (27.9 cm^3) consisting of 48 infrared beams (MedAssociates) for 30 minutes. Data were collected with Open Field Activity

Software (MedAssociates) in one minute intervals over the test period, and total ambulatory time and distance were determined.

Gene Expression Analysis.

Protocols were performed as described (Lucas, Markwardt et al. 2010). Briefly, mice were anesthetized with isoflourane and sacrificed by decapitation. Brains were removed, and dissected by anatomical region. Tissues were flash frozen on dry ice and kept in the -80°C freezer until use. At least 12 hours prior to processing, tissues were placed in RNA*later*-ICE (Ambion Austin, TX) cooled to -80°C and then kept at -20°C until homogenization. Homogenization was performed with a Tissue-Tearor (Biospec Bartlesville, OK) homogenizer in Trizol following the manufacturer's instructions (Invitrogen Grand Island, NY). Taqman PCR was conducted with JumpStart Taq Readymix (Sigma St. Louis, MO) and Applied Biosystems (Carlsbad, California) primers for GAD67 (Mm00725661_s1), calbindin (Mm00486645_m1), parvalbumin (Mm00443100_m1), and β-actin (Mm00607939_s1).

Immunofluorescence.

Animals were anesthetized with isofluorane and perfused intracardially with room temperature 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed for an additional 24-72 hours. The samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C. Tissue blocks were sectioned at 30 µm, mounted onto charged slides (Fisher) and allowed to dry overnight before storage at -80°C. Slides were then thawed

and washed with PBS followed by a one hr incubation in 10% serum (from the host of the 2° antibody) and PBS with 3% bovine serum albumin (BSA). The slides were then incubated overnight with a predetermined concentration of 1° antibody and 5% serum in 0.3% Triton X PBS with 3% BSA. This is followed by washing and then a 2 hr incubation with the corresponding fluorescence-conjugated 2° antibody (Jackson ImmunoResearch West Grove, PA) and 5% serum in 0.3% TritonX PBS with 3% BSA. The slides are then immediately mounted using an antifade media containing DAPI, coverslipped and left to dry at room temperature overnight. When necessary, a Vector (Burlingame, CA) Mouse on Mouse Kit (Fluorescein Cat No. FMK-2201) was used to minimize background staining for mouse-made antibodies.

Western blot analysis.

Cerebella were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% sodium dodecyl sulfate, 0.5% deoxycholic acid; pH 8.0) containing a protease inhibitor tablet (Complete Mini, Roche Diagnostics Indianapolis, IN). Total protein concentration was determined with a bicinchonicic acid protein assay kit (Thermo Scientific Waltham, MA), and absorbance was measured at 540 nm. Protein was denatured in sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol; pH 6.8) at 95°C. Equivalent amounts of protein were loaded into precast polyacrylamide NuPage gels (Invitrogen). One interblot control sample was loaded onto every gel to permit comparison among gels. Protein was transferred onto nitrocellulose membranes. Blots were blocked with 5% milk in tris buffered saline (TBS; pH 7.6) with 1% Tween (TBS-T) and probed with primary antibodies in 5% IgG-free BSA (Jackson ImmunoResearch) TBS-T overnight at 4°C and

peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) in 5% milk TBS-T for one hour at room temperature. Blots were incubated in chemiluminescent substrate (Thermo Scientific Pittsburg, PA) and exposed to film. The optical density of bands was calculated after background subtraction using UN-SCAN-IT gel analysis software (Silk Scientific Inc. Orem, UT). All bands were normalized to the interblot control band, then to actin and expressed as mean optical density mean ± SEM.

Antibody Information.

Immunostaining was performed with antibodies specific to the following proteins: mouse monoclonal GAD67 (MAB5406, Millipore Billerica, MA) at a dilution of 1:500 for fluorescence, 1:1000 for western; mouse monoclonal Calb (C9848, Sigma) at a dilution of 1:2000 for fluorescence, 1:5000 for western; rabbit polyclonal PV (PV25, Swant) at a dilution of 1:500 for fluorescence; mouse monoclonal PV (Millipore) at a dilution of 1:500 for western; and goat polyclonal Huntingtin (SC-8767, Santa Cruz) at a dilution of 1:100 for fluorescence.

Stereology

Animals were anesthetized with isofluorane and perfused intracardially with room temperature 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed for an additional 24-72 hours. The samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C. Tissue blocks were sectioned at 30 µm, mounted onto charged slides (Fisher) and allowed to dry overnight before storage at -80°C. Slides were then thawed

and washed with distilled H_2O for 5 minutes followed by a 10 minute incubation in hematoxylin (Sigma). Slides were incubated in running H_2O for 15 minutes followed by a 1 minute incubation in eosin (Sigma). Slides were then dehydrated and cover-slipped using Permount (Fisher).

Cells were counted by an unbiased investigator blinded to animal genotype using StereoInvestigator (MicroBrightField, Inc Williston, VT). Pilot animals were used to determine parameters needed to give optimal counting precision. The optical fractionator method was used to generate an estimate of PCs counted in an unbiased selection of serial sections in a defined volume of the cerebellum. Serially cut 30 µm thick sagittal tissue sections (every twentieth section) were analyzed throughout the entire cerebellum of animals in each cohort (n=3/genotype). The number of PCs within the entire cerebellum was quantified using the optical fractionator method with dissectors placed randomly according to a 263.68 x 212.13 µm grid. The counting frame was 60 x 60 µm and the dissector height was 26 µm. On average, 150 sites were counted per animal. An estimation of cell population number was provided using overall raw counts and mean section thickness.

Cell Attached Loose Patch Recording.

Mice (4 week old) were anesthetized with isoflourane and sacrificed by decapitation. Brains were placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$ 1.25 Na_2HPO_4 and 25 D-glucose with a pH of 7.4 and osmolality of 295 ± 5 mOsm. The ringer solution was bubbled with 95%O_2/5%CO_2. Sagittal cerebellar slices (300 µm thick) were cut using a Vibratome (7000 smz, Campden Instruments: Sarasota, FL). The slices were

allowed to rest for 60 min at room temperature (22–23°C); all experiments were then performed at room temperature. Slices were superfused continuously with oxygenated recording ACSF at room temperature. Slices were viewed with an upright microscope (Zeiss Axio Examiner A1) using infrared-differential interference contrast optics. Loose patch recordings were acquired from visually identified PC using Axio Vision 4.8 software. Cellular activity was recorded using internal solution containing the following (in mM) 140 K-gluconate, 1 EGTA, 10 HEPES and 5 KCL, pH 7.3. Pipette tip resistance was 2-5 MΩ. The extracellular recording pipettes, containing the internal solution, were placed under visual control directly adjacent to the soma of the cell of interest. Positive pressure was applied throughout this process followed by a brief release of pressure to form a seal averaging approximately 45 MΩ. Cell-attached, loose patch-clamp recordings were obtained using an Axon CNS Molecular Devices amplifier (Multiclamp 700B), filtered at 10 kHz and digitized at 20 kHz (Digidata 1440A). A 2 min gap-free protocol on Clampex 10.2 software was used for data collection. Detection and analysis of event frequency and inter-event interval were performed semi-automatically using the program Clampfit 10.2. The detection threshold was set for analysis based on the event amplitude from a given cell.

Data Analyses.

Data analyses for qRT-PCR, Western Blot assays and loose patch electrophysiology were performed using Microsoft Excel. A two-tailed student t-test assuming unequal variance was utilized to assess statistical significance. Values were considered statistically significant when the p value was less than 0.05. For the measurements of transcripts at different stages of the disease, R6/2 values were

normalized to WT values at each independent age, so t-tests were used; thus, the asterisks ($p<0.05$) indicate when R6/2 values were significantly different from values for WTs of the same age.

For the open field behavior data, a two-way ANOVA (Age X Genotype) was performed using PASW 18.0. For post hoc analysis Fisher's least significant difference (LSD) procedure was utilized. Values were considered statistically significant when the p value was less than 0.05.

For the rotarod behavior data, analyses were performed using PASW 18.0. The raw time values measuring latency to fall were binned according to 33rd percentiles (i.e., 0-12.99, 13.00-59.00, and 59.01-60 seconds) for the maximum speed trial (32 rpm). A G-likelihood test was performed on the binned data (binned Proportions were considered statistically significant when the *p* values were less than 0.05.

For the stereological data, a two-way ANOVA (Age X Genotype) was performed using PASW 18.0. For post hoc analysis Duncan's multiple comparison procedure was utilized. Values were considered statistically significant when the p value was less than 0.05.

Results

R6/2 mice exhibit an overt motor phenotype at midlife

In order to validate previous reports of the onset and progression of motor symptoms in the R6/2 mouse, we utilized the open field and rotarod behavior paradigms. The open field analysis revealed a hypoactive phenotype which continues throughout the lifespan of these animals (n = 8/group) (**fig 2.1A**). A two-way ANOVA revealed significant main

effects of genotype and age ($F(1,36) = 16.1$ and $F(2,36) = 11.2$, respectively; $p < 0.01$ for both) but no interaction ($F(2,36) = 2.1$, $p = 0.14$). The rotarod paradigm, which more directly tests balance and motor coordination, revealed coordination dysfunction at 32 rpm at twelve weeks of age (n = 8/group; $\chi^2(2) = 15.02$ $p<0.01$) (**fig 2.1B**). Additionally, there significant differences between WT and R6/2 in the proportion of animals that fell at speeds of 28, 24, and 20 rpm, $\chi^2(2) = 22.18$, 22.18, and 8.00, respectively ($p < 0.05$ for all). There was no difference between WT and R6/2 at 16 rpm at 12 weeks of age ($\chi^2(2) = 2.86$, $p = 0.24$). There were no differences between the two groups at six weeks of age (data not shown) in concordance with previously published data (Mangiarini, Sathasivam et al. 1996).

GABAergic cell markers are decreased in the cerebellum of R6/2 mice around the time of motor onset

To initially determine whether alterations in PCs occur in the R6/2 model, we performed qRT-PCR to evaluate the levels of transcripts that are highly concentrated in PCs. Transcript levels were normalized to the internal control gene, actin, and then reported as fold WT (WT = 1.0). There were no changes in actin between R6/2 and WT mice at any age. There was a decrease in the level of GAD67 ($p < 0.05$) and Calb ($p < 0.05$) transcript at seven and twelve weeks of age (n = 9/group, compared to age-matched WT littermates) and a reduction in PV transcript ($p < 0.05$) by twelve weeks of age (**fig 2.1C**; n = 9/group). Student's t tests were utilized to assess alterations at individual ages since R6/2 values were only compared to values from WT mice of the same age. We then evaluated these changes in expression at the protein level in cerebellum homogenates;

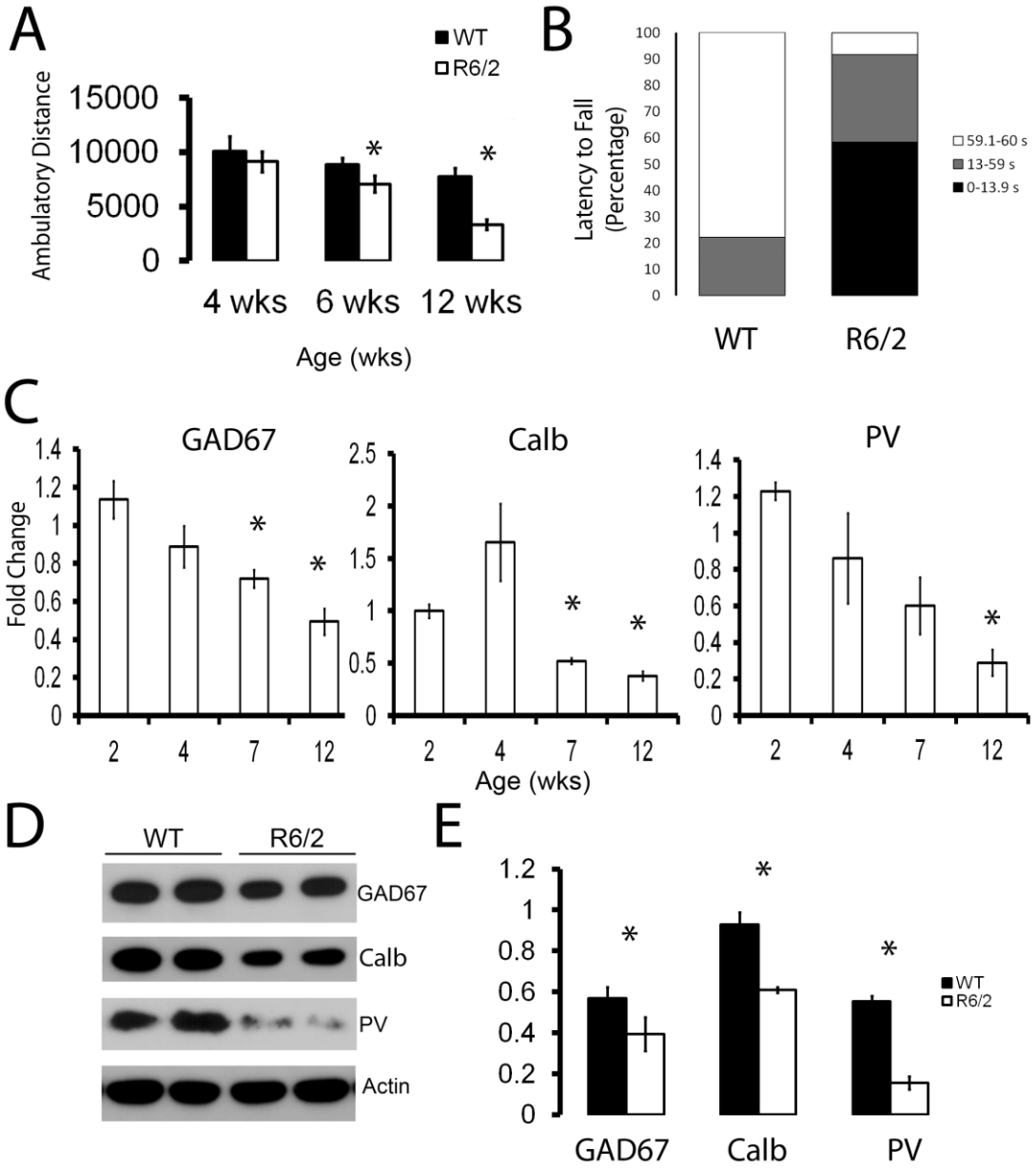

A
WT
R6/2
Ambulatory Distance
15000
10000
5000
0
4 wks
6 wks
12 wks
Age (wks)
B
Latency to Fall (Percentage)
59.1-60 s
13-59 s
0-13.9 s
WT
R6/2
C
GAD67
Calb
PV
Fold Change
Age (wks)
D
WT
R6/2
GAD67
Calb
PV
Actin
E
WT
R6/2
GAD67
Calb
PV

Figure 2.1. R6/2 mice exhibit reductions in Purkinje cell (PC) marker expression around the age of motor symptom onset.

Motor impairments were evident by reduced ambulatory distance in the open field paradigm (**A**) and a deficit in rotarod performance at 32 rpm at 12 weeks of age. Rotarod data is shown as percentage of animals per bin with the highest bin (59.1-60) indicative of the most successful run of a full 60 seconds (**B**). Significant deficits were also seen at 20, 24, and 28 rpm speeds while no difference was observed between groups at 6 weeks of age (data not shown). **C**. Using q-RT-PCR to measure transcript levels on cerebellar homogenates, expression of Purkinje markers was reduced in R6/2 mice, with GAD67 and Calb reduced by 7 weeks and PV reduced by 12 weeks (n=9/group at 7 and 12 weeks, n=5/group at 2 and 4 weeks). Values for mRNA levels were normalized to actin and then compared to WT (fold =1). **D**. Western blot analysis revealed a decrease in protein level of GAD67, Calb and PV at 12 weeks of age. (n=7 per/group). **E**. Quantification of Western blot. Two-way ANOVA tests were performed on the behavioral data. Two-tailed student's t-tests were performed on transcription and Western blot data with significance established as compared to age-matched WT. Error bars: SEM. (*$p < 0.05$).

protein analysis revealed significant decreases in GAD67, PV, and Calb by twelve weeks of age in the R6/2 mice as compared to WT littermates (n = 7/group, two-tailed student's t test, $p < 0.05$; **fig 2.1D & E**). To localize the protein changes to specific cell types, immunohistochemistry was performed; immunoreactivity for GAD67 (**fig 2.2 C&D**), Calb (**fig 2.2 G&H**) and PV (**fig 2.2 K&L**) was reduced markedly in the PC layer of R6/2 cerebellum. Strikingly, the interneurons of the molecular layer retained their basal levels of PV (**fig 2.2L**, arrows). These staining patterns suggest alterations specifically in the PCs within the cerebellum of the R6/2 mice. At four weeks of age, there is no reduction in GAD67, Calb, or PV immunostaining in the R6/2 as compared to WT (**fig 2.2 A&B, E&F and I&J**, respectively).

R6/2 mice exhibit a loss of Purkinje cells by end stage

One explanation for the loss of PC markers could be a reduction in the total cell number in the R6/2 mice. In order to investigate whether decreases in protein and transcript levels stem from localized molecular loss within intact cells or from a loss of PC bodies, we performed stereological investigation on R6/2 mice and WT littermates. Stereological counts revealed no significant decrease in PC number at four weeks of age in the R6/2 mice (**fig 2.3A&B**). A population estimate using mean section thickness gave an estimate of an average of 84,886 cells in 4 week WT and 107,961 cells in 4 week R6/2 animals. There is a reduction in PC number at twelve weeks of age, decreased 55% as compared to WT littermates (**fig 2.3 C&D**). A population estimate using mean section thickness gave an estimate of an average of 97,208 cells in 12-week WT and 43,667 cells in 12-week R6/2 animals. A two-way ANOVA revealed no significant main effects of age or genotype, $F(1,8) = 1.57$ ($p = 0.25$) and $F(1,8) = 4.57$ ($p = 0.07$), respectively. However,

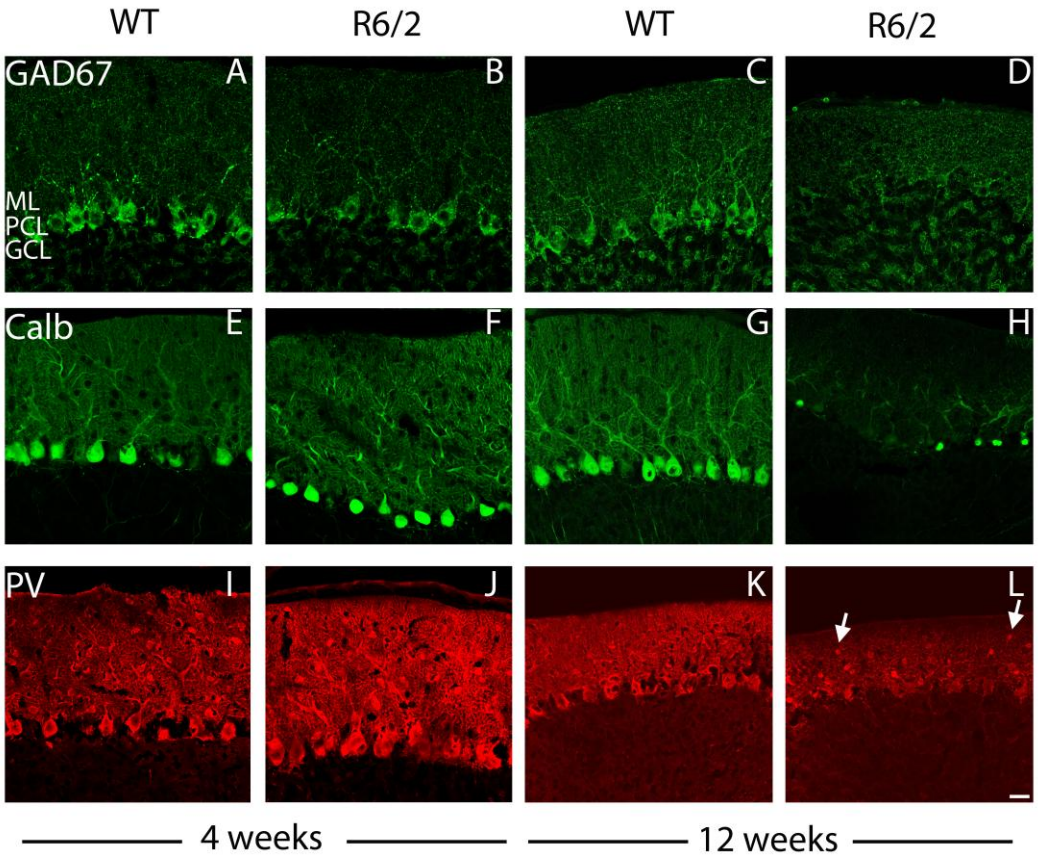


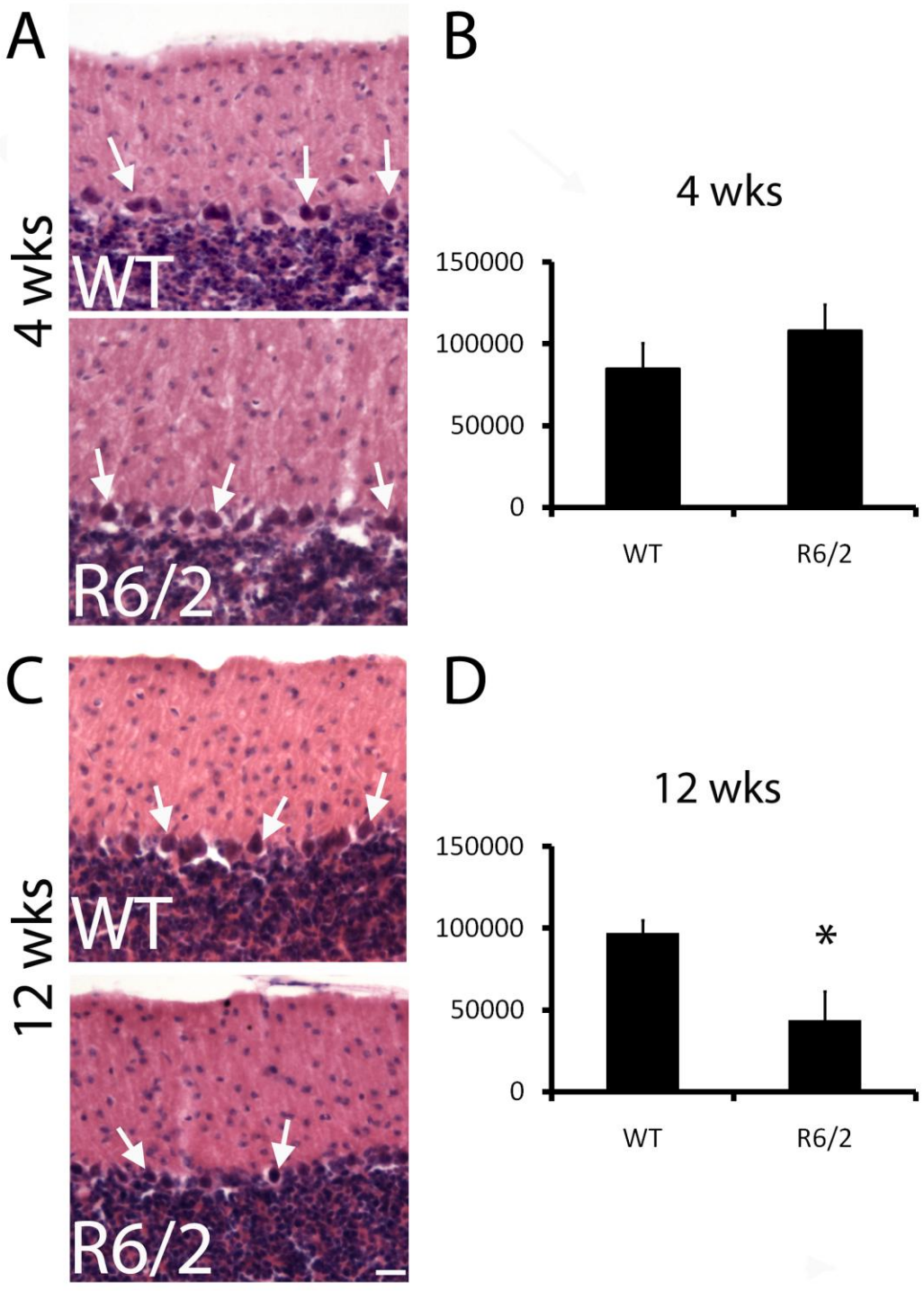
Figure 2.2. Immunoreactivity for GAD67, calbindin (Calb) and parvalbumin (PV) is reduced in the Purkinje cell (PC) layer of the cerebellum at 12 weeks of age but unaltered at 4 weeks of age.

A-D. Immunofluorescence with an antibody specific for GAD67 reveals a loss of staining in the PC layer of 12 week old R6/2 animals (**D**) as compared to 12 week old WT (**C**). There is no difference between groups at 4 weeks of age (**A&B**). **E-H**. An antibody specific for Calb shows a decrease in protein levels throughout the molecular and PC layers of 12-week-old R6/2 animals as compared to WT (**H**). There is no difference between groups at 4 weeks of age (**E&F**). **I-L**. Changes, similar to those seen in GAD67, are seen with an antibody specific to PV. Staining shows a decrease in protein levels in the PC layer of 12-week-old R6/2 animals as compared to WT (**L**). Staining is clearly retained in the interneurons of the molecular layer (arrows in **L**). ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Scale bar, 25 µm.

there was a significant Age X Genotype interaction, $F(1,8) = 9.92$, $p < 0.05$ (n = 3 animals/group). Duncan's multiple comparison procedure showed that the R6/2 12-week old animals had a mean cell count that was significantly reduced from the other three group's mean cell counts. The lack of a difference in cell number between WT and R6/2 mice at 4 weeks of age suggests that the production and migration of these cells is not affected during development.

Purkinje cells exhibit a reduction in firing rate prior to motor symptom onset and transcriptional changes in R6/2 mice

The motor abnormalities observed in the R6/2 mouse model of HD arise prior to an overt loss of PCs in the cerebellum. In order to elucidate the possible role of early alterations in cellular function in this cell population, cell-attached loose-patch recordings were performed in presymptomatic animals (4 weeks of age). Using an acute slice preparation, overall spike rate was recorded from 4-week-old R6/2 mice and WT littermates. Slices were incubated in ACSF and, once patched, allowed to acclimate to the pipette tip for three minutes in order to eliminate any events in response to mechanical stimulation. Spontaneous events were then recorded for three minutes with no stimulation. Cells with fewer than five spikes within the recording interval were excluded. Loose patch recordings revealed a significant reduction in the spike frequency in the R6/2 mice as compared to WT littermates at four weeks of age, prior to the onset of motor symptoms (n = 4 for R6/2 and n = 5 for WT with 15 and 17 cells, respectively; two-tailed student t test, $p < 0.05$; **fig 2.4 A&B**).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WT
R6/2

Figure 2.3. Cell counts reveal neuronal loss in the Purkinje cell (PC) layer of 12-week-old R6/2 mice.

A. Hematoxylin and eosin staining was performed on 30 micron thick cerebellar sections from WT and R6/2 mice. Representative pictures are shown. Staining reveals uniform cell distribution in the cerebellum with no significant differences in stereological counts of PC between WT and R6/2 animals at 4 weeks of age, quantification in **B**. **C**. At 12 weeks there is a significant reduction in the number of PCs by 55%, quantification in **D**. In **C**. representative pictures are shown of 12-week-old animals. Arrows indicate PCs. n = 3/group. Two-way ANOVA, *$p < 0.05$. Error bars represent SEM. Scale bar, 20 μm. n = 3/group at each age tested.

Intranuclear inclusions are not evident in Purkinje cells at the time of electrophysiological deficits

To investigate the pathogenic mechanisms behind the change in firing rate, we questioned whether huntingtin inclusions were present within PCs at the time of electrophysiological deficits. Immunofluorescence using a mthtt-specific antibody revealed the presence of mthtt within the molecular and granule cell layers of the R6/2 cerebellum (**fig 2.5B** arrows) while staining was entirely absent in 12-week-old WT (**fig 2.5 A**). Colocalization of mthtt-positive inclusions with PV (green, **fig. 2.5B**, inset) in the molecular layer of 4-week-old R6/2 mice indicated that molecular layer inclusions were concentrated in molecule layer interneurons**.** Intriguingly, similar staining patterns were observed at 7 weeks of age, though there were more pronounced inclusions in the interneurons (enlarged in fig 2.5E) with few inclusions noted in PCs (**fig 2.5C** arrows). By 12 weeks of age the R6/2 mice show inclusions in most cells throughout the layers of the cerebellum including pronounced inclusions within the PC layer (**fig 2.5D**, enlarged in **2. 5F**).

Discussion

Huntington Disease is a debilitating neurological disorder. The R6/2 mouse is a severe HD model with an early onset of symptoms and an abbreviated lifespan (Mangiarini, Sathasivam et al. 1996). We show that decreases in PC-specific transcript and protein levels occur as part of the disease etiology in the R6/2 mouse model of HD. Furthermore, we demonstrate that PCs are lost during the course of the disease in R6/2 mice and that prior to degeneration- and overt motor symptom onset, PCs are

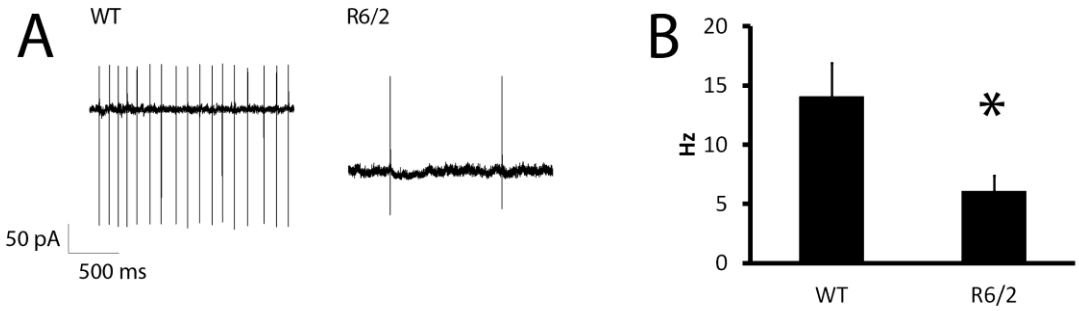
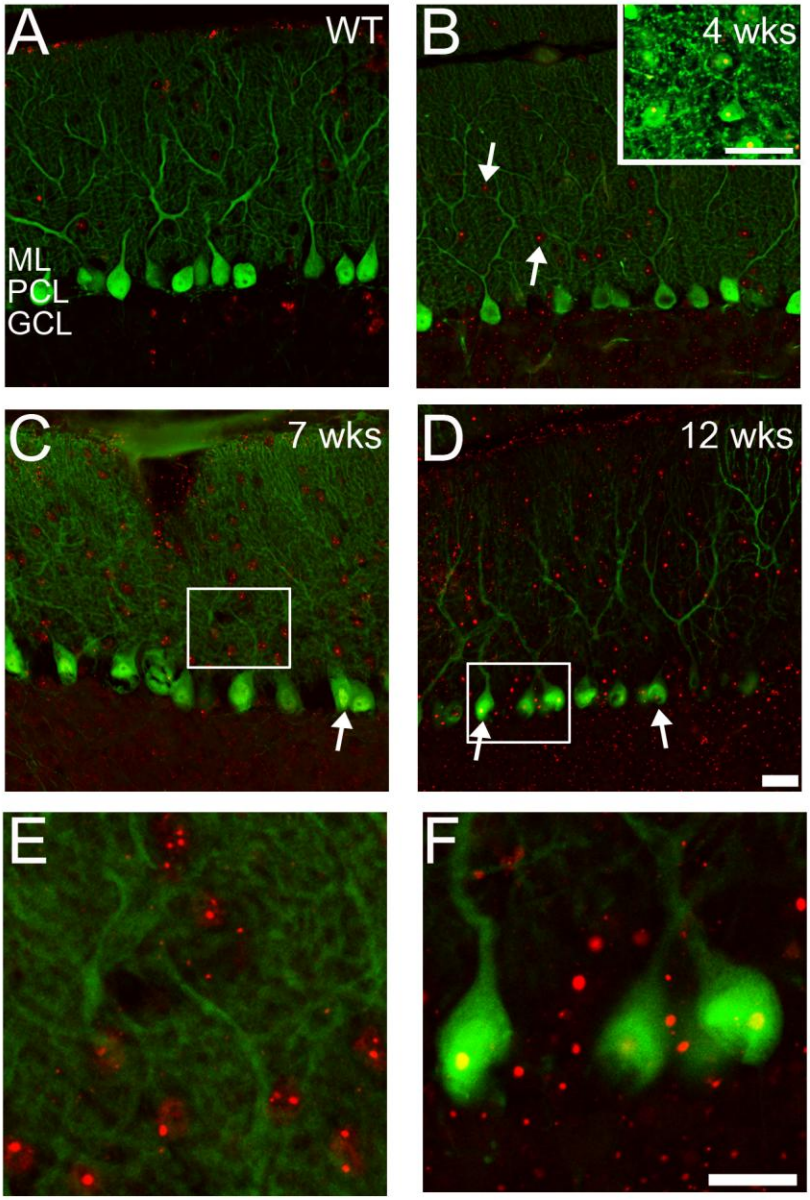


Figure 2.4. *Purkinje cell spike rate is reduced in presymptomatic R6/2 mice.*

A, *Using* presymptomatic R6/2 and age-matched WT mice, loose patch recordings were performed on acute cerebellar slices (300 micron thick); sample traces shown. Recordings lasted for 3 minutes and were then analyzed using clampex software; quantification in **B**. There was a significant difference between event number (spike rate) and interevent interval (data not shown) between the WT and HD mice (n= 5 and 4 animals for WT and R6/2 respectively; *$p < 0.05$). Two-tailed student's t-tests were performed with error bars representing SEM.


A
WT
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12 wks
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F

Figure 2.5. Huntingtin inclusions are present in the Purkinje cells (PCs) of 12 week R6/2 mice but found in interneurons at a younger age.

In order to localize mthtt inclusions to specific cell types in these animals, immunohistochemistry was performed with an antibody specific to mthtt (in red) and calbindin for visualization of the cerebellar layers (in green). In 4-week-old R6/2 animals, mthtt staining is seen in the molecular (arrowheads) and granule cell layers of the cerebellum but not in the PC layer (**B**). (**INSET)** Colocalization of the inclusion pathology in 4-week-old R6/2 with parvalbumin (in green) reveals that these aggregates are found within the molecule layer interneurons. Inclusions are absent in 12-week-old WT animals (**A**). **C**. 7 week old R6/2 mice exhibit staining throughout the cerebellum, including in a few PC (arrows). Inclusions are seen in the interneurons of the molecular layer, enlarged in **E**. In 12 week old R6/2 animals, intranuclear inclusions are seen as punctuate staining throughout the PC layer (arrows) and cerebellum (**D**); representative PCs are highlighted in **F** ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Scale bar (**D**), 25 µm. Scale bar (**F**), 5 µm.

at a physiological level, with a dramatic reduction in firing rate. This demonstration of presymptomatic functional deficits in PCs of R6/2 mice, prior to the appearance of intranuclear inclusions, is novel and suggests that alterations in cerebellar function may contribute to the onset of motor symptoms in this model.

Initial investigations of transcript levels in the cerebellum indicate a loss of the enzyme GAD67 and the calcium-binding proteins PV and Calb by end-stage in the disease time course. Immunohistological staining revealed that these proteins were lost specifically from the PC layer. While a previous report argued for a role in transcriptional downregulations of PV in the cerebellum (Luthi-Carter, Hanson et al. 2002), the observed loss of PC number associated with transcriptional and protein level changes suggest that these changes are due to a loss of PCs, not transcriptional dysregulation. However, while we did not directly investigate this possibility, reductions in transcript/protein levels in the remaining cells could contribute to alterations in calcium homeostasis and signaling (Llano, DiPolo et al. 1994; Schwaller, Meyer et al. 2002), which has already been noted in other brain regions in HD (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011).

That such cell loss is occurring in the cerebellum is striking as ataxia is not a common finding in this model (Mangiarini, Sathasivam et al. 1996), and PC dysfunction has been shown to cause ataxia-like motor deficits (Walter, Alvina et al. 2006). While ataxia may not be a key symptom of HD, alterations in gait and balance abnormalities have been observed in both young (5 weeks of age) and end stage mice (Carter, Lione et al. 1999; Luesse, Schiefer et al. 2001; Chiang, Chen et al. 2010), in addition to symptoms of dystonia (Mangiarini, Sathasivam et al. 1996; Stack, Kubilus et al. 2005; Stack,

Dedeoglu et al. 2007). There are also some reports of ataxia and gait disturbances in HD patients (Koller and Trimble 1985; Grimbergen, Knol et al. 2008), although it is possible that more overt manifestations of ataxia are masked by the traditional motor symptoms. Recent work has suggested that disruption of Purkinje cell function by mutations in the α3 isoform of the sodium-potassium ATP-pump cause the dystonia seen in a Dystonia-Parkinsonism mouse model through aberrant interactions with the basal ganglia (Calderon, Fremont et al. 2011). It is intriguing to postulate that the cerebellum is playing a similar role in the R6/2 model to influence basal ganglia function.

Previously it has been shown that cells of the deep cerebellar nuclei precisely encode spike timing of synchronous PC firing (Person and Raman 2011). Reductions in GABAergic output from the inhibitory PC could lead to over-excitation in these deep cerebellar nuclei and propagation of misfired cellular signals. Additionally, alterations in spiking patterns could contribute to dysfunctional motor output as the cerebellum forms disynaptic connections with the basal ganglia through the thalamus (Bostan, Dum et al. 2010; Bostan and Strick 2010). Interestingly, reports have shown that PC spiking activity is associated with altered levels of GAD67 mRNA (Litwak, Mercugliano et al. 1990; Drengler and Oltmans 1993); it follows that reductions in firing rate could lead to reductions in the mRNA and protein levels that we observed. Further, as PC have been shown to be particularly dependent on activity for survival (Morrison and Mason 1998), it is possible that a reduction in overall PC activity could contribute to cell death.

To determine what mechanisms could underlie PC loss, we investigated alterations in cellular function and cell-specific accumulation of huntingtin prior to cell loss and the onset of overt motor symptoms. At 4 weeks of age, there was a marked

reduction in firing rate in R6/2 mice as compared to the WT. Intriguingly, these firing abnormalities occurred without evidence for huntingtin positive inclusions in PCs. Therefore, the observed reductions in firing rate could reflect cell-autonomous effects of soluble mthtt on membrane properties or, since mthtt staining was readily observed in molecular layer interneurons, non-cell autonomous effects of interneuron dysfunction (Garden, Libby et al. 2002). Notably, we observed relative preservation of the molecular layer interneurons, so it is possible that alterations in interneuron function could influence PC firing (increased inhibition). A similar pattern of cell loss (loss of PCs, preservation of interneurons) has been shown previously in a model of spinocerebellar ataxia-1 (Vig, Subramony et al. 1998). Regarding the toxicity of huntingtin, both soluble and aggregated huntingtin have the potential to be toxic (Lajoie and Snapp 2010; Weiss, Kimura et al. 2011); the preservations of the interneurons through the disease time course supports previous reports suggesting the intranuclear inclusions may be protective and not causative in cell death (Kuemmerle, Gutekunst et al. 1999; Arrasate, Mitra et al. 2004).

As previously stated, the R6/2 mouse model is a severe HD mouse model that may more closely replicate a juvenile-onset (JOHD) disease progression (Sawiak, Wood et al. 2009). The most severe cases of HD are JOHD and have the longest recorded repeat length. Intriguingly, case studies have shown that JOHD presents not only with the cardinal features of adult onset HD (rigidity, chorea, dementia) but also additional symptoms including a higher occurrence of seizure activity (Hattori, Takao et al. 1984; Ruocco, Lopes-Cendes et al. 2006). In JOHD, there are multiple reports of cerebellar involvement, atrophy and dysfunction, including fMRI studies showing an incidence of

cerebellar atrophy in these most severe patients (Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). Therefore, it is possible that these results may not extend to adult-onset HD mouse models; experiments with HD adult-onset mouse models are required to address this issue.

Our study indicates that the PC population is particularly vulnerable in the R6/2 mouse model. Further experiments are required to determine the extent to which different cell types contribute to PC dysfunction and cell loss, with the goal of designing approaches to rescue this cell population and improve motor function in HD.

Acknowledgments

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CHAPTER II: B

PURKINJE CELL DYSFUNCTION AND LOSS IN A KNOCK-IN MOUSE MODEL OF HUNTINGTON DISEASE

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Introduction

Huntington Disease (HD) is an autosomal-dominantly inherited neurodegenerative disorder characterized by a triad of symptoms including motor, cognitive, and psychiatric disturbances (Group 1993). Expression of the pathogenic protein, mutant huntingtin (htt), is ubiquitous throughout the brain and has been suggested to be involved in a number of aberrant processes including transcriptional dysregulation (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009), disruption of calcium signaling (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), and alterations of synaptic physiology (Klapstein, Fisher et al. 2001; Morton, Faull et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009).

Studies have shown that the motor circuits of the basal ganglia are severely affected in this disease (Roos, Pruyt et al. 1985; Reiner, Albin et al. 1988; Andre, Cepeda et al. 2011) though there is evidence for morphological alterations in the cerebellum, including alterations in Purkinje cells (PCs), the main output neuron of the cerebellar cortex (Jeste, Barban et al. 1984; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004) in both patients (Rodda 1981; Jeste, Barban et al. 1984) and animal

models of HD (Turmaine, Raza et al. 2000). Additionally, in an animal model of HD, deficits have been found in the levels of the neurotransmitter GABA in PCs (Reynolds, Dalton et al. 1999). Recent work using single cell RT-PCR has shown that there are transcriptional alterations specifically within PCs in an animal model of HD (Euler, Friedrich et al. 2012). However, there is little information regarding the extent to which PCs are lost in mouse models of HD and whether PC function is compromised.

We recently provided evidence for PC abnormalities in the R6/2 model of HD (Dougherty, Reeves et al. 2012); changes include a reduction in transcript and protein levels of GABAergic cell population components (glutamic acid decarboxylase 67 and parvalbumin) and a specific purkinje cell marker (calbindin). Furthermore, we found that PC loss was preceded by a significant reduction in PC spike rate. Taken together these data provide evidence that PC dysfunction is an important neuropathological phenotypic component of the R6/2 mouse model of HD.

Of note, the transgenic R6/2 line expresses an exon 1 fragment of mutant htt, resulting in a HD mouse model that displays an early disease onset and aggressive phenotype (Mangiarini, Sathasivam et al. 1996), so it is not clear whether the PC abnormalities we observed would be relevant for the understanding of adult-onset HD. Thus, we sought to determine whether PC loss and dysfunction are a part of the pathophysiology of HD in a mouse model of adult-onset HD. The knock-in HdhQ200 is particularly useful for this kind of study, as it expresses mutant htt in an appropriate genomic and protein context and recapitulates midlife onset and key progressive behavioral and pathological features of HD (Lin, Tallaksen-Greene et al. 2001). These include well-characterized progressive, measurable, and disease-relevant behavioral

deficits and neurological abnormalities (Heng, Tallaksen-Greene et al. 2007; Heng, Duong et al. 2010) and, notably, robust striatal neuronal intranuclear inclusions by 20 weeks of age, a progressively deteriorating motor phenotype beginning at 50 weeks of age, and gross motor impairment present by 80 weeks of age (Heng, Duong et al. 2010). Previous studies have shown that a similar knock-in mouse (HdhQ150) develops a phenotype reminiscent of that seen in the commonly used R6/2 mouse model of HD albeit on a different time scale (Woodman, Butler et al. 2007). Additional reports have illustrated similarities in disease pathology in these mouse models including: aggregate formation (Sathasivam, Lane et al. 2010), motor abnormalities (Heng, Tallaksen-Greene et al. 2007), and physiological alterations in synaptic molecules (Heng, Detloff et al. 2009). It remains important to characterize similarities between these models as it is the knock-in model that seems to hold the most physiological relevance for the adult onset form of HD. Further, as the production of these knock-in mice involved a two-step gene targeting approach, only the size of the CAG repeat length differentiates the WT mouse from its knock-in littermates (Lin, Tallaksen-Greene et al. 2001).

In order to investigate whether PC survival and function are compromised in the HdhQ200 model, we performed molecular and electrophysiological characterizations of the alterations within the cerebellum. Here we show a reduction in transcript and protein levels of a specific PC marker (calbindin) and a PC-specific decrease in glutamic acid decarboxylase 67 and parvalbumin in symptomatic HdhQ200/Q200 mice. We also present data showing a reduction in PC number by over 50%, mirroring PC loss in the R6/2 mouse. Additionally, we see a reduction in spike rate in remaining PCs in symptomatic mice. These data indicate that PCs are vulnerable in the HdhQ200/Q200

knock-in model of HD and that this mouse model would be useful in determining the factors that contribute to PC loss and dysfunction in adult HD.

Methods

Animals.

The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all experimental protocols. The HdhQ200 mice were maintained as described previously (Heng, Duong et al. 2010). Mice were bred using heterozygous HdhQ100 or HdhQ200 mating pairs in order to yield homozygous, heterozygous and wildtype (WT) mice. 50-week-old male and female mice were utilized for all experiments. These mice were housed in groups of up to 5 animals per cage with food and water *ad libitum*

Genotyping

Genotyping was performed as described in (Heng, Tallaksen-Greene et al. 2007). Briefly, DNA was isolated from tail samples using DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR reactions utilized a Taq polymerase kit (Invitrogen, Carlsbad, CA) and primers (Invitrogen) specific to the CAG repeat in the *Hdh* locus. PCR products were visualized using agarose gel electrophoresis with ethidium bromide and read on a Gel Doc system (Bio-Rad, Hercules, CA). Periodically, tail samples were sent to Laragen (Laragen Inc., Los Angeles, CA, USA) for CAG sizing to confirm CAG stability and consistency of the CAG repeat number.

Gene Expression Analysis.

Protocols were performed as described (Lucas, Markwardt et al. 2010). Briefly, mice were anesthetized with isoflourane and sacrificed by decapitation. Brains were removed and dissected by anatomical region. Tissues were flash frozen on dry ice and kept in the -80°C freezer until use. At least 12 hours prior to processing, tissues were placed in RNA*later*-ICE (Ambion Austin, TX), cooled to -80°C, and kept at -20°C until homogenization. Homogenization was performed with a Tissue-Tearor homogenizer (Biospec Bartlesville, OK) in Trizol following the manufacturer's instructions (Invitrogen Grand Island, NY). Taqman PCR was conducted with JumpStart Taq Readymix (Sigma St. Louis, MO) and Applied Biosystems (Carlsbad, California) primers for calbindin (Mm00486645_m1) and β-actin (Mm00607939_s1). Actin values were not different between groups ($p>0.05$).

Immunofluorescence.

Mice were anesthetized with isofluorane and perfused intracardially with ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed for an additional 24-72 hours. The samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C until use. Tissue blocks were sectioned at 30 μm, and sections were mounted onto charged slides (Fisher) and allowed to dry overnight before storage at -80°C. Slides were then thawed and washed with PBS followed by a one hour incubation in 10% serum

(from the host of the secondary antibody) and PBS with 3% bovine serum albumin (BSA). The sections were then incubated overnight with a predetermined concentration of primary antibody and 5% serum in 0.3% TritonX PBS with 3% BSA. This was followed by washing and then a 2 hour incubation with the corresponding fluorescence-conjugated secondary antibody (Jackson ImmunoResearch West Grove, PA) and 5% serum in 0.3% TritonX PBS with 3% BSA. The slides were then immediately mounted using an antifade media containing DAPI, coverslipped and left to dry at room temperature overnight. When necessary, a Vector (Burlingame, CA) Mouse on Mouse Kit (Fluorescein Cat No. FMK-2201) was used to minimize background staining for experiments involving mouse-made antibodies. Immunofluorescence was performed on 3-5 slides per mouse with 4 brain sections per slide. For imaging, intensity settings were set for clear visualization of PCs in the WT mice. The images provided were collected on a Leica confocal microscope and are representative or overall changes observed throughout experimental sections.

Primary Antibody Information.

Immunostaining was performed with the following antibodies: mouse anti-GAD67 (MAB5406, Millipore Billerica, MA) at a dilution of 1:500; mouse anti-Calb (C9848, Sigma) at a dilution of 1:2000; rabbit polyclonal anti-PV (PV25, Swant) at a dilution of 1:500; and goat polyclonal anti-mutant huntingtin (SC-8767, Santa Cruz) at a dilution of 1:100.

Stereology

Mice were anesthetized with isofluorane and perfused intracardially with ice-cold 4% paraformaldehyde in PBS. Brains and sections were prepared as described above for immunofluorescence. Sections were thawed and washed with distilled H_2O for 5 minutes followed by a 10 minute incubation in hematoxylin (Sigma). Slides were washed in running H_2O for 15 minutes followed by a 1 minute incubation in eosin (Sigma). Slides were then progressively dehydrated and cover-slipped using Permount (Fisher).

Cells were counted by an unbiased investigator blinded to animal genotype using StereoInvestigator (MicroBrightField, Inc Williston, VT). The optical fractionator method was used to generate an estimate of PCs counted in an unbiased selection of serial sections in a defined volume of the cerebellum. 30 µm thick sagittal tissue sections were serially cut with analysis performed on every twentieth section throughout the entire cerebellum of mice in each cohort (n=3/genotype). The number of PCs within the entire region was attained using the optical fractionator method with dissectors placed randomly according to a 263.68 x 212.13 µm grid. The dissector height was 26 µm and the counting frame was 60 x 60 µm. On average, 150 sites were counted per mouse. An estimation of cell population number was provided using overall raw counts and mean section thickness.

Cell-Attached Loose Patch Recording.

Mice were anesthetized with isoflourane and sacrificed by decapitation. Brains were placed in ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$ 1.25 Na_2HPO_4 and 25 D-glucose with a pH of 7.4 and osmolality of 295 ± 5 mOsm. This external solution was

bubbled with 95%O_2/5%CO_2. Sagittal cerebellar slices (300 µm thick) were cut using a Vibratome (7000 smz, Campden Instruments: Sarasota, FL). The resultant slices were allowed to rest for 60 min at room temperature (22–23°C); all experiments were then performed at room temperature. Slices were superfused continuously with oxygenated recording ACSF and viewed with an upright microscope (Zeiss Axio Examiner A1) using infrared-differential interference contrast optics. Loose patch recordings were acquired from visually-identified PCs using Axio Vision 4.8 software. Cellular activity was recorded using internal solution containing the following (in mM): 140 K-gluconate, 1 EGTA, 10 HEPES and 5 KCL, pH 7.3. Pipette tip resistance was 2-5 MΩ. The extracellular recording pipettes, containing the internal solution, were placed directly adjacent to the soma of the cell of interest. Positive pressure was applied throughout this process followed by a brief release of pressure to form a seal averaging approximately 45 MΩ. Recordings were obtained using an Axon CNS Molecular Devices amplifier (Multiclamp 700B), digitized at 20 kHz (Digidata 1440A), and filtered at 10 kHz. A 3 min gap-free protocol on Clampex 10.2 software was used for data collection. Event detection and analysis of event frequency and inter-event interval were performed semi-automatically using the program Clampfit 10.2. The detection threshold was set for analysis based on the event amplitude from a given cell.

Data Analyses.

Data analyses for qRT-PCR, stereology, and loose patch electrophysiology were performed using Microsoft Excel and GraphPad Prism software. One-way ANOVAs were performed followed by post-hoc Tukey's multiple comparison tests were utilized to

assess statistical significance. Values were considered statistically significant when the p value was less than 0.05.

Results

Calbindin mRNA and protein is reduced in a knock-in model of HD

To determine whether PC loss was a possibility in the HdhQ(n) models of HD, we first determined whether there were alterations in transcript levels of the PC marker and calcium buffer calbindin (Calb) in mice homozygous for the mutant htt gene and with either 100 CAG (HdhQ100/Q100) or 200 CAG (HdhQ200/Q200) (n=10/group) at 50 weeks of age. There was a decrease in Calb transcript in the HdhQ200/Q200 mice as compared to WT littermate controls (**fig 2.6**; $p<0.05$). Additionally, there was a trend towards a reduction in the levels of Calb transcript in HdhQ100/Q100 compared to WT littermate controls ($p<0.1$). Assessment of haploid expression of the HdhQ200 mutant htt transgene revealed that heterozygotic expression resulted in a reduction in Calb (**fig 2.6**) from WT levels with no statistical difference between HdhQ200/+ and HdhQ200/Q200 mice. Considering that no alterations in Calb expression were observed in the HdhQ100/Q100, we chose to focus on the characterization of HdhQ200/Q200 and heterozygous (HdhQ200/+) mice as compared to age-matched WT mice.

To determine whether Calb expression was reduced at the protein level, we performed immunofluorescence on WT and HdhQ200/Q200 mice with an antibody against Calb. Staining patterns revealed a reduction in staining intensity throughout the purkinje and molecular layers (**fig 2.7A&B**). To determine whether this could be due to

a loss of the PC bodies themselves, we evaluated the intensity of staining for two other proteins concentrated in PCs: glutamic acid decarboxylase (GAD67) (**fig 2.7 C&D**) and parvalbumin (PV) (**fig 2.7 E&F**). There was a marked reduction in staining intensity of these proteins in the HdhQ200/Q200 mice as compared to WT mice for both GAD67 and PV. These decreases were especially pronounced in the PC layer. Interestingly, while PC bodies were still visible with the Calb and GAD67 immunofluorescence, PV-positive cell bodies in the PC layer were almost non-existent, suggesting that PV is reduced within intact cell bodies. Additionally, as evidenced by the PV staining, interneurons of the molecular layer were still present (arrows; **fig 2.7F**). This staining pattern is strikingly similar to the one we previously observed in the R6/2 model (Dougherty, Reeves et al. 2012) as well as to the pattern reported in a model of spinocerebellar ataxia-1 (Vig, Subramony et al. 1998). Importantly, at this age, mutant htt inclusions were present within PCs (**fig. 2.8**) of HdhQ200/Q200 mice.

Homozygous HdhQ200 mice exhibit a reduction in PC number at 50 weeks of age

The loss of multiple PC markers in HdhQ200/Q200 mice suggested that the number of PCs may be reduced in this mouse model. In order to investigate whether alterations in PC-specific proteins were the result of reductions in cell numbers, we performed unbiased stereological counts on Hemotaxylin & Eosin-stained serial sections of the cerebellum (n=3/group). Counts revealed a significant reduction in the overall PC population in 50 week old HdhQ200/Q200 mice as compared to age-matched WT controls (**fig 2.9**; $p<0.05$), while PC number was not significantly reduced in the heterozygous HdhQ200/+ mice. Assessment of the population number using mean

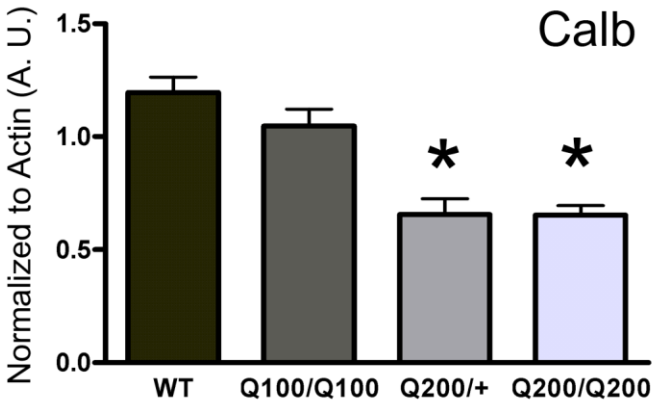

Figure 2.6. HD mice exhibit reductions in calbindin mRNA expression.

Using q-RT-PCR to measure transcript levels in cerebellum homogenates, expression of the purkinje marker calbindin (Calb) was significantly reduced in homozygous HdhQ200/Q200 mice at 50 weeks (n = 10/group, $p<0.05$). There was a trend towards reduction in the transcript level of Calb in homozygous HdhQ100/Q100 mice when compared to WT controls (n= 10/group; $p<0.1$). Assessment of haploid expression of the mutant htt transgene revealed that heterozygous expression resulted in a reduction in Calb from WT levels with no statistical difference between HdhQ200/+ and HdhQ200/Q200 mice. One-way ANOVA was performed followed by post-hoc Tukey's Multiple Comparison Tests with error bars representing SEM.

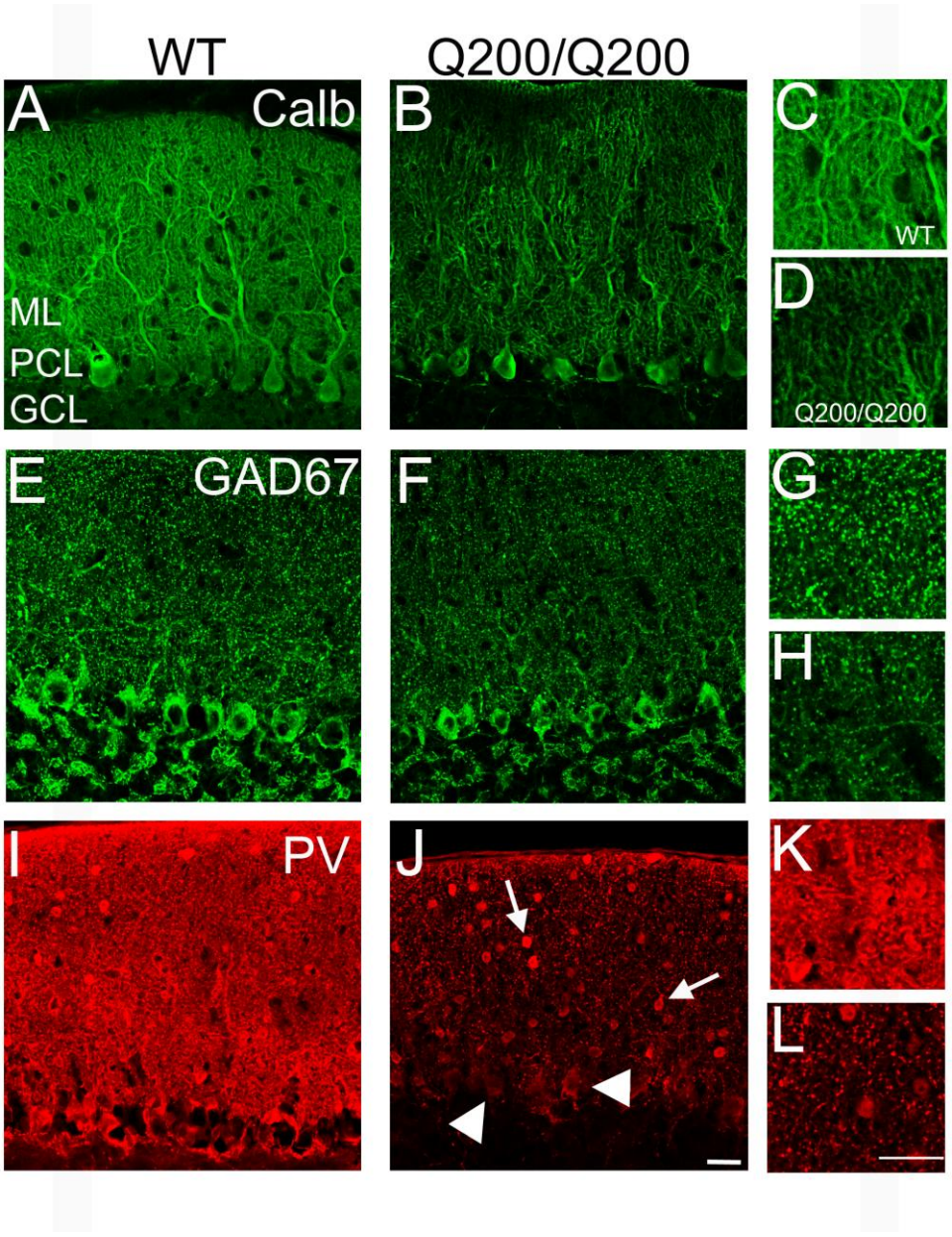

Figure 2.7. Immunoreactivity for calbindin (Calb), GAD67, and parvalbumin (PV) is reduced in the Purkinje cell (PC) layer of the cerebellum of 50 week-old HdhQ200/Q200 mice.

In order to assess protein level changes in Calb, immunofluorescence was performed on HdhQ200/Q200. Staining revealed reductions of the protein levels of Calb (**A&B**) in HdhQ200/Q200 mice as compared to WT. Reductions in other GABAergic cell markers, GAD67 (**C&D**) and PV (**E&F**), were also evident in the PC layer as compared at WT. As exhibited by the intense staining pattern, the interneurons of the molecular layer remain PV+, arrows in **F**. ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Representative images shown. (n=3 animals/group) Scale bar: 25 µm.

section thickness gave an estimation of an average of 103018 cells in WT, 73559 cells in HdhQ200/+ and 64315 cells in HdhQ200/Q200 mice (38% reduction in PC number in HdhQ200/Q200 as compared to WT) (**fig. 2.9B**).

Spike Frequency is reduced from the PCs of 50 week old homozygous HdhQ200 mice

As it is possible that the remaining PCs could compensate on a functional level for the loss of PCs, we next sought to determine whether the functional activities of the remaining PCs were altered in the HdhQ200 mice. Given that a change in firing frequency was an early robust measure of PC dysfunction in the R6/2 mice (Dougherty, Reeves et al. 2012), cell-attached loose patch electrophysiology was performed in 50 week old HdhQ200/Q200 and HdhQ200/+ mice (n=3-6 mice/group; 10-12 cells/group). Loose patch recordings revealed a significant reduction in spike frequency in the HdhQ200/Q200 mice as compared to age-matched WT controls (**fig 2.10**; $p<0.05$), while there was no significant difference in firing rate in the HdhQ200/+ mice as compared to age-matched WT controls.

Discussion

HD is a devastating neurological disorder characterized by cognitive, psychiatric and motor dysfunction. The motor component, termed chorea, along with the early observation of overt cell loss in the striatum, has previously led to a focus on the motor pathways of the basal ganglia as the source of this dysfunction (Lange, Thorner et al. 1976; Vonsattel, Myers et al. 1985). However, emerging evidence suggests that the main

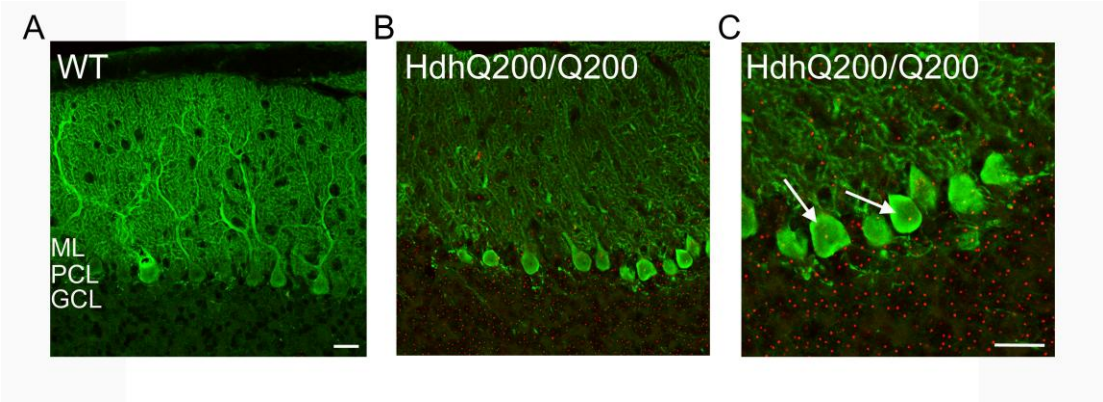

Figure 2.8. Huntingtin-positive inclusions are found in the PCs of HdhQ200 mice.

In order to localize mutant htt inclusions to specific cell types in these mice, immunofluorescence was performed with an antibody specific to mutant htt (in red) and calbindin (in green) for visualization of the PC layer. Very limited huntingtin staining was seen in the age matched WT (**A**). 50 week- old HdhQ200/Q200 mice exhibit staining throughout the cerebellum (**B**). Inclusions are seen in the interneurons of the molecular layer and throughout the PC layer (arrows in **C**). ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Representative images shown. (n=3 animals/group) Scale bars: 25 µm

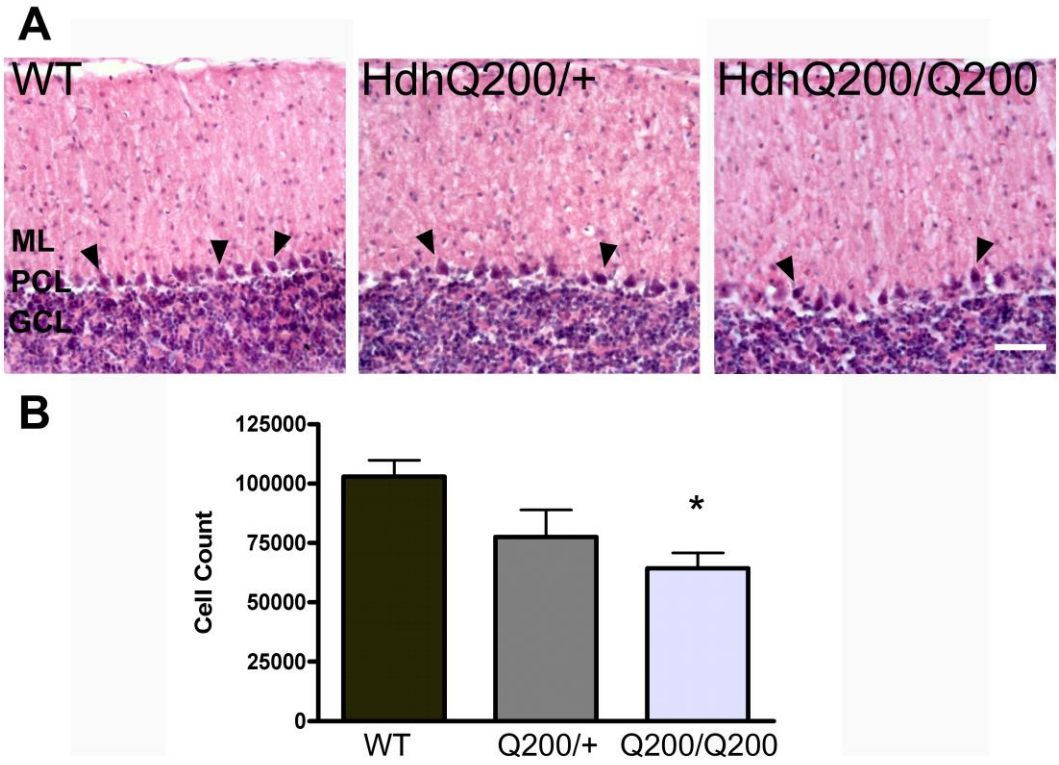

Figure 2.9. HDhQ200 mice exhibit reductions in PC number.

A, Hematoxylin and Eosin staining was performed on 30 micron thick cerebellar sections from WT, HdhQ200/+, and HdhQ200/Q200 mice. Representative pictures are shown; arrowheads highlight positively stained PCs. Stereological analysis reveal a significant reduction in the number of PCs between WT and HdhQ200/Q200 mice at 50 weeks of age, quantification in **B** (n=3/group; *$p<0.05$). One-way ANOVA was performed followed by post-hoc Tukey's Multiple Comparison Tests with error bars representing SEM. Scale bar: 100 µm.

output neuron of the cerebellar cortex, the Purkinje Cell (PC), is dysfunctional in early onset HD. A recent study from our laboratory found that PCs are lost in the R6/2 model of HD (Dougherty, Reeves et al. 2012); in light of postmortem data indicating that signs of cell death are observed in PCs (Turmaine, Raza et al. 2000) and PC numbers are reduced in HD (Jeste, Barban et al. 1984), we hypothesized that PC number, and potentially function, would also be affected in mouse models of adult-onset HD.

We show that decreases in PC-specific transcript and protein levels occur as part of the disease etiology in the HdhQ200 mouse model of HD. Furthermore, we demonstrate that PCs are lost in symptomatic mice and that PCs are dysfunctional at a physiological level, with a dramatic reduction in firing rate. This demonstration of molecular and functional deficits in PCs directly mirrors what was observed in the severe R6/2 line that displays an early onset and aggressive phenotype. Importantly, these findings are novel and suggest that alterations in cerebellar function may contribute to the motor symptoms characteristic of this adult onset HD model.

Within the field, early studies suggested a role for the cerebellum in HD as shown by cerebellar atrophy in both patients and mouse models (Kageyama, Yamamoto et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). However, due to striatal atrophy and the marked loss of medium spiny neurons observed in HD patients and animal models, there has been a focus on the striatum in many studies of HD etiology. One major reason that the cerebellum is overlooked may be the lack of an overt cerebellum-associated motor phenotype. However, some work has shown that ataxia is

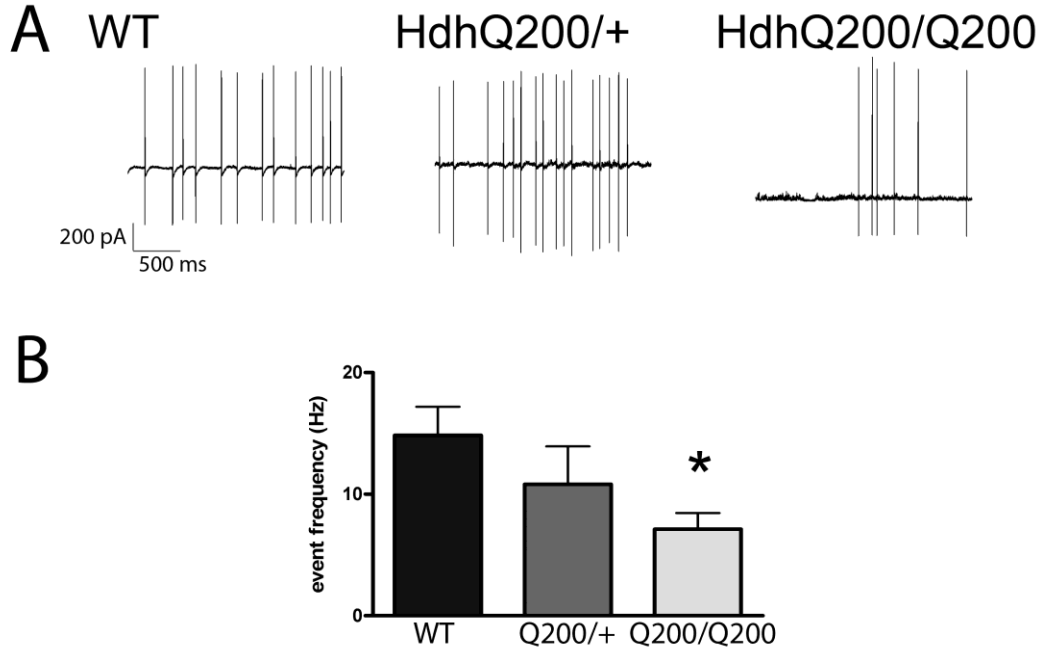

Figure 2.10. Decreased firing frequency from PCs is observed in HdhQ200 mice.

A. Using 50 week-old HdhQ200/+, HdhQ200/Q200 mice and age matched WT controls, loose patch recordings were performed on acute cerebellar slices (300 micron thick); sample traces are shown. Recordings lasted for 3 minutes and were then analyzed using clampex software, with quantification in **B**. There was a significant decrease in event number and interevent interval (data not shown) between the WT and HdhQ200/Q200 mice (*$p<0.05$). There was no significant difference between HdhQ200/+ and either group, though a trend was present between each ($p<0.1$; n= 3 animals and 10 cells/group). One-way ANOVA was performed followed by post-hoc Tukey's Multiple Comparison Tests with error bars representing SEM.

present as part of the motor pathology (Koller and Trimble 1985). Further, it has been suggested that an ataxia-like dysfunction may be masked by the more obvious striatal motor pathologies. The vast majority of studies performed on the cerebellum explore alterations at the regional level. One caveat to this approach is that cerebellar neuronal populations are very diverse and, as a result, whole homogenate studies could dilute effects occurring in restricted cell populations. Recent PC single-cell transcription studies have shown mirroring reductions in mRNA levels of a transcription factor essential for PC development in HD and spinocerebellar ataxia type 1 (Serra, Duvick et al. 2006; Euler, Friedrich et al. 2012).

In addition PC loss, reductions in the levels of the calcium buffer PV within intact cells would be expected to have major implications for the overall function of PCs. Studies have suggested that mature PCs lack functional NMDA receptors (Farrant and Cull-Candy 1991; Llano, Leresche et al. 1991) and therefore rely heavily on Ca^{2+} influx through voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores. As a potent calcium buffer, PV is an essential cytosolic moderator of excitotoxicity in the PCs. Loss of this buffer has been shown to result in an alteration in PC firing (Servais, Bearzatto et al. 2005) and a concomitant dysfunction in locomotor behavior (Farre-Castany, Schwaller et al. 2007). The reduction in molecules observed in the HdhQ200/+ suggests that haploid expression of the mutant htt allele is sufficient to cause the transcriptional alterations; though with the population reduction only trending towards a difference from WT, haploid mutant htt expression is not sufficient to cause the loss of cells or functional abnormalities.

The functional consequence of a reduction of PC firing, while not investigated here, could lead to alterations in firing from the deep cerebellar nuclei. Alterations in PC firing have been shown to result in propagation of misfired signals to the downstream cerebellar nuclei in other CAG repeat disorders involving the cerebellum (reviewed in (Shakkottai and Paulson 2009). Importantly, PCs have been shown to be particularly dependent on activity for survival (Morrison and Mason 1998); it is possible that a reduction in overall PC activity could contribute to the observed alteration in cell viability. While it is intriguing to postulate that alterations in PC number and function contribute to the motor deficits seen in the HdhQ200/Q200 model, further characterization of the pathological timeline would be necessary to support that conclusion.

Taken together, these data highlight the vulnerability of the PC population in a mouse model of adult-onset HD. Further investigation into the cell-autonomous versus non cell-autonomous effects of mutant htt on PC function and viability would increase our understanding of the role of PC dysfunction in the motor phenotype in HD with the ultimate goal of improving motor function and quality of life in people with HD.

Acknowledgments

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CHAPTER III

RESTRICTED EXPRESSION OF MUTANT HUNTINGTIN TO PARVALBUMIN-POSITIVE CELLS CAUSES HYPERACTIVITY AND ALTERED SYNAPTIC FUNCTION IN THE MOTOR CORTEX

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Introduction

Huntington Disease (HD) is a devastating neurological disorder characterized by motor, psychiatric, and cognitive disturbances. HD is caused by an aberrant expansion of the CAG repeat domain (glutamine coding) within exon one of the huntingtin (htt) gene (Group 1993). On the cellular level, HD is characterized by alterations in various cellular functions including the maintenance of calcium homeostasis (Panov, Gutekunst et al. 2002; Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), synaptic physiology (Klapstein, Fisher et al. 2001; Morton, Faull et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009), and transcriptional regulation (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009).

Though the mutant protein is ubiquitously expressed, specific neuronal populations are especially vulnerable to the toxic effects of mutant huntingtin (mthtt). Within the striatum, a brain region prominently affected in HD, the medium spiny projection neurons undergo severe neurodegeneration while some regional populations of interneurons are relatively spared (Hodgson, Agopyan et al. 1999; Shelbourne, Keller-

McGandy et al. 2007). Multiple studies have indicated that areas of the cerebral cortex are affected as well (Hedreen, Peyser et al. 1991; Gu, Li et al. 2005; Spampanato, Gu et al. 2008; Nopoulos, Aylward et al. 2010; Wolf, Sambataro et al. 2012; Gray, Egan et al. 2013; Shin, Kim et al. 2013) and that reduced trophic support from the cortex may contribute to medium spiny neuron vulnerability (Zuccato, Ciammola et al. 2001). Interestingly, cell-specific expression of mthtt in all neurons is sufficient to cause hypoactivity and cortical alterations, while expression only in pyramidal neurons has no impact on behavior or cortical dysfunction, leading investigators to hypothesize that a component of cortical pathology requires the involvement of interneurons (Gu, Li et al. 2005). In support of this hypothesis, early alterations in excitatory input onto parvalbumin (PV)-positive interneurons are observed in the BACHD mouse model, implicating involvement of this subpopulation in the development of symptoms (Spampanato, Gu et al. 2008).

PV+ interneurons are critical in synchronizing the output of pyramidal neurons (Perney, Marshall et al. 1992; Du, Zhang et al. 1996; Yeung, Thompson et al. 2005), with the activation of PV+ interneurons alone being sufficient to drive cortical oscillations (Sohal, Zhang et al. 2009). Synchronization is disrupted in a number of psychiatric and neurodegenerative disorders (Hutchison, Dostrovsky et al. 2004; Cunningham, Hunt et al. 2006; Gonzalez-Burgos and Lewis 2008; Lewis, Hashimoto et al. 2008; Lodge, Behrens et al. 2009), including HD (Thiruvady, Georgiou-Karistianis et al. 2007; Walker, Miller et al. 2008), making it critical to elucidate the contribution of PV+ interneuron dysfunction to the pathogenesis of HD.

To investigate the role of the PV+ subclass of GABAergic neurons in HD-associated motor and synaptic dysfunction, we utilized a cre/lox system of conditional gene expression (Gu, Li et al. 2005). We bred mice with expression of a floxed stop codon preceding exon one of the human mutant htt gene to mice with a PV promoter-driven cre recombinase enzyme. The resultant mice had expression of the mutant htt gene only in PV+ cells. Here we report that this mouse model exhibited a hyperactive phenotype at twelve months of age, including alterations in both spontaneous and evoked inhibitory transmission in the motor cortex. Furthermore, the sequestration of mthtt into inclusions in PV+ processes at 24 months of age was associated with normalization in locomotor behavior and evoked GABA release. Taken together, these data suggest that the mthtt-mediated dysfunction of the PV+ subclass of interneurons in the motor cortex may be sufficient to drive hyperactivity in HD and that the formation of mthtt inclusions may protect cortical circuitry from soluble mthtt-mediated dysfunction.

Methods

Animals.

The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all experimental protocols. The "PVcre" and "Rosa" mouse line was obtained from Jackson Laboratories and maintained through breeding to WT hybrid (B6CBA) animals. The "Rosa" strain was B6.129-*Gt(ROSA)26Sor*$^{tm1(HD*103Q)Xwy}$/J, order number 007708**.** The "PVcre" was B6; 129P2-*Pvalb*$^{tm1(cre)Arbr}$/J, order number 008069. All experiments were conducted with both male and female animals at all ages. The "Rosa" mice carried a repeat length of 103Q. For all experiments, PVcre littermate mice were used as controls.

The R6/2 mouse line was obtained from Jackson Laboratories and maintained through breeding male F1 generation offspring to WT hybrid (B6CBA) females (B6CBA-Tg (HDexon1)62Gpb/3J). The mice were obtained from Jackson Laboratories by way of an ovarian transplant female with ovaries from a sexually unviable R6/2 +/- female. All experiments were conducted with both male and female animals at 12 weeks of age. R6/2 mice carried a repeat length of 167 as genotyped by Laragen, Inc (Culver City, CA). All animals were housed in groups of up to 5 animals per cage with food and water *ad libitum.*

Behavioral Assessment.

Behavioral analyses were conducted on littermates at six (n=10/group), eight (n=10/group), ten (n=10/group), twelve (n=15/group), and twenty-four months of age (n=8/group) during the lights on period (6am – 6pm). All experiments were conducted blind to the genotype of the animals.

Rotarod.

The rotarod apparatus (MedAssociates, St. Albins, VT) consisted of a 5-station treadmill with a computer-controlled stepper motor-driven rod with constant speed or accelerating speed modes of operation. Animals were trained on the rotarod for four days. On the first day of the training period, animals were placed on the treadmill at an accelerating speed of 2-24 rotations per minute (rpm) for a maximum of 60 seconds and a total of 5 trials. On the second through fourth days, the animals were placed on the treadmill at a constant speed of 16 rpm for a maximum of 60 seconds and a total of 5

trials per day. On the fifth day, animals underwent two trials each at rotating speeds 16, 20, 24, 28, and 32 fixed rpm. Each trial lasted for a maximum of 60 seconds, during which latencies to fall were recorded. Mice were allowed to rest for at least five minutes between each trial.

Open field.

Animals were placed in a square apparatus (27.3 cm x 27.3 cm x 20.3 cm) consisting of 48 infrared beams (MedAssociates) for 30 minutes. Data were collected with Open Field Activity Software (MedAssociates) in one-minute intervals over the test period, and total ambulatory time and distance were determined.

Immunofluorescence.

Animals were anesthetized with isoflurane and perfused intracardially with ice cold 4% paraformaldehyde in phosphate buffered saline (PBS). Animal numbers for all immunostaining experiments were: 6/group at 12 months, 3/group at 18 months, and 8/group at 24 months of age. Brains were removed and postfixed for an additional 24-72 hours. For chromogenic staining of mounted sections, the samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C. Tissue blocks were sectioned at 30 µm, mounted onto charged slides (Fisher Scientific; Pittsburg, PA) and allowed to dry overnight before storage at -80°C. Slides were then thawed and washed with PBS followed by a one hr incubation in 10% serum (from the host of the 2° antibody) and PBS with 3% bovine serum albumin (BSA).

The slides were then incubated overnight with a predetermined concentration of 1° antibody and 5% serum in 0.3% Triton X PBS with 3% BSA. This was followed by washing and then a 2 hr incubation with the corresponding fluorescence-conjugated 2° antibody (Jackson ImmunoResearch; West Grove, PA) and 5% serum in 0.3% TritonX PBS with 3% BSA. The slides were then immediately mounted using an antifade media containing DAPI, coverslipped and left to dry at room temperature overnight. When necessary, a Vector (Burlingame, CA) Mouse on Mouse Kit (Fluorescein Cat No. FMK-2201) was used to minimize background staining for mouse-made antibodies. Nuclear localization was established with Topro3 nuclear stain (Invitrogen). For floating sections, the samples were drop frozen in 2-methylbutane and stored at -80°C. Brains were then sectioned on a sliding microtome (Leica Microsystems; Buffalo Grove, IL) at 40 microns and stored in a sterile 50% glycerol/PBS buffer. For use, slices were incubated for 30 minutes in 0.3% H_2O_2 in methanol followed by a 30-minute incubation in citrate buffer (for antigen retrieval). Slides were then incubated for 48 hrs in a predetermined concentration of 1° antibody and 5% serum in 0.3% Triton X PBS with 3% BSA. This is followed by washing and then a 24 hr incubation with the corresponding fluorescence-conjugated 2° antibody and 5% serum in 0.3% TritonX PBS with 3% BSA. Finally, slices are mounted onto slides and coverslipped using an antifade media containing DAPI. Negative controls included either absence of primary antibody or species-matched IgG in the place of the primary antibody. Representative images were captured on a Leica confocal microscope and processed with Adobe Photoshop CS3; adjustments in contrast and brightness were applied to images where appropriate. In many cases, fluorophores were chosen based on maximal separation (FITC and CY5), but when rhodamine and

CY5 channels were used, emission filters were set to <615 nm for rhodamine to limit potential leak through of strong CY5 signals into the rhodamine channel; likewise, with that combination of secondary antibodies, the CY5 emission range was restricted to 640-700 nm to limit any contribution from strong rhodamine intensity.

Antibody Information.

Immunostaining was performed with these antibodies: mouse anti-GAD67 (MAB-5406, Millipore Billerica, MA) at a dilution of 1:500 for fluorescence; mouse anti-Calb (C-9848, Sigma) at a dilution of 1:2000 for fluorescence; rabbit polyclonal anti-PV (PV25, Swant) at a dilution of 1:500 for fluorescence; mouse anti-huntingtin (MAB-5492, Chemicon) at 1:5000 for fluorescence, rabbit anti-huntingtin at 1:500 for fluorescence (Chun, Lesort et al. 2001; Lin, Tallaksen-Greene et al. 2001), and goat polyclonal huntingtin (SC-8767, Santa Cruz) at a dilution of 1:200 for DAB and 1:100 for fluorescence.

Whole Cell Recordings.

Mice were anesthetized with isoflurane and then killed by decapitation (n=3-5 animals and 10-12 cells per condition). Brains were placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 3.5 KCl, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 26 $NaHCO_3$ and 10 D-glucose. The saline was bubbled with 95%O_2/5%CO_2. Coronal brain slices (300 µm thick) were cut using a Vibratome (Ted Pella, Inc., Riverside, CA). The slices were kept for 30 minutes at 37±1°C and then stored at room temperature (22±1°C). Slices were superfused continuously with oxygenated recording ACSF at room

temperature. Whole-cell patch clamp recordings were acquired from visually identified pyramidal neurons in layer five of the motor cortex. Position in cortex was verified with strepavidin staining for biocytin filled cells. Recordings were conducted on a Zeiss upright microscope (Carl Zeiss, Thornwood, NY). Cells were voltage clamped at -70 mV, using internal solution containing the following (in mM) 129 CsCl, 2 MgATP, 10 EGTA, 10 HEPES, 0.2 GTP and 2 QX-314, pH 7.2. Pipette tip resistance was 2-5 MΩ. Voltage clamp recordings were obtained using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) controlled by Clampex 8.0 software via a Digidata 1322A interface (Molecular Devices), filtered at 5 kHz and digitized at 10 kHz. Input resistance and series resistance were monitored by applying a 10 mV voltage step. Spontaneous and evoked IPSCs were pharmacologically isolated with CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM) and D-APV (DL-2-amino-5-phosphonoovaleric acid, 50 μM). Spontaneous effects were then further isolated with TTX (tetrodetoxin,1 μM). Analysis of spontaneous IPSCs (sIPSCs) was performed using Clampfit 8.0 software, which focused on amplitude, interevent interval and decay time of events. All sIPSCs that fit the template and passed visual inspection were inspected in the analysis. To calculate the paired-pulse ratio, the amplitude of the second IPSC was measured relative to the baseline set immediately before the second stimulus, and normalized to the amplitude of the first IPSC relative to the baseline set immediately before the first stimulus. CNQX, APV and TTX were purchased from Sigma-Aldrich and diluted from stock solutions to final concentrations in ACSF.

Stimulation.

Synaptic responses were evoked with a bipolar stimulating electrode consisting of a twisted pair of 25 μm Formvar insulated nichrome wires. The electrode was positioned in layer 5 of the motor cortex. A series of paired stimulations at 20, 30 and 100 ms intervals were applied to elucidate pair-pulse ratios. For 66 Hz train recordings, 34 stimulations were applied over 500 ms to evoke a response in patched pyramidal cells. Recordings were taken at 32±1°C.

Data Analyses.

Data analyses were performed using GraphPad Prism software. A two-tailed student t-test was utilized to assess statistical significance when two groups were compared. For the measurements of multiple groups, one-way ANOVAs were performed followed by post hoc Tukey's Multiple Comparisons test. For longitudinal behavioral analysis repeated measures two-way ANOVA tests were performed. Values were considered statistically significant when the p value was less than 0.05.

Results

Immunoreactive Mutant Huntingtin-Positive Inclusions in the Cortex of Mice with Conditional Expression of mthtt.

To determine how PV+ neuron dysfunction contributes to HD-related behavioral and synaptic changes, we generated mice with mthtt expression restricted to PV+ cell populations. In order to verify the expression of the mthtt protein in the expected cell populations, we first performed immunohistochemical studies using antibodies against

mthtt. Using chromogenic detection, mthtt immunoreactivity was seen throughout the cortical layers of conditional HD mice at 12 months of age, both in a diffuse, cytosol-filling form (arrows) as well as in the punctate form (arrowheads), suggesting the presence of inclusion bodies in restricted cell populations in the cortex of our model (**fig 3.1A-C**). The greatest intensity of staining was visible in the cortex, with little to no staining in other regions with PV+ neurons.

To confirm this staining pattern and colocalize mthtt with PV immunoreactivity, we used double-labeling immunofluorescence in floating sections from 12-month old mice. The most evident pattern of immunoreactivity with this approach was the inclusions, since sections from this age show cytosolic autofluorescence, even with autofluorescence eliminator reagents (not shown). Using double-labeling immunostaining, we observed that the mthtt-positive inclusions invariantly colocalized with GAD67 (marker for inhibitory cell terminals; **fig 3.1D**) and PV (**fig 3.1E**). Further, longitudinal characterizations of these inclusions using immunofluorescence revealed an increase in the presence of the mthtt+ inclusions with age, with the greatest occurrence at 24 months of age (**fig 3.1F**). Interestingly, punctate mthtt immunoreactivity was never observed within the nucleus, but was concentrated in PV+ processes, some of which were MAP2+ (**fig 3.1G**) and others that were contacting the perisomatic regions of pyramidal neurons (**fig 3.1E**) indicating localization of inclusions to both dendritic and axonal terminals.

To determine whether the localization of mthtt to PV+ processes is observed in other HD models, we investigated whether the same pattern exists in the R6/2 model of HD (Mangiarini, Sathasivam et al. 1996). We found that mthtt-positive inclusions are

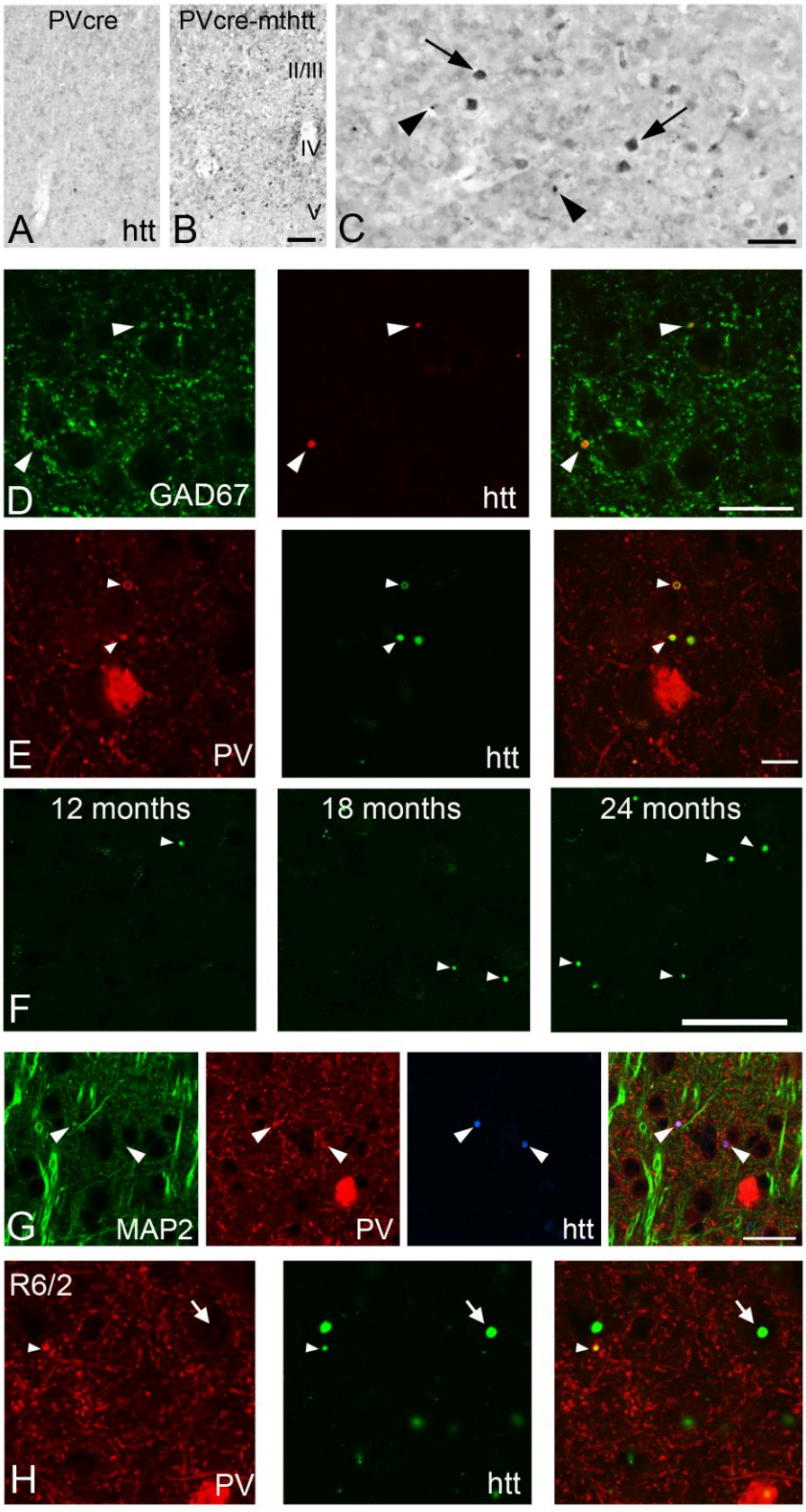

PVcre
PVcre-mthtt
II/III
IV
V
A
htt
B
C
D
GAD67
htt
E
PV
htt
12 months
18 months
24 months
F
G
MAP2
PV
htt
R6/2
H
PV
htt

Figure 3.1. Immunoreactivity of Mutant Huntingtin (mthtt) Colocalizes with Parvalbumin in the Cortex of Mice with Conditional Expression of mthtt.

Using DAB immunostaining for mthtt, positive staining is detected throughout the motor and somatosensory cortex of conditional HD mice at 12 months of age (**A-C**). These positively stained cortical cell populations exhibit both diffuse cytoplasmic staining patterns (arrows) as well as inclusion type punctuate staining (arrowheads, enlarged in **C**). This staining is absent in the control PVcre littermate (**A**). Mthtt-positive staining colocalizes with GAD67 (**D**) and varicosities along the processes of PV+ cells (E; arrowheads) in the cortex. Mthtt+ inclusion density increases with age from 12 months to 24 months of age (**F**). Mthtt is found in MAP2+ cell processes (**G**). Similar aggregate staining is seen in the R6/2 mouse model at 12 weeks of age (arrowheads, **H**), with numerous intranuclear inclusions being negative for PV immunoreactivity, as expected (arrows, **H**). Scale bars B: 250 µm, C: 100 µm, D: 25 µm, E: 10 µm, F&G= 25 µm. Representative images were selected from 3-8 animals per group at each age.

abundant in all cellular nuclei, including PV+ neuronal nuclei, but are also found in PV+ processes (**fig 3.1G, arrowhead**). In addition to demonstrating that non-nuclear PV+ inclusions occur in the R6/2 mice, the lack of PV-immunoreactivity overlap with mthtt in numerous other cells demonstrates that leak-through from the mthtt fluorescence channel was not contributing to the overlap observed in our colocalization experiments. Interestingly, the highest prevalence of inclusions was noted in the cortex, with no inclusions detected in the striatum. Results were confirmed with three different mthtt antibodies from three different host species; all showed the same staining pattern (data not shown), and no staining was observed when species-matched IgG was used in place of the primary antibody.

Conditional HD Mice Exhibit Hyperactivity at 12 months of age

To determine whether the expression of mthtt in PV+ cell populations was sufficient to cause motor dysfunction, we evaluated locomotor activity and coordination using open field analysis and rotarod testing, respectively. Behavior was evaluated at 4, 6, 8, 10, and 12 months of age. Longitudinal analysis of motor behavior in conditional HD mice revealed hyperactivity at 10 and 12 months of age as compared to PVcre littermates (**fig 3.2A**). Further investigation of motor performance at 12 months revealed an increase in total time spent jumping and walking as well as a reduction in time spent at rest (**fig 3.2B**), indicating a generalized increase in activity. However, rotarod analysis at 12 months of age revealed no significant difference in the latency to fall between genotypes at all speeds tested (**fig 3.2C**), demonstrating that gross motor coordination was not affected. Ambulatory counts for other control groups (PVcre-negative ROSA-

positive and complete wild type mice) were not different from PVcre ROSA-negative mice (not shown). mthtt fluorescence channel was not contributing to the overlap observed in our colocalization experiments. Interestingly, the highest prevalence of inclusions was noted in the cortex, with no inclusions detected in the striatum. Results were confirmed with three different mthtt antibodies from three different host species; all showed the same staining pattern (data not shown), and no staining was observed when species-matched IgG was used in place of the primary antibody.

PV+ Cell Population Expression of mthtt is Sufficient to Alter GABA Release in the Motor Cortex.

Considering the high expression of mthtt in cortical PV+ neurons, we hypothesized that cortical inhibition would be altered in these mice. To test this hypothesis, we used whole cell patch clamp electrophysiology to isolate inhibitory postsynaptic potentials on layer V pyramidal neurons in the motor cortex. Initial investigations revealed alterations in basal GABA release as evidenced by increases in both amplitude and frequency of sIPSC (**fig 3.3 A-C**). Further, with bath application of TTX, miniature IPSCs (mIPSCs) exhibited an increase in average event amplitude and a reduction in frequency (**fig 3.3 D-F**). Taken together these data suggest that there are alterations in both action potential mediated inhibitory transmission in the motor cortex as well as spontaneous vesicle fusion events.

Given the restricted expression of the mthtt protein to PV+ neurons, which contact pyramidal neurons primarily on the soma, we performed evoked recordings in the

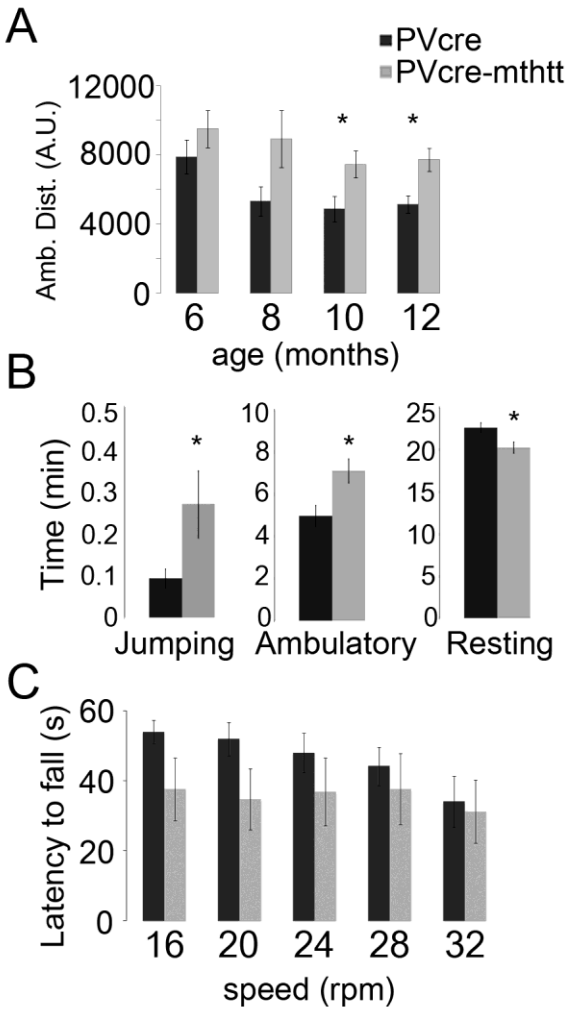


Figure 3.2. Conditional HD Mice Exhibit Hyperactivity at 12 months of age.

Mice with conditional expression of mthtt in PV+ neurons showed hyperactivity in the open field paradigm at 10 and 12 months of age (**A**). Additional analysis of open field data at the 12 month time point revealed increased jumping time and ambulatory time and reduced resting time in the HD mice as compared to the littermate PVcre mice (**B**). Additional time spent in the arena was accounted for by time spent rearing which was not significantly different between the groups. Motor coordination is unaffected in the HD animals at 12 months of age as tested by the rotarod paradigm (**C**). Two-way ANOVA tests were performed on the behavioral data. Error bars: SEM. (n=8-15 animals/group; *$p < 0.05$).

motor cortex using the paired-pulse paradigm to confirm presynaptic involvement. We observed an increase in the paired-pulse ratio (PPR) in HD mice as compared to their PVcre littermates (**fig 3.3 G&H**). As PPR is usually inversely related to presynaptic release (Dobrunz, Huang et al. 1997), this suggests a reduction in GABA release probability in the cortex of conditional HD mice, consistent with a reduction in mIPSC frequency.

Reductions in Evoked GABA Release in the Motor Cortex of Conditional HD mice.

PV+ interneurons in the cortex entrain regional pyramidal neurons at the gamma frequency (40-100 Hz) (Bartos, Vida et al. 2002). To investigate whether conditional expression of mthtt in these neuronal populations results in alterations in the evoked GABA release at that frequency, we performed repetitive stimulation in the motor cortex at 66 Hz and recorded the resultant IPSC response. Measurement of the area under the curve revealed a statistically significant reduction in evoked GABA release in the motor cortex (**fig 3.3 I&J**) with this stimulation protocol, suggesting physiologically relevant reductions in GABA release in the cortex of these mice.

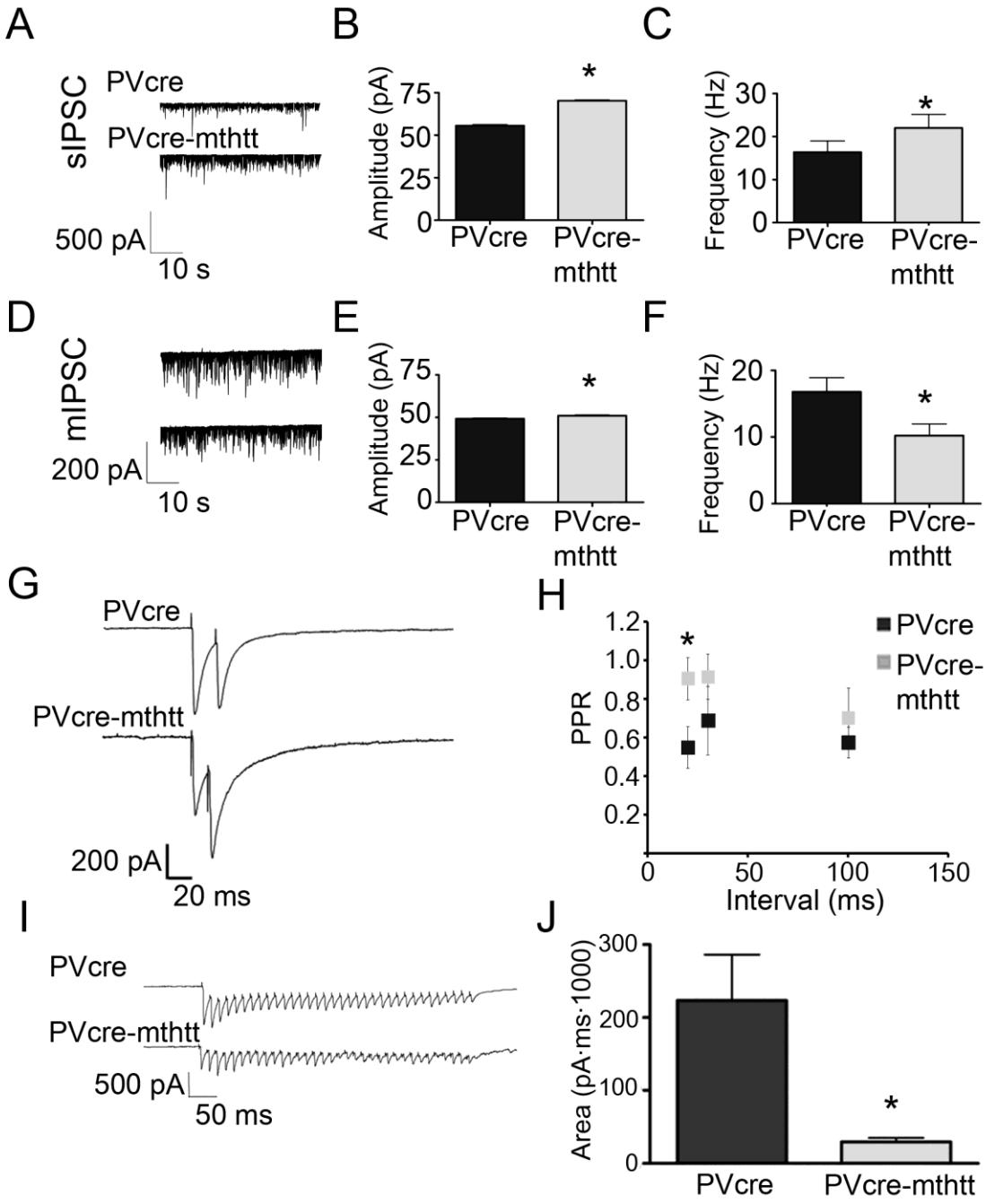
Conditional HD Mice Show no Alteration in Transcript Levels at 12 months of age

Studies have shown that mthtt interacts with peroxisome proliferator-activated receptor gamma coactivator-1α or (PGC-1α) (Cui, Jeong et al. 2006) which has been shown to be highly concentrated in GABAergic cell populations (Cowell, Blake et al. 2007). To determine whether alterations in behavior and neurotransmission were

accompanied by changes in transcription, we performed qRT-PCR on anterior cortex homogenates for PGC-1α in PVcre and PVcre-mthtt mice. There were no alterations in the levels of the coactivator (1.0±0.13, 1.2±0.14 Arbitrary Units (A.U.) PVcre & PVcre-mthtt, respectively; p= 0.46). Further, there were no alterations in the transcript levels of PV (1.0±0.06 & 1.1±0.12 A.U., PVcre & PVcre-mthtt, respectively; p= 0.53) or GAD67 (1.1±0.12 & 0.9±0.17 A.U., PVcre & PVcre-mthtt, respectively; p= 0.41; all normalized actin), suggesting that mthtt expression in PV+ neurons does not cause overt cell death.

Phenotypical Alterations at Midlife are Absent in Conditional HD mice at 24 Months of Age.

Considering the neurodegenerative nature of HD, we postulated that the alterations in motor performance and synaptic physiology would continue throughout life and worsen over time, especially considering the increased accumulation of mthtt inclusions with age. In order to explore this possibility, we aged a cohort of animals to 24 months of age. Alterations in basal GABA release were evident and included an increase in sIPSC amplitude with no change in sIPSC frequency, as well as a reduction in mIPSC amplitude with no change in mIPSC frequency (**fig 3.4A-F**). These alterations differ from those observed at 12 months of age and indicate that GABAergic dysfunction is still evident at the synaptic level; importantly, the unaltered mIPSC frequency suggests that PV+ neurons have not been lost over time in this model, as a loss of this interneuron subtype would result in decreases in sIPSC and mIPSC amplitude and frequency. However, surprisingly, upon paired stimulation, the PPR of the evoked IPSC was normal (**fig 3.4G**), suggesting that there is no longer any difference in release probability of


A
sIPSC
PVcre
PVcre-mthtt
500 pA
10 s
B
Amplitude (pA)
75
50
25
0
PVcre
PVcre-mthtt
C
Frequency (Hz)
30
20
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PVcre
PVcre-mthtt
D
mIPSC
200 pA
10 s
E
Amplitude (pA)
75
50
25
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PVcre
PVcre-mthtt
F
Frequency (Hz)
20
10
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PVcre
PVcre-mthtt
G
PVcre
PVcre-mthtt
200 pA
20 ms
H
PPR
1.2
1.0
0.8
0.6
0.4
0.2
0
50
100
150
Interval (ms)
PVcre
PVcre-mthtt
I
PVcre
PVcre-mthtt
500 pA
50 ms
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Area (pA·ms·1000)
300
200
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PVcre
PVcre-mthtt

Figure 3.3. PV+ Cell-specific Expression of mthtt is Sufficient to Cause Alterations in GABA Release in the Motor Cortex.

In acute slices, sIPSCs were recorded from layer 5 pyramidal neurons in the motor cortex (sample traces in **A)**. At 12 months of age there is an increase in average event amplitude and an increase in event frequency (**B&C**). Addition of TTX to the bath allowed for isolation of mIPSCs (sample traces in **D)**. Under these conditions there is a slight increase in average event amplitude and a reduction in frequency (**E&F**). **G.** Sample traces of evoked IPSCs in layer 5 pyramidal neurons in the motor cortex from 12 month old animals show an increase in the ratio of the second response to the first upon paired stimulation. **H.** Upon paired-pulse stimulation at multiple intervals, there is an increase in paired pulse ratio at the 20ms interval in the conditional HD mice, suggesting a reduction in GABA release probability from the presynaptic interneuron. **I.** Sample traces of evoked IPSCs in layer 5 pyramidal neurons in the motor cortex from 12 month old animals show a reduction in the overall area under the curve upon repetitive stimulation at the gamma frequency. **J.** Upon repetitive stimulation at gamma frequency (66 Hz), there is a reduction in GABA release as measured by area under the curve in 12 month old conditional HD mice. Error bars: SEM. (n=3-5 animals and 10-12 cells per condition; *$p<0.05$, Student's t-test).

GABA upon stimulation. Remarkably, this same normalization was seen upon repetitive train stimulation at the gamma frequency (**fig 3.4H**), indicating that differences were no longer present with a physiological pattern of stimulation. Accordingly, at this same age, these mice no longer demonstrated a hyperactive phenotype (**fig 3.4I**).

Discussion

Huntington Disease is a devastating neurological disorder, with no disease-altering treatments at present. In order to investigate the role of PV+ neurons in the disease etiology of HD we utilized a novel mouse model with PV+ cell-specific expression of exon 1 of the human htt gene with 103 CAG repeats. This restricted expression of mthtt to PV+ cell populations results in a unique HD-like phenotype characterized by hyperactivity and alterations in spontaneous and evoked GABA release in the motor cortex at 12 months of age. Taken together, these data suggest a cellular mechanism by which mthtt causes hyperactivity and cortical dysfunction.

HD patients commonly present with chorea, or erratic, involuntary movements. While it is not possible to model true "chorea" in mice, we present one of the few murine models with mthtt overexpression to exhibit overt hyperactivity, as most mouse models of HD typically demonstrate reduced locomotion and impaired coordination (Carter, Lione et al. 1999; Gu, Li et al. 2005; Hickey, Gallant et al. 2005; Albertson, Yang et al. 2011). Electrophysiological characterizations revealed a reduced probability of GABA release in the motor cortex as well as an overall reduction in gamma frequency stimulation-evoked GABA release, and we propose that these alterations could enhance

cortical output to the spinal cord and/or the striatum. In fact, imaging studies in patients have shown that there are strong correlations between alterations in excitatory/inhibitory balance in the cortex and motor abnormalities (Ridding, Sheean et al. 1995; Brown, Ridding et al. 1996). As such, this model could be useful for understanding the neurobiological mechanisms underlying the uncontrollable movements associated with HD.

Changes in motor function and evoked GABA release normalized by 24 months of age, when the most mthtt-positive inclusions were observed in the cortex. There are conflicting theories on the role of inclusion bodies in the pathology of HD; some studies have shown pathological links to inclusion formation, including a direct correlation between inclusion density and CAG repeat length (Becher, Kotzuk et al. 1998), as well as an increased propensity towards cell death (Cooper, Schilling et al. 1998; Martindale, Hackam et al. 1998). Other reports have suggested that the presence of inclusions be considered protective (Saudou, Finkbeiner et al. 1998; Arrasate, Mitra et al. 2004; Bowman, Yoo et al. 2005). We hypothesize that the non-aggregated form of mthtt causes the observed dysfunction at the earlier time points while sequestration of mthtt in inclusions normalizes local inhibition, potentially by preventing soluble mthtt's effects on synaptic function (Li, Wyman et al. 2003; Milnerwood and Raymond 2007).

Based on the evidence that action potential-dependent and evoked mechanisms of GABA release are affected at 12 months of age but normalize at 24 months of age in this model, it is intriguing to postulate that soluble htt differentially affects the protein machinery responsible for the exocytosis of synaptic vesicles for spontaneous versus evoked release. A multitude of proteins involved in the control of neurotransmitter

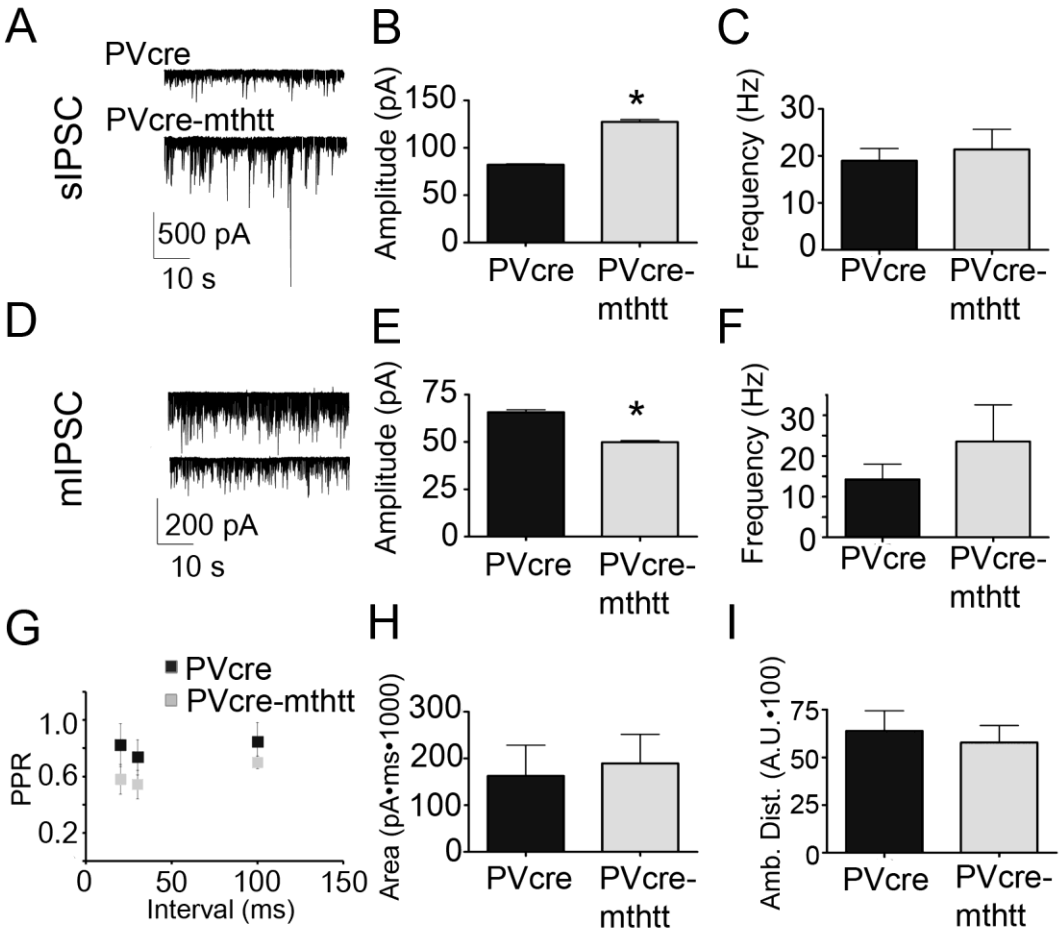

Figure 3.4. Phenotypical Alterations Shown at 12 Months of Age are Absent in Conditional HD mice at 24 Months of Age.

Spontaneous recordings were repeated at 24 months of age; the sIPSCs exhibited an increase in average amplitude (**B**) and with no change in frequency (**C**), while mIPSCs displayed a reduced average amplitude (**E**) and no change in frequency (**F**). Sample traces shown in **A&D**. **G**. Application of the paired-pulse stimulation paradigm in 24 month old animals revealed no significant alteration in the release probability of GABA in the conditional HD mice. **H.** Application of the repetitive stimulation paradigm in 24 month old animals revealed no significant alteration in evoked GABA release in the conditional HD mice. Hyperactivity was not evident at this age as measured by ambulatory distance in the open field paradigm (n= 10/group; **I**). Error bars: SEM. (n=3-5 animals and 10-12 cells per condition; *$p<0.05$, Student's t-test).

release can interact with mthtt, including (but not limited to) VAMPS, SNAREs, complexins, synaptotagmins, myosin, and pacsin (Deitcher, Ueda et al. 1998; Young and Neher 2009; Jorquera, Huntwork-Rodriguez et al. 2012; Peng, Rotman et al. 2012). We hypothesize that soluble mthtt interacts with synaptic release proteins in PV+ interneurons to impair vesicle release as has been demonstrated in excitatory neurons (Li, Wyman et al. 2003), but that the sequestration of the soluble protein into insoluble aggregates could reduce the interaction of mthtt with synaptic vesicles, thereby normalizing neurotransmission at 24 months of age (see Figure 3.5).

One unexpected result was the finding that the mthtt inclusions were never nuclear in our model. A previous report, using similar cre-driven models of conditional expression, showed that immunoreactivity was not only present in the nucleus, but was high there relative to the rest of the neuron (Gu, Li et al. 2005). The lack of intranuclear inclusions in the PV+ neurons could suggest that these neurons may handle the mutant protein differently then excitatory neurons; in fact, even in the R6/2 model, mthtt+ inclusions appear later in PV+ interneurons than in other cell types (data not shown; Meade CA, Reiner et al. 2002). Considering the lower frequency of inclusions at younger ages, it is possible that PV+ neurons are exposed to the soluble form of mthtt for a longer period of time than other neurons, before sequestration into inclusions. Also, a lack of intranuclear inclusions would suggest that the effects of mthtt in these mice are unlikely due to interference of mthtt with transcriptional processes; in fact, a lack of transcriptional alterations in levels of PGC-1α, PV, and GAD67 in our model supports this theory. Alternatively, it is possible that dysfunction of glutamatergic neuronal populations influences the processing of mthtt in interneurons, causing it to translocate to

the nucleus, accounting for the nuclear localization of mthtt in PV+ neurons in other HD models.

One surprising finding was a lack of inclusions in PV+ interneurons of the striatum, despite previously demonstrated cre activity in this PVcre mouse line (Janssen, Yasuda et al. 2011). While we cannot rule out the possibility that soluble mthtt is causing neuronal dysfunction in these cells, it is important to note that alterations in synaptic activity in the motor cortex can be sufficient to produce changes in motor behavior in the absence of striatal dysfunction (Ridding, Sheean et al. 1995; Brown, Ridding et al. 1996). Furthermore, no deficits were observed in rotarod performance, and no marked striatal atrophy or lateral ventricle enlargement was evident (not shown), indicating that this pattern of mthtt expression is not sufficient to cause overt striatal damage.

While the tools were not available to investigate the impact of overexpression of normal huntingtin on the measures evaluated here, we hypothesize that since the majority of the mthtt exon 1 protein is made up of the CAG repeat, the effects we are seeing are directly due to the trinucleotide repeat and not the residual effects of the "normal" portion of the protein. As such, we would predict that CAG repeats alone (or as part of another protein) would have the potential to affect GABAergic transmission and behavior similar to what we have observed with our conditional model. Along those lines, fMRI studies have shown reductions in cortical activation in other repeat disorders (Hashimoto, Backer et al. 2011) and overexpression of the CAG repeat alone was shown to cause abnormal cortical EEG recordings (Ordway, Tallaksen-Greene et al. 1997). It would be interesting to see whether overexpression of the trinucleotide repeat alone is able to cause the same pathology described here and whether overexpression of normal huntingtin can rescue

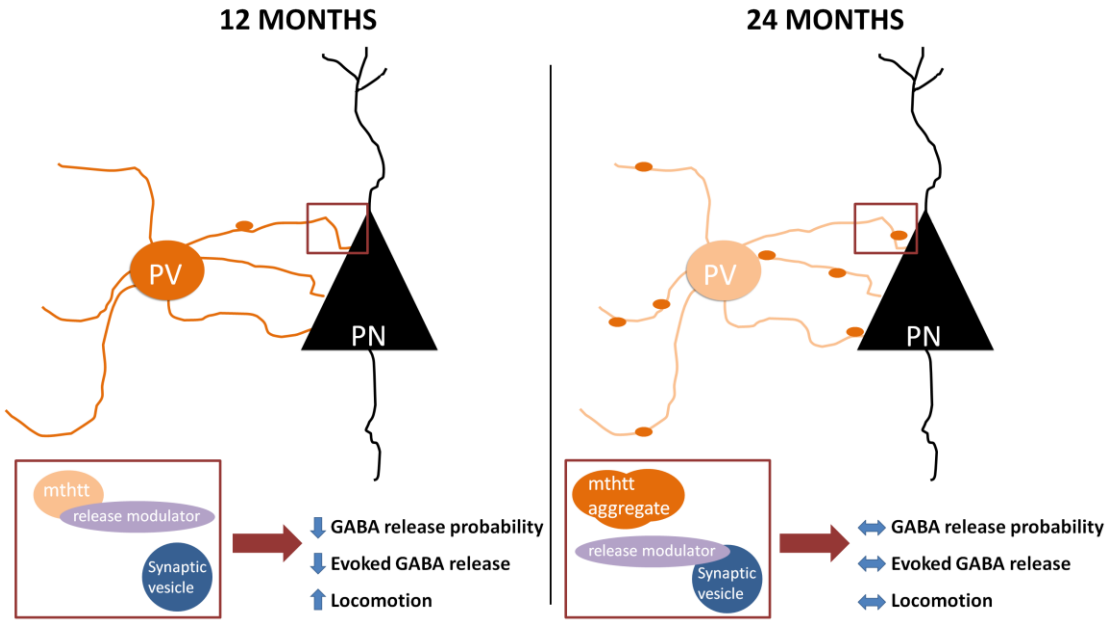

Figure 3.5. Model for Mutant Huntingtin-Induced Cortical Interneuron Dysfunction.

From the data presented here and additional literature support (see *Discussion*) we propose the following model. At 12 months of age, soluble mthtt (dark orange) within the presynaptic PV+ interneuron is able to interact with molecular modulators of synaptic release (purple) at the level of the local synapse. This would cause reductions in inhibitory transmission (GABA release probability and overall evoked release) from interneurons in the cortex, manifested by an increase in locomotion. By 24 months of age, a higher proportion of the mthtt load has been sequestered into insoluble aggregates, allowing the release modulators to bind their intended substrates and properly regulate GABA release, resulting in normalization of cortical inhibition and locomotor activity.

these effects, in light of the evidence that normal huntingtin can prevent some of the toxic effects of mthtt (Ho, Brown et al. 2001; Sun, Savanenin et al. 2001).

In summary, we have found that restricted expression of exon one of mutant huntingtin to PV+ cell populations is sufficient to cause hyperactivity at 12 months of age. This conditional expression also results in reduced inhibitory transmission in the motor cortex. Taken together these data suggest not only a role for PV+ interneurons in the etiology of HD but also a specific niche for these GABAergic cell populations in overall motor control. Further, with the unanticipated longitudinal results in this model, this work also supports the previously suggested protective role of inclusion bodies in this neurodegenerative disorder. This is an exciting finding as it suggests that inclusions alleviate cellular strain and also sequestration of the mutant protein may help improve local circuit function. Future studies with this model have the potential to shed light on the molecular mechanisms by which mthtt interferes with interneuron function, with the goal of improving motor function in patients with HD.

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CHAPTER IV

LOSS OF THE TRANSCRIPTIONAL COACTIVATOR PGC-1α LEADS TO ALTERATIONS IN SYNAPTIC TRANSMISSION IN THE MOTOR CORTEX

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Introduction

Peroxisome proliferated-activated receptor gamma coactivator one alpha (PGC-1α) is a transcriptional coactivator which, by interacting with different transcription factors, initiates cell and tissue-specific gene programs. Since the discovery of the coactivator in 1998 (Puigserver, Wu et al. 1998), many studies have suggested that a reduction in its levels and/or activity plays a role in multiple neurological disorders including PD (Zheng, Liao et al. 2010), AD (Qin, Haroutunian et al. 2009; Sheng, Wang et al. 2012), HD (Cui, Jeong et al. 2006; Taherzadeh-Fard, Saft et al. 2009; Chaturvedi, Calingasan et al. 2010), schizophrenia (Christoforou, Le Hellard et al. 2007; Jiang, Rompala et al. 2013), anxiety (Hettema, Webb et al. 2011) and multiple sclerosis (Witte, Nijland et al. 2013). Studies with whole body and neuron-specific PGC-1α -/- mice indicate that PGC-1α is required for the expression of a subset of metabolic and neuronal transcripts (Lin, Wu et al. 2004; Lucas, Markwardt et al. 2010; Ma, Li et al. 2010; Lucas, Dougherty et al. 2012), but the physiological consequences of these transcriptional changes are not clear. Elucidating the impact of PGC-1α deficiency on neuronal function will give us insight into its contribution to neuronal dysfunction in these disorders.

PGC-1α has been shown to be most highly concentrated in GABAergic cell populations throughout the brain (Cowell, Blake et al. 2007), and PGC-1α -/- mice exhibit deficiencies in the expression of the calcium buffer parvalbumin (PV) in forebrain regions including the cortex, hippocampus, and striatum(Lucas, Markwardt et al. 2010). In these regions, PV is expressed by a subset of GABAergic interneurons that exhibit fast-spiking and non-adapting properties (Kawaguchi 1993; Kawaguchi and Kondo 2002; Tepper and Bolam 2004) and entrain local pyramidal neurons to generate gamma oscillations (Wang and Buzsaki 1996; Bartos, Vida et al. 2002; Vreugdenhil, Jefferys et al. 2003; Sohal, Zhang et al. 2009). A previous study from our laboratory demonstrated that mice lacking PGC-1α show alterations in short-term synaptic plasticity and enhanced evoked GABA release in the dentate gyrus of the hippocampus. Additionally, stimulation at the gamma frequency revealed an increased variability in inhibitory postsynaptic current responses (IPSCs) and increased frequency of asynchronous IPSCs. Considering that hippocampal interneuron networks function differently from cortical interneuron networks, it is particularly important to evaluate the impact of PGC-1α deficiency in the cortex with relevance for disorders in which cortical PGC-1α deficits have been reported (PD (Zheng, Liao et al. 2010) and AD).

In light of the deficiency in PV expression in the cortex of PGC-1α -/- mice and the previously observed alterations in GABA release in the hippocampus, we sought to determine the physiological impact of PGC-1α deletion on cortical GABA release. We hypothesized that PV+ interneurons would primarily be affected by the loss of PGC-1α and that alterations in interneuron function would influence inhibitory transmission onto cortical pyramidal neurons. In order to investigate the potential role of PGC-1α in

cortical synaptic functioning, we utilized a PGC-1α -/- mouse model (Lin, Wu et al. 2004). Our data show that, in contrast to results from the PGC-1α -/- hippocampus, a loss of PGC-1α leads to reductions in basal GABA release, concurrent with a reduction in presynaptic GABA release probability and reduced GABA release upon gamma frequency stimulation. Further, in PGC-1α -/- mice expressing eGFP specifically in PV+ cells, we found that fast-spiking interneurons from PGC-1α -/- mice have a reduced firing rate, suggesting that PGC-1α functions in a cell-autonomous manner to regulate interneuron excitability. Taken together these data suggest that reductions in PGC-1α expression are associated with deficiencies in inhibitory neurotransmission and synaptic function in the cortex. These results have implications for cortical network signaling, as synchronization of firing by PV+ interneurons is critical for normal cortical output and higher cognitive processing.

Cortical network synchrony is an essential biological regulator of regional output. The ability to entrain global firing and coordinate synaptic physiology allows for higher level functioning. This synchronization of neuronal activity may underlie optimal cortical function as it has been shown to be lost in a number of psychiatric and neurodegenerative disorders (Hutchison, Dostrovsky et al. 2004; Cunningham, Hunt et al. 2006; Gonzalez-Burgos and Lewis 2008; Lewis, Hashimoto et al. 2008; Lodge, Behrens et al. 2009).

Recent evidence from the lab has identified other putative targets for the regulator including the calcium sensors, synaptotagmin 2 (SYT2) (Kochubey and Schneggenburger 2011) and complexin 1 (CPLX1) and the structural protein neurofilament heavy chain (NEFH; unpublished observation). PV and SYT2 have been shown to be concentrated in

overlapping neuronal populations (Sommeijer and Levelt 2012). Our lab has shown that CPLX1 and NEFH also colocalize to this neuron subset (unpublished observation).

SYT2 functions as a calcium sensor, and in conjunction with CPLX1 interacts with SNARE proteins to influence synaptic release (McMahon, Missler et al. 1995; Chicka and Chapman 2009). These molecules are essential for appropriate vesicle release upon calcium efflux. It follows that with reductions in the levels of these proteins, synaptic firing will be dysfunctional. Studies have shown that ablation of CPLX1 results in increased spontaneous and asynchronous vesicle exocytosis but reductions in asynchronous vesicle release (Yang, Kaeser-Woo et al. 2010). Further, independent loss of SYT2 results in a similar phenotype (Kochubey and Schneggenburger 2011). Taken together these data suggest that each of these proteins autonomously affect synaptic physiology and that in conjunction could potentially result in further dysfunction in neuronal firing. Additional studies are required to elucidate whether these interactions are inconsequential, additive, or synergistic.

In order to investigate the potential role of PGC-1α in cortical synaptic functioning we utilized a PGC-1α null mouse model (Lin, Wu et al. 2004). Our data shows that loss of PGC-1α leads to alterations in basal GABA release, concurrent with a reduction in presynaptic GABA release probability and reduced GABA release upon gamma frequency stimulation. Taken together these data suggest that ablation of PGC-1α, and by extension its downstream transcriptional targets, results in deficiencies in synaptic function. These changes have implications for cortical network signaling, as synchronization of firing by interneurons is critical for normal cortical output and global brain function.

Methods

Animals.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. PGC-1α -/- mice (generous gift of Jiandie Lin, University of Michigan, (Lin, Wu et al. 2004)) were maintained on a C57BL/6J genetic background and housed two to five in a cage at 26 ±2°C room temperature with food and water ad libitum. All experiments were conducted with male and female mice at 4 weeks of age unless otherwise specified.

For targeted interneuron recordings, mice from the PGC-1α -/- line were crossed with mice expressing eGFP under the control of the GAD67 promoter (G42 line; JAX#7677). In this mouse line, eGFP is only expressed in the PV+ subset of inhibitory interneurons, most strongly in the cortex (Chattopadhyaya, Di Cristo et al. 2004; Bartley, Huang et al. 2008). All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* adopted by the U.S. National Institutes of Health.

For Pyramidal Neurons:

Whole Cell Recordings.

Mice (P27 to P33) were anesthetized with isoflourane and then decapitated. Brains were placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 3.5 KCl, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 26 $NaHCO_3$ and 10 D-glucose. The saline was bubbled with 95%O_2/5%CO_2. Coronal brain slices (300 μm thick) were cut using a Vibratome (Ted Pella, Inc., Riverside, CA). The slices were kept for 30 minutes at 37±1°C and then stored at room temperature (22±1°C). Slices were superfused

continuously with oxygenated recording ACSF at room temperature. Whole-cell patch clamp recordings were acquired from visually identified pyramidal neurons in layer five of the motor cortex. Position in cortex was verified with strepavidin staining for biocytin filled cells. Recordings were conducted on a Zeiss upright microscope (Carl Zeiss, Thornwood, NY). Cells were voltage clamped at -70 mV, using internal solution containing the following (in mM) 129 CsCl, 2 MgATP, 10 EGTA, 10 HEPES, .2 GTP and 2 QX-314, pH 7.2. Pipette tip resistance was 2-5 MΩ. Voltage clamp recordings were obtained using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) controlled by Clampex 8.0 software via a Digidata 1322A interface (Molecular Devices), filtered at 5 kHz and digitized at 10 kHz. Input resistance and series resistance were monitored by applying a 10 mV voltage step. Spontaneous and evoked IPSCs were pharmacologically isolated with CNQX (10 μM) and D-APV (50 μM). Spontaneous effects were then further isolated with TTX (1 μM). Analysis of spontaneous IPSCs (sIPSCs) was performed using Clampfit 8.0 software which focused on amplitude, interevent interval and decay time of events. All sIPSCs that fit the template and passed visual inspection were inspected in the analysis. To calculate the paired-pulse ratio, the amplitude of the second IPSC was measured relative to the baseline set immediately before the second stimulus, and normalized to the amplitude of the first IPSC relative to the baseline set immediately before the first stimulus. CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), APV (DL-2-amino-5-phosphonoovaleric acid) and TTX (tetrodetoxin) were diluted from stock solutions to final concentrations in ACSF.

Stimulation.

Synaptic responses were evoked with a bipolar stimulating electrode consisting of a twisted pair of 25 µm Formvar insulated nichrome wires. The electrode was positioned in layer 5 of the motor cortex. A series of paired stimulations at 20, 30 and 100 ms intervals were applied to elucidate pair-pulse ratios. For gamma train recordings, 34 stimulations were applied to evoke a response in patched pyramidal cells. Recordings were taken at 32±1°C.

Data Analyses.

Data analyses for all electrophysiological experiments were performed using Microsoft Excel. A two-tailed student t-test assuming unequal variance was utilized to assess statistical significance. Values were considered statistically significant when the p value was less than 0.05.

For Interneurons:

Slice Preparation.

Experiments were conducted in 300 µm acute brain slices prepared from the frontal cortex of P27 to P33 mice. The mice were anesthetized with isoflurane and decapitated, and their brains were removed rapidly. Coronal slices of the brain were cut using a vibrating microtome (VT1000S; Leica, Bannockburn, IL). Slicing and dissection of the cortex was done in ice-cold (1–3°C) dissecting solution containing the following (in mM): 87 NaCl, 3 KCl, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 75 sucrose and 20 glucose, bubbled with 95% O_2-5% CO_2, pH 7.35–7.45. Slices were stored in a holding chamber containing a slightly modified artificial cerebral spinal fluid (ACSF, see below) for approximately 30 minutes at 30° to 32° C and then transferred to room

temperature. Modified ACSF contained (in mM) 1 $CaCl_2$ and 2 $MgCl_2$. Slices were bubbled with 95% O_2-5% CO_2 for ≥1 h before recording.

Intrinsic Firing Assessment

During the experiment, slices were held in a submersion recording chamber perfused (3-4 mLs/ min) with ACSF composed of (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose. The solution was bubbled with 95% O2/5% CO2, and the pH was between 7.35 to 7.45. Picrotoxin (100 μM) was added to the external solution to block inhibitory synaptic responses mediated by $GABA_A$ receptors; 50 μM D-APV (D-2-amino-5-phosphonopentanoic acid) was added to prevent NMDA receptor mediated currents. All intrinsic firing experiments were performed at 28° to 30° C. Interneurons expressing eGFP were identified visually using infrared differential inference contrast optics and epifluorescent optics on a Nikon (New York) E600FN upright microscope. Targeted interneurons were patched in the voltage-clamp configuration and recorded in current-clamp configuration while maintaining a holding potential of −60 ± 1mV using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Patch electrodes (4–6MΩ) were filled with internal solution composed of the following (in mM): 150 K-gluconate, 0.1 EGTA, 3 NaCl, 6 KCl, 10 HEPES, 10 Na-ATP, and 0.3 GTP. pH was adjusted to 7.3 with KOH. The resting potential was measured immediately after break-in, and input resistance was measured in voltage-clamp with a 400-ms, –8-mV step from a –60-mV holding potential. Firing frequency versus injected current plots (F–I plots) were made by measuring the initial firing frequency of a spike train evoked by a series of incremental 600-ms current steps at intervals of 50 or 100pA. The spike threshold potential was defined as the membrane potential, in a 5-ms window

preceding spike peak, at which the third derivative was a maximum (an inflection point). The action potential (AP) amplitude was calculated from the spike threshold to the peak of the AP. The AP half-width is the duration of the AP at the half way point of the AP amplitude. Afterhyperpolarization (AHP) was calculated from the spike threshold to the peak of the AHP. The access resistance and holding current (<200 pA) were monitored continuously. Recordings were rejected if either access resistance or holding current increased ~20% during the experiment.

Electrophysiological Interneuron Classification

To distinguish fast-spiking (FS) cells from non-FS (NFS) interneurons, depolarizing current steps were used to analyze the firing response of each interneuron. Single spike properties were determined on spikes elicited by near threshold current injection. Spike-frequency adaptation was quantified by the ratio between the last and first interspike intervals in spike trains evoked by 600 ms depolarizing steps. Cells were classified as FS if they had the following (Rotaru et al., 2011): (1) at the half-way point of the F-I plot the firing frequency reached at least 150 Hz; (2) narrow spikes (duration at half peak amplitude $\leq$ 0.6 ms); (3) large afterhyperpolarizing potentials (amplitude $\geq$ 14 mV); and (4) absence of significant spike-frequency adaptation (adaptation ratio $\leq$ 1.5). These criteria may exclude some FS neurons.

Data Analyses.

All statistics were performed using Origin software (Origin Lab Corporation, 2002) and statistical significance was $P < 0.05$. Data are presented as means ± SE and sample number (n) refers to cell number for electrophysiological experiments. Statistical

comparisons for electrophysiological data were made using the Student's t-test or one-way ANOVA followed by Tukey's posthoc analysis. In figures and table, * indicates a statistically significant difference.

Results

Basal GABA Release is Altered in the Motor Cortex of PGC-1α -/- Mice.

To assess overall inhibitory activity at the basal level, we initially evaluated both spontaneous and miniature IPSCs in the motor cortex from PGC-1α -/- mice around postnatal day 30. This age was chosen based on evidence that these mice exhibit age-related structural changes in the brain (Lucas, Dougherty et al. 2012) and that PGC-1α expression in the cortex peaks around postnatal day 14-21 (Cowell, Blake et al. 2007). The motor cortex is of particular interest because decreases in PV expression were very robust in this region in these mice (Lucas et al. 2010). Whole cell voltage clamp recordings were performed on layer 5 pyramidal neurons in the motor cortex in the presence of APV and CNQX (sample traces **fig 4.1 A&B**). The observed sIPSCs exhibited increased amplitudes (**fig 4.1 C&E**) and decreased frequency (**fig 4.1 D&F**) in PGC-1α -/- mice as compared to those measured in WT littermates. These alterations suggest that loss of PGC-1α may lead to changes in both spontaneous and action potential (AP)-mediated activity.

As studies have shown that AP-mediated activity and spontaneous vesicle fusion events can be differentially regulated, we pharmacologically isolated the spontaneous events with bath application of TTX to eliminate AP-mediated activity (sample traces **fig 4.2 A&B**).. The observed mIPSCs exhibited decreased amplitudes (**fig 4.2 C&E**) and

decreased frequencies (**fig 4.2 D&F**) as those measured in WT littermates. These alterations suggest that loss of PGC-1α differentially affects spontaneous vesicle release and AP-mediated activity. mIPSCs are smaller and less frequent whereas AP-regulated events are larger in magnitude but still less frequent. Overall, there are alterations in basal inhibition in the motor cortex; however the origination of these changes is as yet undetermined. Alterations in frequency support the supposition that the change is a result of presynaptic changes in release however the observed changes in amplitude could stem from mechanisms on both the pre- and postsynaptic neuron. In order to further investigate the origin of these changes we performed paired stimulation, which has been shown to indirectly measure presynaptic release activity (Dobrunz, Huang et al. 1997; Dobrunz and Stevens 1997).

PGC-1α -/- Mice Exhibit a Lower Release Probability of GABA the Motor Cortex.

Further characterization of evoked activity is required to evaluate the roles for PGC-1α in presynaptic properties. In order to evaluate presynaptic alterations in neurotransmitter release, we utilized a paired-pulse paradigm. Alterations in the ratio of the second response to the first suggest changes in presynaptic release (Dobrunz and Stevens 1997; Zucker and Regehr 2002). With loss of PGC-1α (and by extension its downstream targets), there is a loss of paired-pulse depression in eIPSCs into the layer 5 pyramidal neurons of the motor cortex at the 20ms paired-pulse interval (**fig 4.3 A&B**). This suggests a reduction in presynaptic release probability of GABA.

Global Ablation of PGC-1α Results in Reduced Evoked Cortical GABA Release

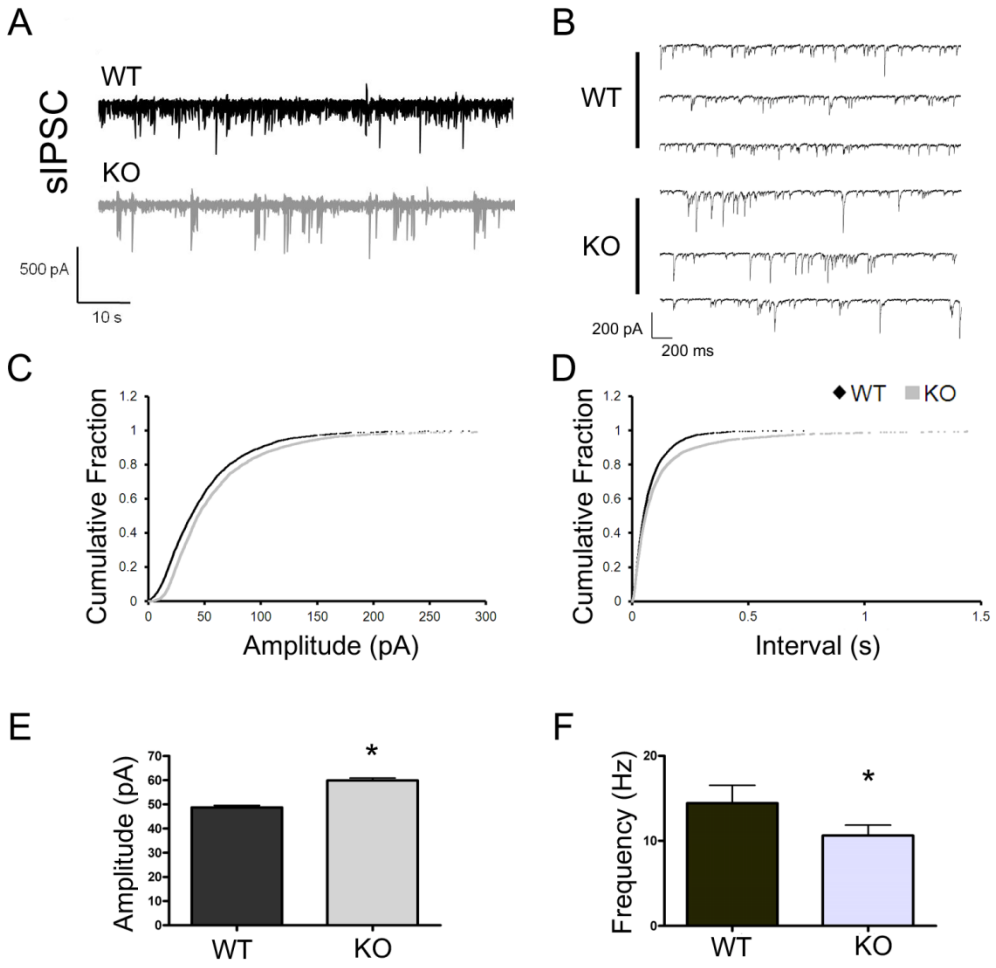

Figure 4.1: Global ablation of PGC-1α results in alterations in basal GABA release

A. Representative traces of sIPSCs in both WT and KO animals. **B**. Higher Power representative traces of sIPSCs in both WT and KO animals. **C, D.** Cumulative probability plots of amplitude and interval, respectively. **E**. Quantification revealed that the sIPSC amplitude was increased in the KO animals as compared to WT. **F**. sIPSC frequency (extrapolated from interevent interval) was reduced in the KO animals as evidenced by an lengthening of the interevent interval. The observed changes in sIPSC activity are indicative of alterations in basal GABA release from the cortical interneurons. Student's t-test *$p<0.05$ n=3 animals/group, approx. 10 cells per condition.

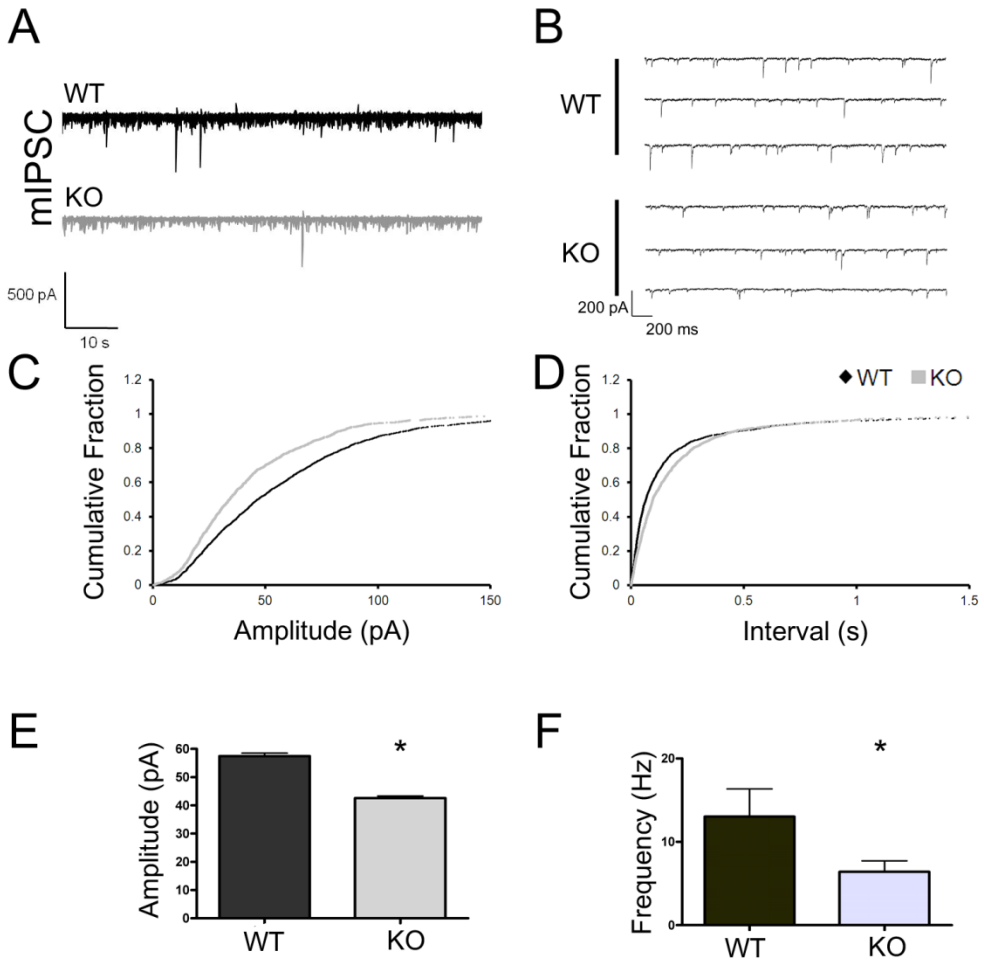

Figure 4.2: Global ablation of PGC-1α results in alterations in spontaneous inhibitory vesicle fusion events

A. Representative traces of sIPSCs in both WT and KO animals. **B**. Higher Power representative traces of sIPSCs in both WT and KO animals. **C, D.** Cumulative probability plots of amplitude and interval, respectively. **E**. Quantification revealed that the mIPSC amplitude was reduced in the KO animals as compared to WT. **F**. mIPSC frequency (extrapolated from interevent interval) was reduced in the KO animals as evidenced by a lengthening of the interevent interval. The variations in mIPSC activity are suggestive of dysfunctional spontaneous vesicle fusion and release independent of action potential related events. Student's t-test *p<0.05 n=3 animals/group, approx. 10 cells per condition.

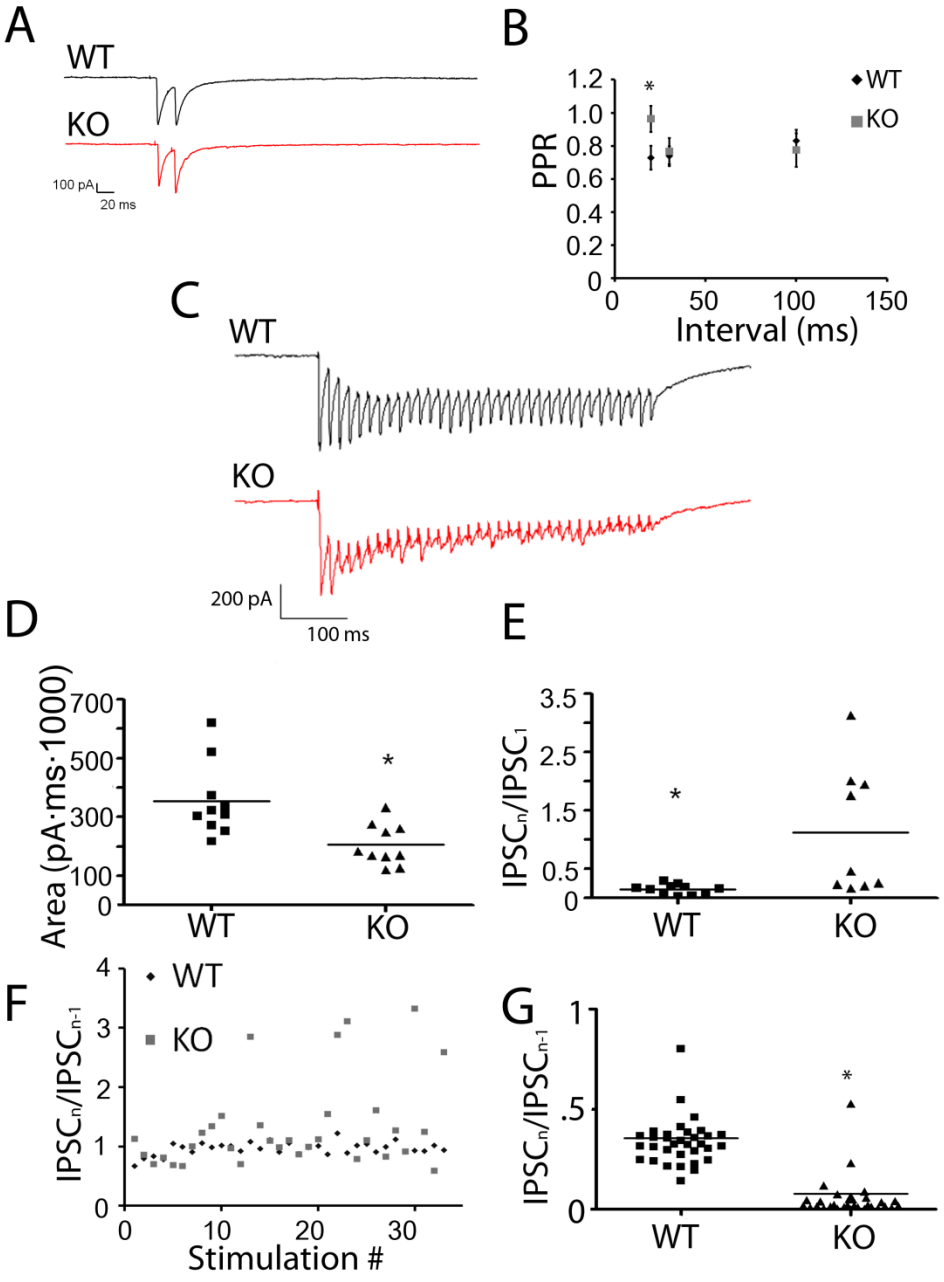
A
WT
KO
100 pA
20 ms
B
PPR
Interval (ms)
WT
KO
C
WT
KO
200 pA
100 ms
D
Area (pA·ms·1000)
WT
KO
E
IPSCn/IPSC1
WT
KO
F
IPSCn/IPSCn-1
Stimulation #
WT
KO
G
IPSCn/IPSCn-1
WT
KO

Figure 4.3: Loss of PGC-1a alters evoked activity through the cell populations in the motor cortex

A. Representative traces of paired pulse responses in both WT and KO animals. **B**. Paired pulse depression was lost in the KO animals at the 20ms interval. There was no difference between WT and KO at the longer intervals examined. **C**. Representative traces of train stimulation and response in both WT and KO animals (average of 10 sweeps per trace). **D**. Total charge during the train (area under curve) was significantly reduced in KO animals as compared to WT. **E**. Alterations in short-term plasticity over the length of the train were also evident. **F, G.** Variability in decay was observed in the KO animals by examining the plasticity ratio. Plasticity ratios were determined by the formula: $event_n/event_{n-1}$. Student's t-test *$p<0.05$ n=3 animals/group, approx. 10 cells per condition.

To further evaluate how a lack of PGC-1α compromises evoked GABA release, we applied repetitive trains of stimuli at gamma frequency which mimic the frequency at which PV+ interneurons entrain the cortical network(Traub, Whittington et al. 1996; Wang and Buzsaki 1996). We observed a reduction in overall area under the response curve in the PGC-1α -/- mice (**fig 4.3D**). Additionally, quantification of the amplitude of the final response over the first resulted in a bimodal distribution of the ratio in the PGC-1α -/- mice (**fig 4.3E**). Upon analysis of short-term plasticity throughout the response train, an alteration in the persistent decay of events is observed in the PGC-1α -/- mice. As exhibited by $IPSC_n/IPSC_{n-1}$, the WT responses represent approximately 90% of the previous response whereas the responses from the PGC-1α -/- animals are unpredictable (**fig 4.3 F&G**). These observations in train decay might suggest alterations in inhibitory vesicle release upon maintained stimulation. This could result from the exhaustion of the docked vesicles in the -/- mice prior to the onset of this depletion in the +/+ mice.

Global Ablation of PGC-1α Results in Alterations in the Intrisic Firing Properties of Fast-spiking Interneurons

One possible explanation for the lack of integrated inhibitory responses is that interneurons are unable to establish and/or maintain normal synaptic functionality without PGC-1α. It seems probable that with reductions in any number of PGC-1α's putative targets, including PV and SYT2, could result in alterations in calcium responsiveness and therefore alter intrinsic firing from that interneuron population. To allow for visualization of PV+ neurons for whole cell patch clamping, we generated PGC-1α +/+ and -/- mice with eGFP-positive PV+ interneurons by crossing PGC-1α -/-

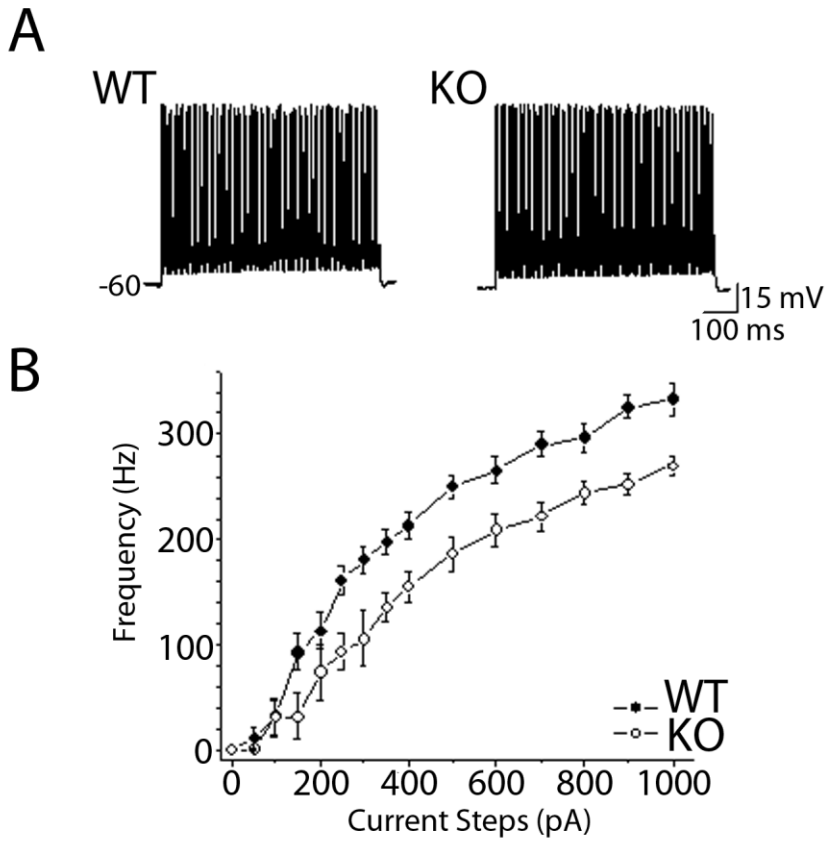

Figure 4.4: Loss of PGC-1α alters Interneuron Intrinsic Characteristics

A. Representative traces of the intrinsic firing due to an injection of a 400 pA current step in parvalbumin positive interneurons from WT and PGC-1α KO animals. **B:** Data is plotted as firing frequency as a function of injected current (F–I). The KO fast-spiking PV+ interneurons overall have decreased excitability compared to WT fast-spiking PV+ interneurons (n=9 WT, 5 KO).

Property	WT (9)	KO (5)	p-value
	FS	FS	
Resting Potential (mV)	-54.78 ± 3.80	-65.6 ± 4.82	0.109
Input Resistance (MΩ)	159 ± 21.80	206.2 ± 27.35	0.211
Spike Threshold Potential (mV)	-42.95 ± 0.76	-44.01 ± 0.52	0.357
Action Potential Amplitude (mV)	57.68 ± 3.00	61.35 ± 3.24	0.451
Action Potential Half-Width (ms)	0.50 ± 0.017	0.54 ± 0.021	0.145
Afterhyperpolarization (mV)	**17.21 ± 0.70**	**21.68 ± 1.06**	**0.003**
Frequency at Maximum Current Step (Hz)	**341.40 ± 19.47**	**268.46 ± 9.33**	**0.021**

Table 4.1. Intrinsic Properties of Parvalbumin Interneurons in motor cortex from WT and PGC-1α KO mice. Values in bold are statistically significant differences.

mice with the G42 GAD-eGFP mouse line (Chattopadhyaya, Di Cristo et al. 2004; Bartley, Huang et al. 2008). Upon current injection, PV+ interneurons of G42-PGC-1α -/- mice show a reduction in excitability as compared to +/+ littermates (**fig 4.4**). Further, G42-KO mice show an increase in afterhyperpolarization and a reduction in frequency at the maximum current step with no alteration in resting membrane potential or input resistance (**table 4.1**). This reduction in interneuron excitability could contribute to the observed reduction in AP-mediated GABA release in the cortex.

Conditional Ablation of PGC-1α Results in Alterations in Cortical GABA Release

To determine the physiological impact of PGC-1α deletion from PV+ neurons, we evaluated basal and evoked inhibitory postsynaptic currents in layer 5 pyramidal neurons from anterior cortex using whole cell voltage clamp recordings. Between 3 and 4 months of age, no differences were seen between PV-cre +/+ and PV-cre Fl/Fl mice in amplitude or frequency of sIPSCs, indicating that basal GABA neurotransmission was unaffected at this age by a loss of PGC-1α. However, as hypothesized, there was a increase in interevent interval (decrease in frequency; **fig 4.5A**) of mIPSCs, consistent with a presynaptic loss of Syt2 and/or Cplx1 .

To assess further potential alterations in synaptic synchrony that could occur with the combined loss of PV, Syt2, and Cplx1 expression in PV+ fast-spiking interneurons, we replaced the calcium in the extracellular recording medium with strontium during the measurement of evoked IPSCs. Bath application of strontium results in desynchronized release caused by inefficient molecular buffering relative to calcium, as evidenced by a reduction in event amplitude and an increase in late asynchronous synaptic events

(Behrends and ten Bruggencate 1998; Rumpel and Behrends 1999). As expected, upon stimulation of axons in Layer 5 in PV-cre +/+ mice, strontium significantly reduced evoked IPSC amplitude (**fig 4.5B, quantified in 4.5C**). There was also an increase in event frequency post-stimulation; this appeared as continued low amplitude events over the train decay (**fig 4.5B, arrowheads**). Interestingly, similar late IPSC events were observed in slices from PV-cre Fl/Fl even in the presence of calcium (**fig 4.5C, arrowheads**), and application of strontium reduced evoked IPSC amplitude to a much greater extent than in the PV-cre +/+ mice (**fig 4.5B; quantified in 4.5C**). Importantly, these alterations in peak amplitude of evoked responses in strontium were not due to a change in total GABA release (the overall area under the curve; **fig 4.5D**), suggesting that the reduction in amplitude of individual events could not be accounted for by a general reduction in GABA release. The reduction in eIPSC amplitude upon strontium replacement of calcium in PV-cre Fl/Fl mice indicates an increased susceptibility to strontium-mediated asynchrony, suggesting that mice lacking PGC-1α specifically in PV+ neurons exhibit abnormalities in synchronous neurotransmitter release.

While these results are supportive of the hypothesis that PGC-1α drives a program for synchronous neurotransmitter release in PV+ interneurons, we questioned whether PV-cre Fl/Fl mice would exhibit a decline in spontaneous and evoked GABAergic transmission with age, potentially due to reductions in Nefh or other unidentified PGC-1α-dependent genes. In contrast to 3 month-old PV-cre Fl/Fl mice, 8 month-old PV-cre Fl/Fl mice showed dramatic reductions in spontaneous GABA release as evidenced by reduced miniature IPSC amplitude and frequency (**fig 4.5E**). Furthermore, while no differences were seen between PV-cre +/+ and Fl/Fl mice in evoked GABA release in a

paired pulse protocol at 3 months of age (**fig 4.5F**), the paired pulse ratio was significantly reduced in 8 month-old PV-cre Fl/Fl mice, indicating a requirement for PGC-1α in the maintenance of normal GABAergic neurotransmission.

Discussion

PGC-1α has also been shown to be concentrated in GABAergic cell populations (Cowell, Blake et al. 2007). Considering that GABAergic interneurons are an integral part of the neuronal circuitry through the brain and that these neurons have been shown to modulate the activity of regional output neurons, entrain network activity, and inhibit over-excitation throughout neuronal pathways (Cobb, Buhl et al. 1995; Wang and Buzsaki 1996; Bartos, Vida et al. 2002; Mann and Paulsen 2007); it is not unexpected that alterations in PGC-1α and by extension its downstream targets found in GABAergic interneurons result in dysfunction of synaptic properties.

PV interneurons are described as fast-spiking and non-adapting (Kawaguchi 1993; Kawaguchi and Kondo 2002; Tepper and Bolam 2004). These intrinsic properties, as well as the expression of the voltage gated potassium channel, Kv3.1, and their perisomatic targeting, make PV+ interneurons ideal modulators of the systemic activity of the cortex known as cortical network oscillations (Perney, Marshall et al. 1992; Du, Zhang et al. 1996; Yeung, Thompson et al. 2005). These oscillations, entrained by the PV+ interneurons, result in synchronized coordinated responses of the global cortical network. With loss of PGC-1α, PV is brought to negligible levels (Lucas, Markwardt et al. 2010) and as such it follows that alterations in the PGC-1α -/- mice might mirror those

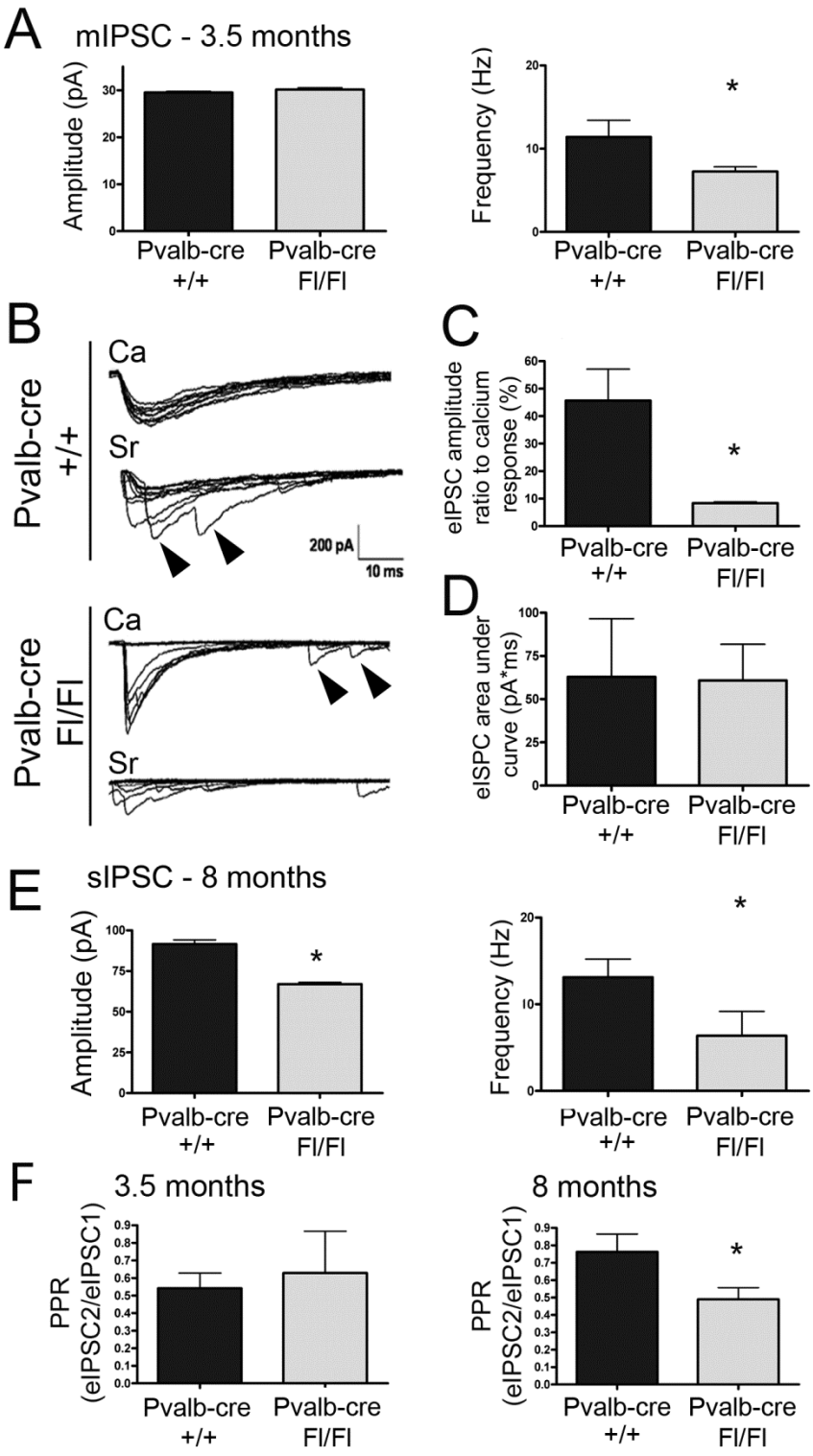
A
mIPSC - 3.5 months
Amplitude (pA)
Frequency (Hz)
Pvalb-cre +/+
Pvalb-cre Fl/Fl
B
Pvalb-cre +/+
Ca
Sr
200 pA
10 ms
Pvalb-cre Fl/Fl
C
eIPSC amplitude ratio to calcium response (%)
D
eISPC area under curve (pA*ms)
E
sIPSC - 8 months
F
3.5 months
8 months
PPR (eIPSC2/eIPSC1)

Figure 4.5. Conditional interneuron-specific deletion of PGC-1α causes abnormalities in evoked synchronous GABA release.

Selective deletion of PGC-1α from PV-positive neurons does not affect the amplitude of miniature inhibitory postsynaptic potentials (mIPSCs) in Layer 5 pyramidal neurons but causes a decrease in interevent interval (**A**) in three and ½ -month old mice, consistent with a decrease in the expression of presynaptic synchronous release machinery. Replacement of extracellular calcium with strontium (to increase asynchronous release) causes late synaptic events (arrowheads) in PV-cre +/+ mice after stimulation and significantly reduces the amplitude of IPSCs in PV-cre Fl/Fl mice (**C**, representative traces), while overall evoked GABA release is not altered over a 1 second period (**D**). Spontaneous, late synaptic events are even present in PV-cre Fl/Fl in the presence of calcium (arrowheads). In contrast, basal GABA neurotransmission is reduced in 8 month-old PV-cre Fl/Fl mice as evidenced by decreased amplitude and increased interevent intervals of mIPSCs (**E**) and decreased paired pulse ratio at short intervals (15 ms; f). n = 10 cells/group for all measures. *$p < 0.05$.

seen in the PV -/- mice (Schwaller, Dick et al. 1999) or mice lacking physiologically functional PV+ cell populations. These mice, lacking PV, exhibit a loss of regional synchrony in the hippocampus and deficits in working memory (Korotkova, Fuchs et al. 2010). The knock-out mice display alterations in regional GABA release (Vreugdenhil, Jefferys et al. 2003). PGC-1α -/- mice show similar alterations to the PV -/- mice in the hippocampus including the increase in GABA release upon gamma frequency stimulation (Lucas, Markwardt et al. 2010). However, in our previous report electrophysiological exploration of the PGC-1α -/- animals was only performed in the hippocampus. As studies have shown that physiological changes can be region specific, characterization of the cortical implications of loss of PGC-1α is essential.

It is possible that the reduction in GABA release is due to a reduction in PV+ cell number in the motor cortex. This appears unlikely as GAD67 immunostaining in normal in the cortex (Lucas, Markwardt et al. 2010) and also there is no appreciable difference observed in the number of eGFP+ interneurons in PGC-1α -/- mice, however a focused analysis of interneuron density within the motor cortex would be necessary to fully address that question.

In light of the developmental regulation of PV by PGC-1α, it would be interesting to determining whether PV+ interneurons are making fewer contacts with target neurons and whether this is normal at earlier ages. Due to the peak of PGC-1α around P14, it is possible that PV+ interneurons never form normal functional contacts within their target neurons. In order to assess this possibility, morphological anaylsis could be performed at different ages first to assess normal development and then examine whether connections deteriorate with age.

PGC-1α regulates a variety of gene programs including those playing a role in metabolism, calcium buffering, and synaptic functioning. Taken together our data describes the consequences of global loss of the co-activator, which include alterations in inhibitory transmission in the cortex (see figure 6). These animals display an overt motor phenotype, including reduced motor coordination and an increase in the occurrence of resting tremor (Lucas, Dougherty et al. 2012). It is intriguing to postulate that the alterations in the motor cortex could contribute to this motor phenotype but as PGC-1α is expressed throughout the body, including in the heart and muscle, we are unable to elucidate the role of these neurons independent of peripheral pathology. However, neuron-specific ablation of PGC-1α, utilizing a cre-lox system of conditional deletion, is capable of causing a coordinated deficit, in support of the idea that neuronal populations play a causative role in the motor dysfunction.

SYT2 functions as a calcium sensor, in conjunction with CPLX1, these molecules interaction with SNARE proteins to influence synaptic release (McMahon, Missler et al. 1995; Chicka and Chapman 2009). These molecules are essential for appropriate vesicle release upon calcium efflux. It follows that with reductions in the levels of these proteins synaptic transmission will be dysfunctional. Studies have shown that ablation of CPLX1 results in increased spontaneous and asynchronous vesicle exocytosis but reductions in asynchronous vesicle release (Yang, Kaeser-Woo et al. 2010). . Further, independent loss of SYT2 results in a similar phenotype (Kochubey and Schneggenburger 2011). These data suggest that each of these proteins autonomously affect synaptic physiology and that the loss of both in conjunction could potentially result in exacerbated dysfunction in

synaptic transmission. Further studies are required to elucidate whether these interactions are inconsequential, additive, or synergistic.

PV, SYT2, CPLX1, and NEFH localize to a similar subset of GABAergic neurons. PGC-1α has also been shown to be concentrated in GABAergic cell populations (Cowell, Blake et al. 2007). Considering that GABAergic interneurons are an integral part of the neuronal circuitry through the brain and that these neurons have been shown to modulate the activity of regional output neurons, entrain network activity, and inhibit over-excitation throughout neuronal pathways (Cobb, Buhl et al. 1995; Wang and Buzsaki 1996; Bartos, Vida et al. 2002; Mann and Paulsen 2007); it is not unexpected that alterations in PGC-1α and by extension its downstream targets found in GABAergic interneurons result in dysfunction of synaptic properties.

Beyond these putative synaptic functioning targets of PGC-1α there are additional protein candidates that are reduced with loss of PGC-1α that could play a role in the alteration of cellular physiology and functional firing. These include metabolic regulators as well as calcium and potassium channels. Further transcriptional and electrophysiological investigations could shed light on the role, if any, the loss of these molecules plays in the observed phenotype of PGC-1α -/- mice.

Cortical synchrony at the regional network level is essential for high level functioning and it is through inhibitory interneurons that these network patterns are established and maintained. PGC-1α has been shown to be concentrated in those GABAergic cell populations responsible for maintain the delicate balance of excitation

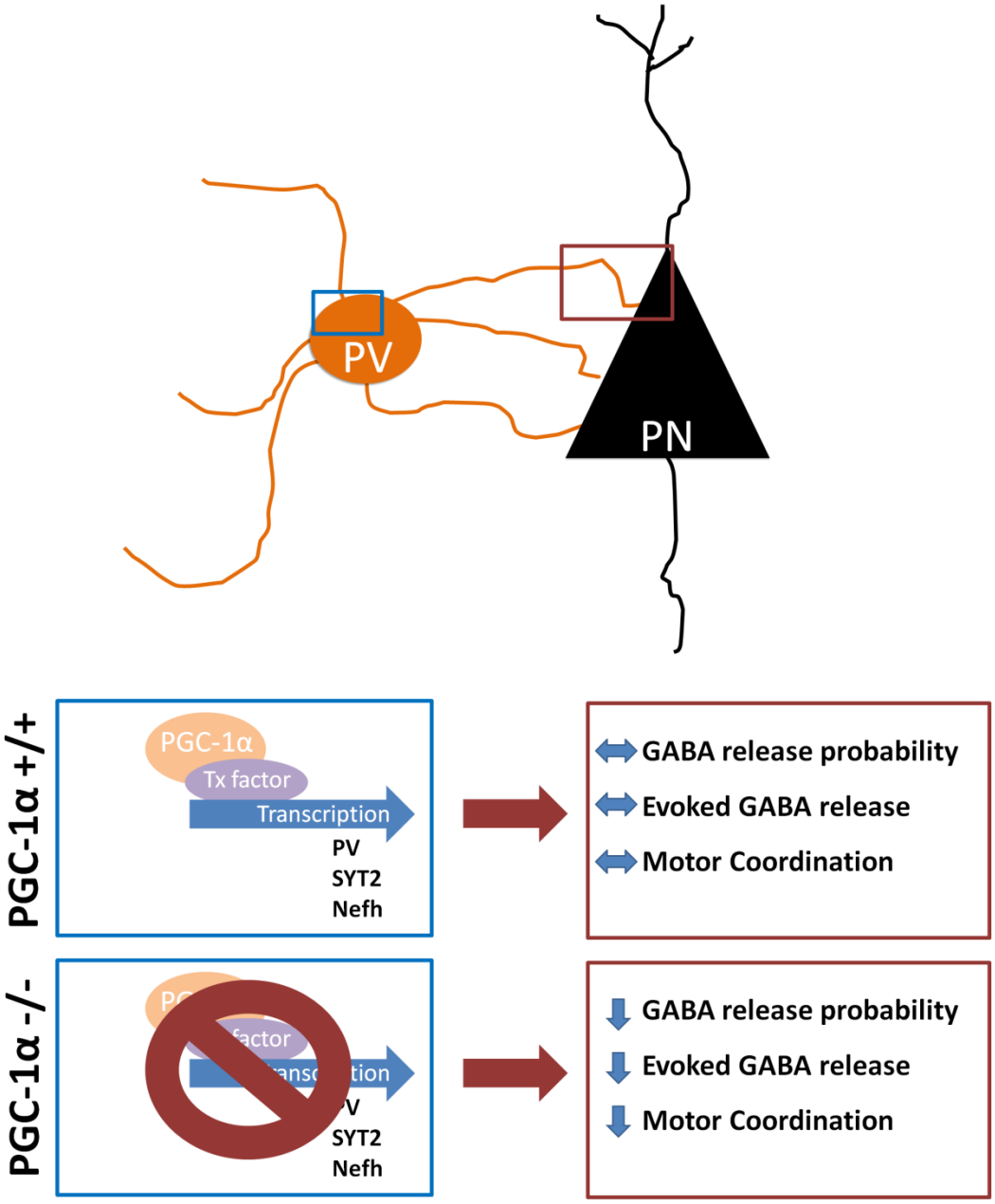

Figure 4.6. Model for Interneuron Dysfunction in PGC-1α -/- mice.

From the data presented here we propose the following model. In normal animals, presynaptic PV+ interneuron is able carry on normal transcriptional processes and properly regulates GABA release, resulting in normalization of cortical inhibition and locomotor activity. With loss of PGC-1α a reduction in its targets is observed this would cause reductions in inhibitory transmission (GABA release probability and overall evoked release) from interneurons in the cortex, manifested by alterations in coordination.

and inhibition. Here we show the synaptic dysfunctions that are the consequence of global loss of PGC-1α.

Acknowledgments

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CHAPTER V

DISCUSSION AND CONCLUSIONS

Huntington Disease (HD) is a devastating neurological disorder characterized by cognitive, psychiatric, and motor dysfunction. HD presents around midlife in most patients with debilitating neurodegeneration, which is ultimately fatal. At present, there are no disease altering or progression halting treatments available. The work presented here describes the role of select GABAergic cell populations in HD with the aim of better understanding potential mechanisms of cellular dysfunction and pathogenic occurrences throughout these cell populations.

Neurophysiological processes throughout the brain are tightly regulated by a balance of excitation and inhibition. It is through this balance that complex signaling and higher-level brain function are accomplished. The vast neuronal networks rely on the ability to fire in groups with temporal and regional control. Often, this fine control is lost in neurological disorders, and HD is no exception. In order to gain insights into the pathological functional changes that occur through the brain we have utilized a number of mouse models to answer distinct questions. These questions relate to the central theme of

GABAergic cell functioning in HD and whether alterations in inhibitory neuron function and/or viability play a role in the pathogenesis and progression of HD.

Chapter Highlights & Key Findings

In **chapter II**, we have assessed the transcriptional, protein, and firing abnormalities that occur within the cerebellum throughout the disease progression of HD. This work centers on the hypothesis that: *dysfunction in the cerebellum plays a role in the motor phenotype of HD through alterations in purkinje cell (PC) function and viability.* The main questions to be addressed in this section were: Where is the mutant protein expressed in the cerebellum? Along what timeline are inclusions present? Are cells lost throughout the disease progression? Do the output neurons continue firing as normal?

The cerebellar cortex is comprised of uniquely organized layers, which integrate at the point of the PC, which then provides the sole output of the region to the deep cerebellar nuclei and then the rest of the central nervous system. The general function of the cerebellum is motor control and so it was logical to postulate that any dysfunction occurring in the region in HD could contribute to the motor alterations seen as part of the disorder. In order to investigate this possibility we utilized multiple mouse models of HD and performed biochemical and electrophysiological analysis on the GABAergic cell populations of the region.

In both the R6/2 trangenic model and the HdhQ200 knock-in mouse model of HD, we observed alterations in levels of calbindin, a calcium binding protein, which is found in the PCs of the cerebellum. We also observed a high density of mutant huntingtin (mthtt)+ inclusions throughout the cerebellum including in the PC layer. We also

observed a decrease in overall cell numbers of PCs accompanied by a marked reduction spontaneous firing from the remaining PCs in both models.

The presence of mthtt+ inclusions in the cerebellum was not, in and of itself, novel. Studies have previously reported finding htt there (Bhide, Day et al. 1996); however, the expansion on the role that the mthtt is playing in the region is quite novel. In the R6/2 model one of the most stunning findings was the reduction in firing from the PCs prior to accumulation of inclusions in that cell population. The physiological firing deficits actually coincided with the formation of inclusions within the interneurons of the molecular layer within the region. It is intriguing to postulate that this could define a role for a non cell-autonomous role of mthtt in the cerebellum whereby disruption of normal cellular function in the interneurons results in downstream alterations in signaling to the PCs and be extension the further propagation of those signals from the PCs.

Beyond the potential input from the interneurons, another possibility is that it is the soluble form of mthtt that is playing the pathogenic role in PC dysfunction. Mthtt staining revealed what is potentially soluble mthtt, in the form of a diffuse soma-filling pattern, in the PCs at the time of the firing abnormalities, which could supply evidence for the theory that it is the soluble form of the protein that is pathogenic in the disease. This idea of soluble versus insoluble toxicity of mthtt will be addressed later in this discussion.

The cerebellum chapter also offered insight into the transcriptional alterations that can be caused by mthtt. There have been a number of reports on the potential interactions of mthtt with transcriptional machinery and interference with transcriptional

function (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009). While the HdhQ200 mice only displayed a reduction in calbindin, the more severe R6/2 model showed reductions in the transcript levels of calbindin as well as parvalbumin and glutamic acid decarboxylase 67. These markers of GABAergic cell populations are key for normal functioning within the cell and act as calcium buffers (CB and PV), as well as enzymatic producers of GABA (GAD67) the neurotransmitter necessary for inhibitory transmission. Loss of these transcripts could have meaningful ramifications for cellular functioning and viability. Previous studies have shown that changes in GAD67 can result in changes in cell firing which could be a contributing factor to the pathological firing changes observed here (Litwak, Mercugliano et al. 1990; Drengler and Oltmans 1993).

Cell loss is a common finding in HD, with the most pronounced cell loss occurring in the MSNs of the striatum (Vonsattel, Myers et al. 1985; Reiner, Albin et al. 1988; Aylward, Sparks et al. 2004). We quantified cell death in the PC layer and observed a reduction in the PC population in both the transgenic and knock-in models. Cerebellar atrophy has been reported previously in HD patients (Jeste, Barban et al. 1984; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004) and is especially common in the most severe cases of the disease, juvenile HD (Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). The recapitulation of this loss in a mouse model provides the opportunity to access the impact of these changes on the disease progression. Interestingly, even with the loss of a large percentage of the PC population, neither of these models exhibited an overt cerebellar-associated

motor phenotype, including ataxia, though some have reported mild ataxia in the R6/2 (Mangiarini, Sathasivam et al. 1996). We postulate however that the extreme motor dysfunction that is observed by the time of cerebellar atrophy might mask a behavioral phenotype that would otherwise be present if these changes were isolated from other regional pathologies.

Recapitulation of the cerebellar alterations found in the severe R6/2 model in the HdhQ200 knock-in model allowed for an extension of the findings into adult onset HD. One caveat to using the R6/2 model was the question of potential relevance of the findings for adult-onset HD. The knock-in mouse more closely models the pathogenesis of the more commonly presented from of the disease. The confirmation of the sensitivity of PCs in that model offers additional support to the suggested validity of the hypothesis of cerebellar dysfunction in HD.

It is not clear from theses descriptive studies how PC dysfunction contributes to the motor phenotype. In order to elucidate the role of the cerebellum in HD, we could utilize the same floxed gene approach followed by region specific cre recombinase. In order to test this hypothesis we could make use of the PC specific cre mouse model, L7/PCP2cre (Barski, Dethleffsen et al. 2000). This mouse would allow for PC specific expression of mthtt. If PC restricted expression of mthtt recapitulates any of the pathologies associated with HD than PCs must play a role in the disorder. One potential mechanism might be through the disynaptic connection of the cerebellum with the cerebral cortex through the ventrolateral thalamus (Kelly and Strick 2003). If cerebellar expression of mthtt results in reduced firing rates from the PCs, this could result in altered inhibition to PC downstream targets including the deep cerebellar nuclei and the

ventral lateral nucleus of the thalamus. This could result in propagation of the misfired signals throughout the downstream network. The thalamic nucleus further connects to the motor cortex. Altered neuromodulation of the cortex via the thalamus could contribute to the motor dysfunction observed in HD.

Based on our data, concerning soluble and insoluble mthtt, it is possible that the appearance of mthtt+ diffuse staining suggesting the soluble (or soluble intermediate) form, in PCs could be the aberrant form of the protein and therefore supports a cell-autonomous PC based location of pathology. PC firing occurs spontaneously in a pace-making manner, and which can either be increased or decreased in frequency to relay cerebellar messages to downstream targets (Nam and Hockberger 1997). Alteration in the precision of this pace-making has been shown to play a role in some disease states (Walter, Alvina et al. 2006). Global expression of mthtt results in a reduction in spike frequency from the PCs in both a transgenic (R6/2) and knock-in model (HdhQ200). On a cellular level there are a number of occurrences that could affect firing in this manner. These include aberrant interactions of mthtt with mitochondria, the ATP-generating organelle, resulting in a reduction in the overall energy load being produced, compromising the cells' ability to maintain firing.

Another potential mechanism of action would be altered cellular excitability and/or differential responses to calcium levels with alterations in the buffers responsible for calcium mediation. It is the role of buffers, including PV and Calb, to set the kinetics of calcium binding and responsivity. PV specifically has slow binding kinetics which specifically speeds the fast component of internal calcium concentration decay (Chard, Bleakman et al. 1993). This allows for a quick cellular "reset" which by extension allows

for the maintenance of fast firing and fast recovery (Caillard, Moreno et al. 2000). It is possible that alterations in the levels of these calcium regulators results in altered cellular physiology and a reduction in the cells' ability to maintain their normal firing patterns. Additionally, as mthtt has been shown to interrupt normal transcription (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009), the presence of the mutant protein could result in altered levels of transcription of channels that regulate cellular activity and excitability. One direct way to test this would be to examine the transcription levels of PC ion channels, but beyond that it would be intriguing to assess overall PC excitability using electrophysiology. This could be accomplished utilizing the patch clamp technique; in current clamp the individual PCs could be stepped through increasingly depolarizing currents to assess onset of spiking and therefore intrinsic excitability. If the alterations within the cerebellum in HD are PC-centric and if for some reason this is sufficient to result in cell loss, the observed changes in firing rate could be a sign that the PCs are starting to slow in firing as a result of intracellular processes being compromised.

It is also possible that the observed cerebellar dysfunction arises from a non cell-autonomous mechanism including the interneurons and surrounding cell populations of the region. As mthtt is present throughout the cerebellar layers it is possible that pathological dysfunction occurs in the cell populations upstream of the PCs. The granule cells, for instance, provide the main excitatory input to the PCs and it is plausible that altered excitation from the granule layer could result in reduced firing from the PCs. Also possible, is that altered inhibition from the interneurons of the molecular layer could

result in changes in PC firing. In order to fully elucidate the cause of these changes we could perform electrophysiological recordings from each of these cell populations and quantify firing properties. Further characterization of the intrinsic excitability of the PCs themselves would also be enlightening.

Chapter III of this thesis focuses on the parvalbumin subpopulation of cortical interneurons and the role that these neurons play in the etiology of HD. The main questions that were addressed as part of this work are: Could expression of exon 1 of mthtt only in PV+ cells result in an HD-like motor phenotype? Does expression of mthtt in PV+ cells alone result in an altered transcriptional profile? How does restricted expression of mthtt affect inhibitory transmission from the PV+ cells? These experiments are in submission in the manuscript entitled, "Restricted expression of mutant huntingtin to parvalbumin-positive cells causes hyperactivity and altered synaptic function in the motor cortex". This work centers on the hypothesis that: *PV interneuron dysfunction will contribute to the motor and regional physiology alterations seen in HD and that expression of the mutant protein in that cell population will recapitulate some aspects of the HD-associated pathology.*

Since interneurons play an essential role in the regulation of regional output (Jones 1993; Tepper and Bolam 2004; Wang, Tegner et al. 2004; Woo and Lu 2006), it follows that dysfunction in these neuronal populations could result in pathological alterations in network firing. Further, as the Yang laboratory suggested in 2005 (Gu, Li et al. 2005), interneurons could play a role in the cortical dysfunction in HD. These conclusions were based on the use of conditional expression of mthtt via the cre/lox system. They observed that with pan-neuronal expression of the mutant protein there

were alterations in cortical synaptic physiology and pyramidal cell morphology. However, upon pyramidal neuron-specific expression of mthtt, the physiological phenotype was not recapitulated. The overarching conclusion was the interneurons play a role in HD. However, as interneurons are a heterogeneous mixture, further exploration of specific roles for interneuron subtypes is essential for a full understanding of the cellular basis of HD pathology. In order to address this seminal issue, we utilized the same genetic tool of conditional expression and restricted mthtt to PV+ cell populations.

Upon restricted expression of exon one of mthtt the first overt presentation of an HD-like phenotype was hyperactivity at 10 and 12 months of age. This is interesting as reduced motor activity is more commonly found in mouse models of HD (Mangiarini, Sathasivam et al. 1996; Gu, Li et al. 2005). This increase in locomotor activity and time spent in motion is not accompanied by alterations in motor coordination. The conclusion that PV+ cell-restricted expression is sufficient to cause hyperactivity in a mouse model supports the theory of their playing an integral role in the pathogenesis of HD.

For the experiments in this chapter, we evaluated GABA release in the motor cortex by measuring both spontaneous and evoked inhibitory activity. sIPSC are the recordings obtained in a whole cell patch clamp configuration with no stimulation. These currents are the result of both spontaneous vesicle fusion events and action potential-mediated activity from the presynaptic inhibitory neuron. Alterations in sIPSC frequency often arise from changes in the presynaptic neuron. Frequency changes can result from an altered number of terminals connecting onto the post-synaptic cell, or altered excitability of the presynaptic cell. Changes in sIPSC amplitude are less straightforward, in that they can result from alterations at either the pre- or postsynaptic cell. Reduced vesicle load,

temporal summation, or vesicle availability can all result in a presynaptically founded change in sIPSC amplitude. However, changes in postsynaptic responsiveness, through receptor density for example, could result in an alteration in sIPSC amplitude that is postsynaptically mediated. In order to distinguish changes in the two types of activity that contribute to sIPSCs, the neurotoxin TTX can be bath applied to an acute slice and block action potential-mediated activity. This drug treatment allows for the quantification of changes in spontaneous fusion events, independent of action potentials.

As staining studies show, the mthtt protein staining remains colocalized with PV staining whenever found; this, in conjunction with the presence of physiological alterations suggesting presynaptic dysfunction, strongly supports the hypothesis that the root cause of the altered inhibition within the motor cortex stems from the PV+ cell populations. The primary question becomes, how is mthtt acting to cause dysfunction within the PV+ interneuron? At 12 months of age the reduced probability of GABA release could stem from the actions of soluble mthtt within PV+ interneurons. These activities could include aberrant interactions with mitochondria, resulting in reduced energy output for the cell to maintain proper firing. Also, it is possible that soluble mthtt inhibits normal protein-protein interactions at the site of the synapse resulting in reductions in vesicle loading and/or docking. Mthtt has been shown to interect with a number of essential synaptic proteins, including complexins, synaptotagmins, and PASCIN (Modregger, DiProspero et al. 2002; Edwardson, Wang et al. 2003; Li, Wyman et al. 2003). Alterations in GABAergic vesicle properties could result in the observed reduction in GABA release both spontaneously and with exogenous stimulation. Further, upon sequestration of mthtt into insoluble aggregates there is less soluble htt present to

disrupt normal cellular processes. This could be responsible for normal physiological functioning at 24 months of age. These normalizations include the amount of GABA release upon repetitive stimulation at the gamma frequency and GABA release probability.

One intriguing finding at 24 months of age is the continued presence of altered basal GABA release. It is possible that over time homeostatic mechanisms have resulted from ongoing alterations in basal release including postsynaptic receptor changes. It is also plausible that changes in spontaneous activity would be independent of evoked activity as studies have suggested that these processes are carried out by independent pools of vesicles (Sara, Virmani et al. 2005; Fredj and Burrone 2009; Hablitz, Mathew et al. 2009; Andreae, Fredj et al. 2012) and could therefore be differentially regulated.

One caveat to the cre/lox system is the potential genotoxicity of the long-term presence of cre recombinase within the nucleus (Loonstra, Vooijs et al. 2001; Forni, Scuoppo et al. 2006). As the role of the enzyme is to excise DNA fragments it is possible the over time random exclusion events could occur resulting in abnormal gene expression independent of the original target. In order to assess this possibility, we performed initial animal model characterizations on all potential genotypes form our cre/lox breeding paradigm prior to the selection of the most appropriate WT. In behavioral and initial electrophysiological characterizations the three potential WT groups: PVcre-WT, WT-Rosa and WT-WT there were no significant differences between groups. Further, as PVcre has the most potential to alter the phenotype, we selected it to compare without PVcre+ Rosa mice as they exhibit the same expression of enzymatic activity. Even at 24

months of age, PVcre mice did not differ from WT-WT or WT-Rosa mice in motor activity or mthtt+ inclusion formation.

One potential explanation for the amelioration of symptoms over time in our mouse model is that the production of mthtt protein diminishes with age. Though not directly tested, we believe it is unlikely that this is occurring in our mice. In a report published in 2000 from the Hen Laboratory, a mouse with inducible expression of mthtt was characterized. "Tet-off" mediated decreases in mthtt levels resulted in a similar rescue of behavioral phenotypes; however, one overt consequence was a reduction in inclusion density over 16 weeks of the "gene off" condition (Yamamoto, Lucas et al. 2000). As we observed an increase in inclusion density over time, we believe this refutes the supposition that mthtt levels are reduced with age in our model. In order to test this theory, PCR can be used to determine htt quantity at a given time.

Additional investigations that could strengthen our conclusions about the relationship of mthtt expression to inclusion formation include regional and longitudinal gene expression studies to assess levels of mthtt. This would allow for conclusions to be made as to the effectiveness of the cre recombinase enzymatic activity and could provide a potential explanation for variability in inclusion formation, with the cortex presenting the highest density at all time points.

While inclusions were never observed in the striatum, it remains that the soluble form of mthtt could be acting there to produce hyperactivity symptoms. There are multiple ways that we could address this issue. Initially, we could perform electrophysiological recordings, at our different time points, in the striatum of conditional

HD mice. If electrophysiological characterizations reveal changes in inhibition from PV+ cell populations, similar to that observed in the cortex, then our overall mechanistic explanations would change. If a reduction in GABA release was observed in the striatum in the conditional HD mice then we would suspect that there could be an increase in firing from the projection neurons. If, for example, the medium spiny neurons (MSNs) in the direct pathway are firing more frequently, then an increase in movement could result, while if the MSNs of the indirect pathway are more affected, then a reduction in movement might be observed (Albin, Young et al. 1995).

Another potential approach for elucidating the regional specific contribution to behavior in these animals is to utilize a different method of cre expression (Guo, Gopaul et al. 1997; van der Neut 1997). This would allow for specific expression and completely eliminate peripheral effects of mthtt in other tissues including the muscle and spinal cord. Further, this experiment would be an additional way to assess whether the mutant protein is acting in the striatum in any form. If PVcre expression through injection in the motor cortex results in a recapitulation of the phenotype observed in our genetic model than this would implicitly strengthen our conclusions that the point of action is the motor cortex. However, if expression of exon 1 of the mutant protein restricted to the motor cortex does not result in a phenotype, then we must conclude that other regions contribute.

It would be interesting, independent of the comparison to our pan-PV+ cell model, to perform these types of region-specific experiments, with additional models of HD, to elucidate a region-to-region role in the disorder. For instance a variety of cre mouse models exist with expression patterns limited to other cell populations and (through injection of the enzyme) full brain regions, such as the cerebellum. Using these

types of conditional expression models it would be possible to define regional roles in the disorder. However, the main complicating factor is whether one can actually separate the roles of a discrete region from another. That is to say that it is possible that the "whole" of HD, and the mutant protein's pathological actions in the brain, is greater than the "sum of its parts". It is possible that upon independent expression of mthtt, in a restricted fashion by region systematically, would never fully recapitulate what could be observed with pan-neuronal or global expression. If this were the case then the assumption would become that full expression of symptoms requires inter-region interactions.

Chapter IV shifts focus to determine the roles for the mthtt-interacting protein PGC-1α in cortical function. In that section, our hypothesis was that *as PGC-1α is concentrated in GABAergic cell populations, loss of the coactivator, and by extension it downstream targets, will cause dysfunction in the inhibitory signaling throughout the brain*. The main questions that were addressed in this section were: Does loss of PGC-1α result in an electrophysiological phenotype similar to that seen with ablation PV or SYT2? Are changes in the cortex similar to the alterations that have been shown in the hippocampus by Lucas et al 2010? How do these results compare to what was observed with cell-specific expression of mthtt?

Using electrophysiological recordings we observed that both spontaneous and evoked GABA release are reduced in the motor cortex in PGC-1α KO mice. These alterations likely stem from changes in the presynaptic interneuron as PGC-1α is highly concentrated in GABAergic cell populations. There is a reduced probability of GABA release upon paired-stimulation and a greatly reduced charge, or overall GABA release upon stimulation at the gamma frequency. These findings mirror what was observed at 12

months of age in the conditional HD mice utilized in **chapter III**. Importantly, as many groups have postulated that PGC-1α could be used as a potential treatment target for neurodegenerative disorders (McGill and Beal 2006), here we provide evidence that although an HD phenotype is not overtly recapitulated with the PGC-1α -/- mice, there may be some important similarities.

Though not all of the physiological alterations in the PGC-1α null mice mirror those observed in HD, it is still interesting to speculate on a potential role for the coactivator in the disorder. It is possible that while changes in the levels of PGC-1α might not play an essential part of the pathological timeline of the disease that its downstream targets do. These transcriptional targets, including PV (Lucas, Markwardt et al. 2010), might represent potential therapeutic targets. That makes the coactivator a prospective tool for induction of these essential proteins and therefore characterizations of PGC-1α still hold value for neurodegenerative diseases, including HD.

Overall Themes

GABAergic Cell Populations & HD

Throughout these investigations the recurrent theme has been the importance of alterations in inhibitory neuronal populations throughout the brain and their role in the pathology of HD. It has become evident that not only are GABAergic cell populations affected throughout the disease progression of HD but that they may play a causative role in some of the hallmark features (ie- hyperactivity) of the disorder. While region-specific PV+ cell mthtt expression would be required to ascertain whether the cortical interneurons themselves are causing hyperactivity, we can conclude that mthtt expression

globally in PV+ cells is sufficient to cause hyperactivity. What is clear is that the GABAergic cell populations are dysfunctional in both a conditional model and multiple global models of HD.

Additionally, it is not uncommon for GABAergic cell populations to have a high energy demand. This might stem from the intrinsic properties of inhibitory cells in network regulation. They function at the most basic level to limit over-excitation throughout neuronal circuits. Additionally, GABAergic cells include populations that are fast-spiking (ie- PV interneurons), pace-making (PCs), and projection neurons (MSNs). Though not directly tested here, it seems worth noting that all of these high energy requiring cell populations are also vulnerable in HD. It is intriguing to postulate that the cellular specificity observed in HD could be correlated with greater energetic needs.

Soluble versus Insoluble

There is much discussion over whether the pathogenic form of mthtt is the diffuse soluble form of the mutant protein or the aggregated insoluble form (Cooper, Schilling et al. 1998; Martindale, Hackam et al. 1998; Saudou, Finkbeiner et al. 1998; Arrasate, Mitra et al. 2004; Bowman, Yoo et al. 2005). In both **chapter II** and **chapter III**, both forms are observed using immunostaining. In **chapter II**, at the time of firing deficits, the soluble form of mthtt is more commonly found in the PCs of the cerbellum, however the insoluble aggregates are already present in the presynaptic interneurons. In **chapter III**, at the time of pronounced motor and cortical physiological alterations there is evidence for the soluble form (DAB immunohistochemistry), and as the HD-like phenotypes normalize with age the density of the insoluble aggregates increases. Overall, we believe

that our findings support the theory of the soluble protein, or soluble toxic intermediate conformations, interfering with protein-protein interactions potentially at the site of the synapse to cause dysfunction shown here. Our most compelling evidence in support of this theory is the indirect correlation between HD-like phenotype presentation and inclusion density found in our conditional model in **chapter III**.

Cell Autonomous versus Non-Cell Autonomous

Another point of contention in the HD field is whether mthtt functions only within the cell populations in which it is found, in a cell autonomous fashion or whether it can act aberrantly across cell populations to affect the network even with restricted expression (Gu, Li et al. 2005; Thomas, Coppola et al. 2011). In **chapter III**, we clearly demonstrate that restricted expression of the protein results in inclusion bodies that always colocalize with the anticipated neuronal subtype. However, with the overall changes in network functioning both in this conditional model and the global expression transgenic models, it may be perhaps disruption of function in a cell-autonomous manner in the interneurons of the cortex, for instance, is sufficient to cause regional dysfunction as a consequence regardless of direct action in the secondary populations by mthtt.

Implications for Phenotypic Rescue & Treatment

Multiple reports have shown that upon disruption of mthtt expression and/or function, whether by inducible terminal or by injected interference, some HD-associated pathological phenotypes can be ameliorated (Yamamoto, Lucas et al. 2000; Regulier, Trottier et al. 2003). We believe that our findings further cement the finding that HD is not irreversible in mouse models and that interruption of mthtt function; one can rescue

phenotypes ranging from behavioral abnormalities to cellular physiological dysfunction. This is promising as rescue on the most basic level of cellular functioning could hold the true key to taking therapeutic options from symptom-based treatment to disease progression-halting (or reversing) cures.

Additional means of rescue could be attempted on a variety of levels. Physiologically, it would be interesting to examine whether recapitulation of the calcium buffer, PV, itself could rescue the alterations in firing found in both the PCs (**chapter III**) and PV+ interneurons (**chapter IV**). Further, if it is interactions between the mutant protein and mitochondria leading to an energetic deficit, treatment with additional ATP and or agonists of mitochondrial complexes could assess whether meeting the cellular energy demand could rectify the pathological alterations observed. Finally, if it is in fact the soluble form of the mutant protein that is most pathogenic than drug induced inclusion formation could be an additional method of toxicity reduction in HD.

Overarching Conclusions

HD is a destructive genetically inherited neurological disorder with swift and devastating implications for patients and families alike. Treatment options are currently focused on improvement of daily life despite progressive deterioration. Here we present data suggesting that cortical PV+ inteneurons and PCs could represent important and novel cellular targets for treatment avenues. With the continual push toward treatment with antisense oligonucleotides and cell-specific protein agonists and antagonists, perhaps it is PGC-1α itself that could be used as a tool to upregulate its downstream targets, including PV, to promote overall cell health and viability.

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APPENDIX

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: January 14, 2013

TO: RITA COWELL, Ph.D.
SC -729 0017
FAX: (205) 975-4879

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: PGC-1alpha and GABAergic Dysfunction in Huntington Disease
Sponsor: NIH
Animal Project Number: 130109037

As of January 14, 2013, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	0
Mice	B	0
Mice	C	0

Animal use must be renewed by January 13, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 130109037 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
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