Role of Striatal Afferents in Evolution of an Animal Model of Huntington's Disease

Alexandre Boutet
Integrated Program in Neuroscience
McGill University, Montreal

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative autosomal disorder caused by expanded CAG repeats in the mutated Huntingtin gene. It is characterized by predominant loss of striatal neurons. Consequently, patients suffering from this disease demonstrate progressive movement disorders, memory loss, and psychiatric problems until death. Three types of mouse models have been engineered to study this disease: knock-out, transgenic, and knock-in. Many attempts have been done to rescue the neurons in HD based on the neurotrophin hypothesis. The main objective of this project is firstly to test whether the R6/2 mouse is an appropriate anatomical and behavioral model for HD and secondarily to further review the neurotrophin hypothesis as a potential therapy.

Keywords: Huntington`s Disease, Brain-derived neurotrophic factor, Nerve Tissue Proteins, Mice

Transgenic

RÉSUMÉ

La maladie de Huntington (MH) est transmise de façon autosomique dominante causant une dégénérescence neurologique principalement dans le corps strié. Cette dernière est associée à une augmentation de la répétition des acides nucléiques CAG au sein du gène Huntingtin muté. Conséquemment, les patients atteints de cette maladie souffrent de troubles moteurs, cognitifs et psychiatriques jusqu'à leur mort. Trois catégories de modèles de souris ont été conçus pour étudier cette maladie : knock-out, transgénique et knock-in. Plusieurs tentatives utilisant l'hypothèse des neurotrophines ont été employées pour prévenir la dégénérescence neuronale dans MH. Les objectifs principaux de ce projet sont en un premier temps de déterminer si la souris R6/2 est un modèle anatomique et comportemental satisfaisant et en un second temps de pousser l'étude des neurotrophines comme thérapie potentielle.

Mots-Cléfs: Huntington`s Disease, Brain-derived neurotrophic factor, Nerve Tissue Proteins,

Mice Transgenic

ABBREVIATIONS

AAV:	adeno-associated virus
BDNF:	brain-derived neurotrophic factor
CNS:	central nervous system
CM:	centromedian nucleus of the thalamus
C-PK:	peak compression force
ELISA:	enzyme-linked immunosorbent assay
ENK:	enkephalin
EOHD:	early onset Huntington's disease
g:	grams
GABA:	gamma-amino-butyric acid
GPi:	internal segment of the globus pallidus
GPe:	external segment of the globus pallidus
HSV:	herpes simplex virus
HD:	Huntington's disease
HADC2:	histone deacetylase 2
HAP-1:	huntingtin-associated protein-1
htt:	huntingtin protein
MH:	maladie de Huntington
mhtt:	mutant hutingtin protein
mRNA:	messenger RNA
NMDA:	N-methyl-D-aspartate
PF:	parafascicular nucleus of the thalamus
rAAV:	recombinant adeno-associated virus
rAAV2:	recombinant adeno-associated virus serotype 2
rAAV4:	recombinant adeno-associated virus serotype 4
rAAV5:	recombinant adeno-associated virus serotype 5
r.p.m.:	revolutions per minute
SN:	sunstantia nigra
SNc:	substantia nigra pars compacta
SNr:	substantia nigra pars reticulata
STN:	subthalamic nucleus
SP:	substance P
T-PK:	peak tension force
trk:	tropomyosin-related kinase
WT:	wild-type

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Alexandre Boutet, Fall 2014

PREFACE AND CONTRIBUTION OF AUTHORS

Regarding the work presented in the Chapter 3.0, Dr. P. Samadi was the main senior investigator under the supervision of Dr. A. F. Sadikot. She contributed to all facets of the project including literature search, project design, data collection and manuscript writing.

I participated in the design of the project including the hypothesis generation. Under the supervision of Dr. Samadi, I learned the experimental protocols and performed and participated in all data collection and analysis. I was also involved in manuscript writing.

The remainders of the co-authors were involved to a lesser degree in diverse facets of the project.

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CHAPTER 1

THE PROBLEM RATIONALE

1.1 Rationale

One of the major research areas in HD involves the generation of various mouse models that ideally reproduce as accurately as possible the pathological, molecular and cellular abnormalities seen in patients suffering from that particular disease, subsequently allowing testing of different therapeutic strategies. However, controversy remains over what mouse models is the most appropriate to study HD.

Multiple therapeutic strategies have been studied in HD without convincing success. However, most specifically studies (Gharami, Xie, An, Tonegawa, & Xu, 2008; Xie, Hayden, & Xu, 2010) using the neurotrophin hypothesis produced evidence of good outcomes. Recent neuro-anatomic advances make it possible to refine therapies using neurotrophins.

1.2 Research Impediments

Major variability in assessment methods of mouse models may be an impediment to data interpretation. In order to control for variability in assessment, we used well-accepted behavioral tests and neuroanatomical analysis methods, at multiple time points during the HD mouse lifespan. Summary of the different tests used as well as their strengths and weakness is presented in Appendix I. Inherent differences in mice were also controlled using pre-intervention baseline studies.

CHAPTER 2

LITERATURE REVIEW: HUNTINGTON'S DISEASE

2.1 Historical overview

Huntington's disease was initially described in 1872 by a general medical practitioner known as George Huntington. William Osler was positively surprised by the quality of the medical writing (Brody & Wilkins, 1967). Unfortunately, this disease was often associated with a negative stigma (Wexler, 2010). For example, neurologists' recommendations in the 1970s would include sterilization. Stigmatization is often attributed to ignorance, however, in the history of HD, science contributed to strengthening of negative prejudices. After it was established in 1909 that this disease was inherited through generations in a Mendelian fashion, efforts were made to change immigration policy (Wexler, 2010). With ongoing historical work, it has been suggested that the infamous New-England witches of the late seventeenth century could have been afflicted with HD (Loi & Chiu, 2012). It was not until further advances in molecular biology that major scientific breakthroughs regarding Huntington's disease were achieved. In the early 1980s, the diseased gene was mapped to the chromosome 4 and in 1993 the CAG-expansion repeats were discovered (Bates, 2005). From then on, Huntington's disease was partially demystified and conventional scientific approaches using cellular and animal models were developed to further the research towards a cure.

2.2 Clinical manifestations

Classic medical teaching often categorizes Huntington's disease with the rare genetic conditions. However, its prevalence may be as high as 10 per 100 000 (Quinn & Schrag, 1998).

Some countries such as Japan, China, Finland report a considerably lower prevalence of HD (Quinn & Schrag, 1998). Given that a number a people are asymptomatic but at an increase risk to develop the disease, the burden of this disease is considerable. In most cases, these symptoms have a midlife onset but they may begin before 20 years of age (Kernich, 2008). The mean duration of the disease is 15-17 years and patients usually pass away in their 50s (Quinn & Schrag, 1998). The symptoms that patients experience are quite heterogeneous especially at disease onset. HD covers a wide array of clinical medicine in terms of symptomatic findings. Motor manifestations are most commonly manifested initially as clumsiness, balance problems and uncontrolled movements in the extremities and face. This usually progresses to chorea (i.e. constant uncontrollable writhing motions) and dysphagia (Kernich, 2008). Cognitive findings usually include diminished concentration, memory and judgment resulting impaired decision making. Eventually, the patients are unable to care for themselves (Kernich, 2008). Similarly, patients often present with psychiatric issues including early onset of mood swings and later with major depressive episodes and apathy (Kernich, 2008). For most patients, the course of the disease is very debilitating and the quality of life during the late stages is poor. A standardized way to assess severity of symptoms is the Unified Huntington's Disease Rating Scale ("Unified Huntington's Disease Rating Scale: reliability and consistency. Huntington Study Group," 1996). Juvenile Huntington's disease or early onset Huntington's disease (EOHD) refers to patients presenting with symptoms before the age of 20. It accounts for approximately 7% of the patients suffering of HD (Sakazume et al., 2009). Clinically, EOHD differs from conventional adult HD. Young-onset patients show more rigidity, oral motor dysfunction, ataxic gait,

behavioral disturbances and seizures (Sakazume et al., 2009). In general, the earlier the onset of the disease, the poorer is the prognosis (Kernich, 2008). However, even though the archetypal model of chorea is HD, chorea may represent a variety of underlying disorders (Quinn & Schrag, 1998). Therefore, sound clinical practice is always recommended when facing a new diagnosis of HD.

2.3 Genetics

HD is an autosomal dominant disease meaning that one of the parents has to have the mutation in order to have an affected child; hence, the chances of giving birth to an affected child is 50% if one parent is affected. It involves a mutated gene on chromosome 4, more specifically the IT15 region. This gene codes for a protein called huntingtin (htt). In the case of HD, there is an expanded stretch of glutamine residues, CAG repeats, attached to its NH2-terminal. 36 repeats is the commonly accepted threshold to be at risk for developing the disease and those with 40 repeats or more will be symptomatic (Langbehn et al., 2004). The number of repeats predicts the risk of HD following the anticipation model (i.e. subsequent generations may have earlier onset and a more severe disease if transmitted via an affected male) especially if the affected parent is the father. Also, we know that the higher the number of repeats, the earlier and more severe the disease will be (Nahhas, Garbern, Feely, & Feldman, 2009). Of note, CAG repeats are also involved in other disorders such as spinobulbar muscular atrophy and spinocerebellar ataxia type 1 (Trottier et al., 1995).

2.4 Pathology

The hallmark pathological feature of HD brain is the symmetric atrophy of the striatum in 95% of the cases (Vonsattel & DiFiglia, 1998). There is also associated atrophy of other brain areas. The most commonly affected area after the striatum is the frontal lobe in 80% of cases. As well, non-striatal regions (e.g. cingulate gyrus, amygdala, hippocampal formation) can also show increased atrophy with concomitant superimposed morbidity (Vonsattel & DiFiglia, 1998). It seems that there is selective vulnerability of medium spiny neurons as they are the ones most severely affected. This may be a function of specific glutamate receptor subtypes (Vonsattel & DiFiglia, 1998).

2.5 The basal ganglia

By definition, the corpus striatum refers mainly to three nuclei: the caudate nucleus, the putamen and the globus pallidus. The basal ganglia is involved in the so called basal ganglia-thalamocortical circuits. The putamen receives inputs from motor areas of the cortex and sends outputs to the internal segment of the globus pallidus and substantia nigra pars reticulata (SNr) via a direct and an indirect pathway (DeLong, 1990). The direct pathway is composed of GABAergic neurons projecting both to the internal segment of the globus pallidus (GPi) and the SNr. The GABAergic neurons in the indirect pathway project indirectly to the internal segment of the globus pallidus and the SNr via the external segment of the globus pallidus (GPe) and the subthalamic nucleus (Y. Wu, Richard, & Parent, 2000). Then, both GPi and SNr project to the thalamus which in turn completes the circle by sending projections back to the cortical motor

areas. According to the neurochemical nature of the neurons and their basal activity, the direct pathway facilitates cortical motor activity and whereas the indirect pathway inhibits it (Figure 2.0) (Bolam, Hanley, Booth, & Bevan, 2000). And it is the balance of activity between these two pathways that plays an important role in the proper execution of movements. The neurochemical signature of the direct is pathway is the substance P (SP) and the enkephalin (ENK) is the neurochemical marker for the indirect pathway (Katzung, Masters, & Trevor, 2012). In HD, it was found that neurons from the indirect pathway are more vulnerable, allowing unopposed direct pathway actions and therefore an hyperkinetic state (Centonze, Bernardi, & Koch, 2007).

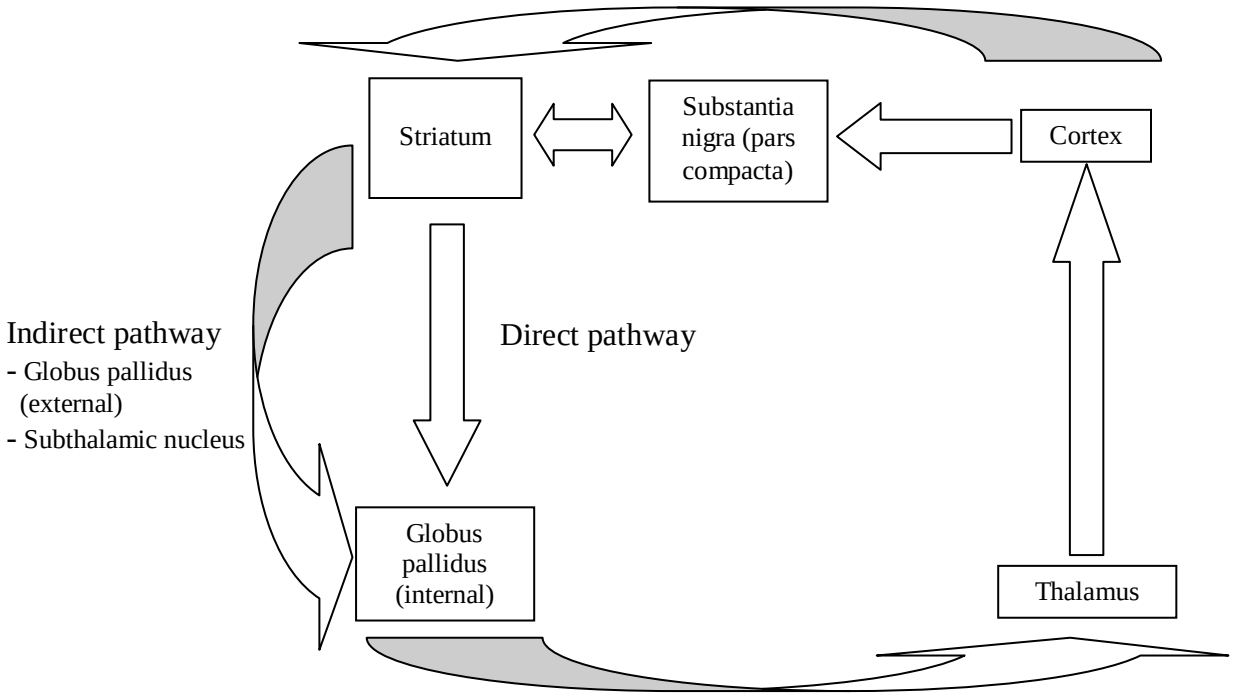

Figure 2.0: Illustration depicting the two major pathways between the cerebral cortex, basal ganglia, and thalamus and their respective neurochemical markers. *Adapted with modifications from* (Katzung, Masters, & Trevor, 2009).

2.6 Pathophysiology

The htt protein is expressed in all regions of the rodent brain with moderate heterogeneity (Strong et al., 1993). Similar results are obtained when studying human brains (Strong et al., 1993). The mutant huntingtin proteins (mhtt) found in HD is also distributed in a similar fashion (Strong et al., 1993). The striatum is not one of the regions with the higher levels of expression of the protein, and therefore levels do not correlate with extent of neurodegneration (Trottier et al., 1995). Furthermore, the htt is widely distributed in non-neuronal tissues including the gastro-intestinal tract, liver and testes (Strong et al., 1993) but the highest level of expression remains in the brain (Trottier et al., 1995). Mainly three pathological hypotheses regarding the mutated proteins have been generated (Trottier et al., 1995):

1. Modification of the structure/function of the protein causing abnormal interactions with other proteins
2. Action of neuronal transglutaminases which use the altered protein as an abnormal substrate, leading to accumulation of toxic cross-linked by products
3. Modification of physiochemical properties of the mutated protein or an alteration of its catabolism, leading to the intracellular deposition of toxic fragments

The most widely studied hypotheses have been the first and third one. For a long time, it was observed that there were cellular aggregates of mhtt, however the connection between HD and these aggregates is not clear (Truant, Atwal, Desmond, Munsie, & Tran, 2008). It was noted that the more advanced stages of HD will contain increased aggregates. Surprisingly, recent studies suggest that larger aggregates may in fact be protective for the neurons (Truant et al., 2008). The explanation put forward initially was the toxic gain-of-function acquired as a result of mHtt. Therefore, aggregates could represent a cellular protective mechanism to localize the toxic

mHtt into an insoluble inactive form. Again, further research refined the hypothesis to a gain-of-function and loss-of-function hypothesis meaning that the inactive insoluble proteins also contribute to loss of important function in the cells. A complex interplay of biological and chemical modulators regulate the equilibrium between the soluble without structure healthy Htt, the soluble with a structure Htt leading to a gain of function and the insoluble with a structure Htt leading to loss of normal function (Figure 2.1) (Truant et al., 2008).

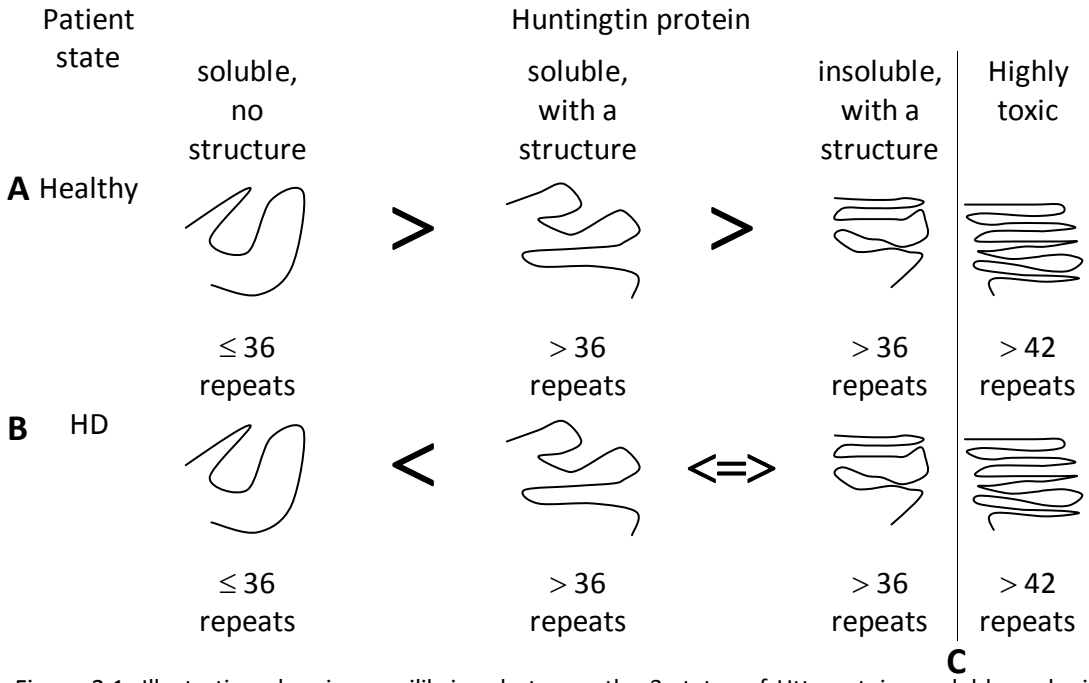

Figure 2.1: Illustration showing equilibrium between the 3 states of Htt proteins: soluble and without structure (healthy), soluble with a structure, insoluble with a structure A: healthy patient with equilibrium skewed towards soluble Htt without structure (healthy) B: HD patient with mHtt that has equilibrium skewed towards unhealthy state, soluble with a structure (gain-of-function) and insoluble without a structure (loss-of-function). Biological and chemical modulators influence the equilibrium between the two latter states. C: HD patients with abnormally long amount of repeats with mHtt essentially being a insoluble structure with loss-of-function or a soluble structure with toxic gain-of-function. *Adapted with modifications from Truant, Atwal, Desmond, Munsie & Tran, 2008.*

HD is therefore the result of a cascade of events that produce multiple insults to the homeostatic process (Rosas et al., 2008). Deficiencies in mitochondrial complexes (Rosas et al.,

2008), protein-protein interactions with secondary toxicity (Subramaniam, Sixt, Barrow, & Snyder, 2009) and even cerebrospinal fluid differences (Fang et al., 2009) have been implicated. From the earliest hypotheses focused around the mHtt proteins, currently, given all the newly implicated factors in this homeostatic process, there are four main hypotheses being studied (Figure 2.2) (Roze, Saudou, & Caboche, 2008) (Vonsattel, 2008). Excessive stimulation of receptors, especially N-methyl-D-aspartic acid (NMDA) subtypes, leads to excitotoxicity and neuronal death. Mitochondrial dysfunction has also been implicated as features of HD are reproduced in animal models after mitochondrial toxin ingestion. Deregulation of large genomic regions in HD models and HD patients is also associated with disease progression. For example, striatal biochemical markers (i.e. ENK and SP) mRNA are altered in HD models (Augood, Faull, Love, & Emson, 1996). Htt has been implicated in the vesicular transport along the cytoskeleton and it is believed that mHtt interferes with axonal transport.

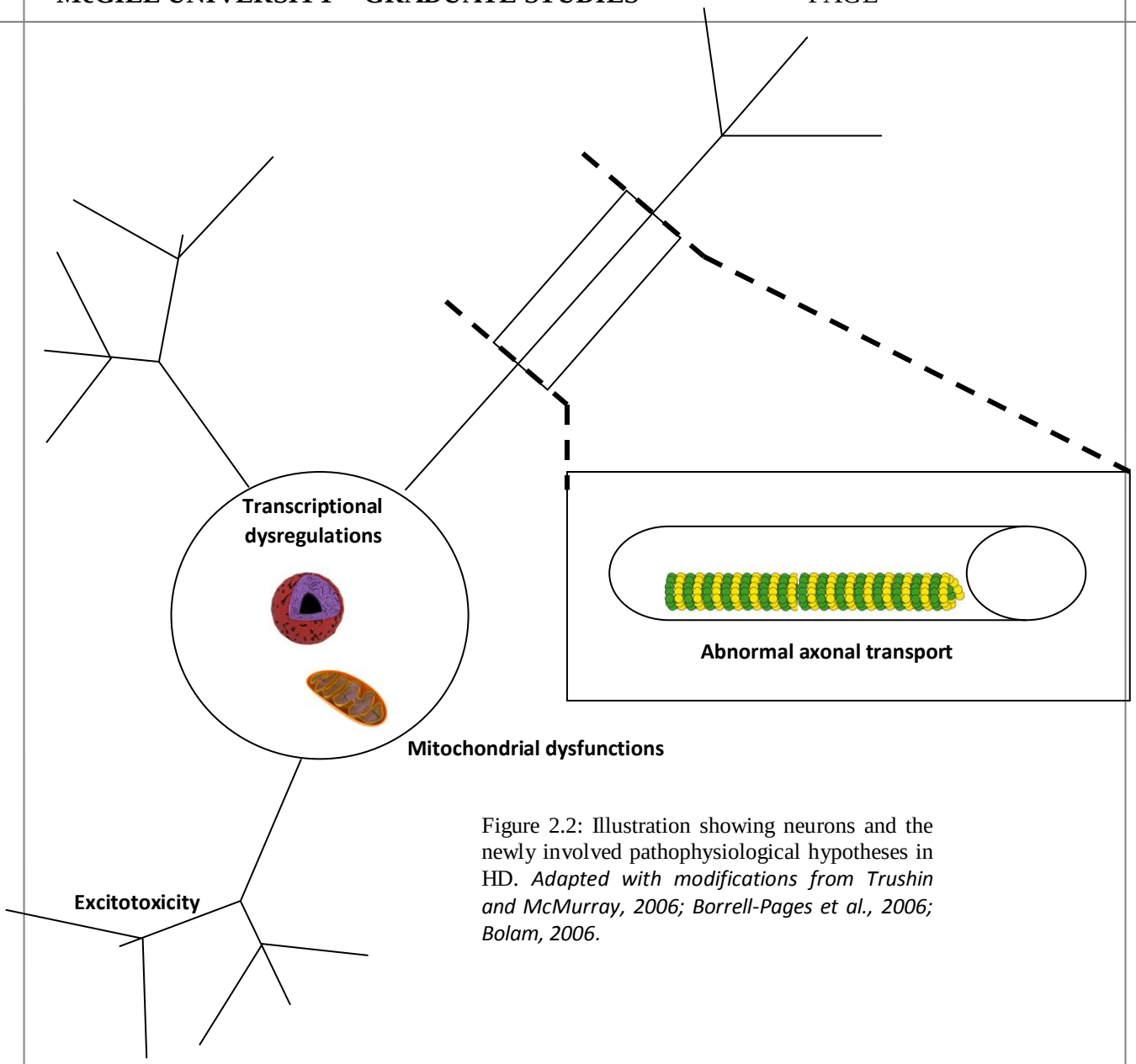

Figure 2.2: Illustration showing neurons and the newly involved pathophysiological hypotheses in HD. *Adapted with modifications from Trushin and McMurray, 2006; Borrell-Pages et al., 2006; Bolam, 2006.*

2.7 Emerging therapies

In view of the new advances in HD research and hence the new hypotheses being generated, emerging therapies are now being studied (Ross & Tabrizi, 2011). Unfortunately, very few breakthroughs in terms of medical therapies and only limited amount of high quality clinical trials have been conducted in the field of HD (Mason & Barker, 2009). Current treatments are symptomatic only. Ultimately, we would hope for a therapy capable of slowing

down and changing the natural history of HD. There is an urgent need for a disease modifying therapy. Current, research focuses on new targets for drug therapies (Figure 2.3). These new targets involve cellular components such as the messenger RNA, histones, the ubiquitin-proteasome system and the autophage-lysosome pathway (Mason & Barker, 2009). Different strategies involving preservation of the affected neurons using neurotrophins or replacing the dying neurons with cell transplantation are also under investigation (Mason & Barker, 2009) (Rosser & Dunnett, 2003). Despite the mitigated pre-clinical results, there is an effort to conduct high-quality trial to assess emerging therapies in HD (Huntington Study Group, 2008).

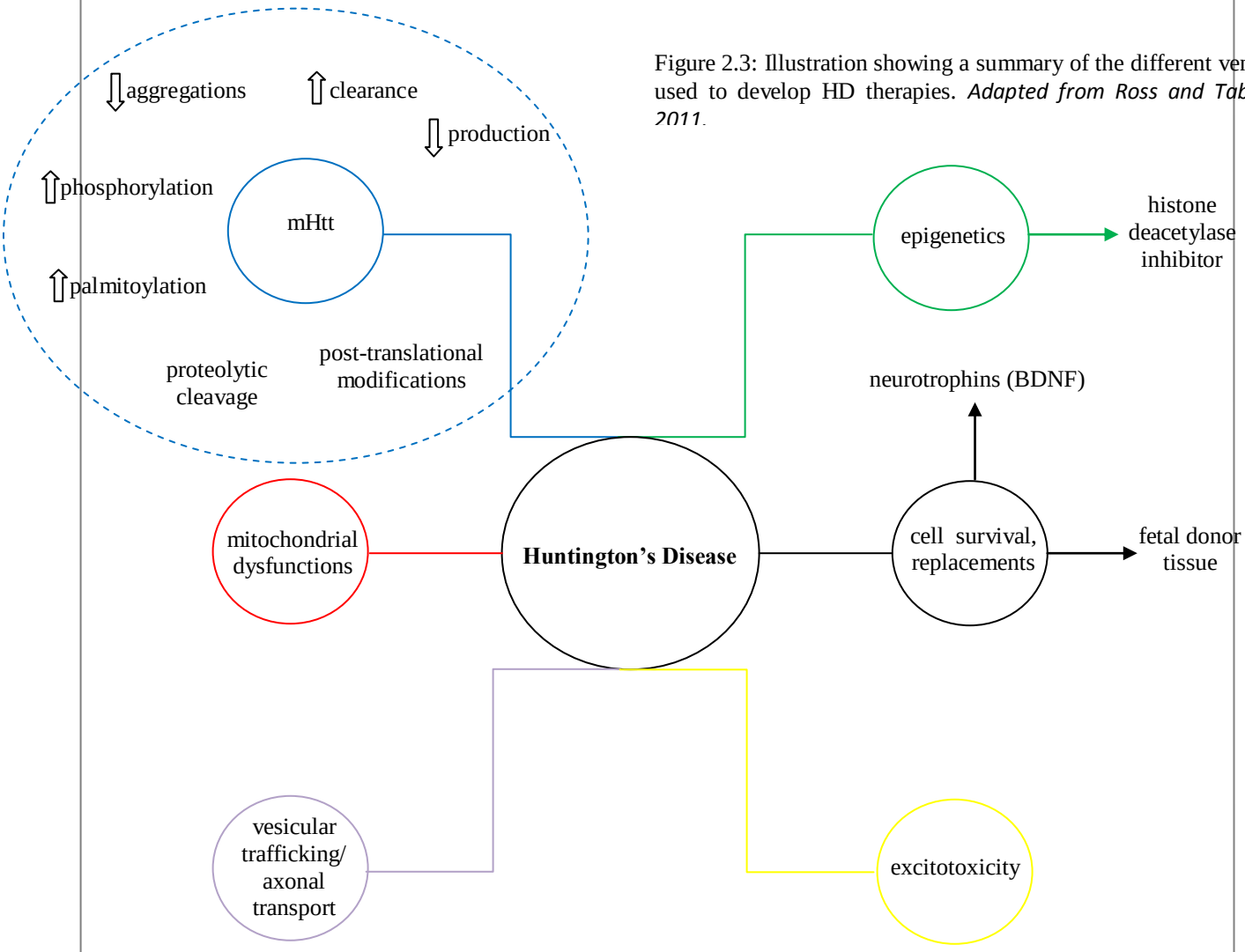

Figure 2.3: Illustration showing a summary of the different venues used to develop HD therapies. *Adapted from Ross and Tabrizi, 2011.*

2.8 Research Questions and Hypotheses

General: We hypothesize that improving behavioral outcomes in an HD mouse model is possible. The project can be separated in two parts. We first performed a literature review of HD mouse models with subsequent focus and assessment of the R6/2 mouse model. We hypothesize that it will an appropriate behavioral and anatomical model. Similarly, after literature review of HD therapeutic strategies, we will use the neurotrophin hypothesis to improve HD phenotypes in R6/2 mice.

2.9 Literature Review : Huntington's disease mouse models

Mouse models, especially the transgenic models, are arguably a powerful tool for understanding neuropathological mechanisms of HD. They also provide an efficient and reliable way to test new compounds aimed to be disease modifying drugs before human trials are undertaken (Mason & Barker, 2009). Over the years, three mouse models have been engineered: knock-out mice, knock-in mice and transgenic mice (L. B. Menalled & Chesselet, 2002) (see table 2.0 for summary).

Mouse Models / Characteristics	Transgenic				Knock-in		
	Truncated IT15 gene		Full-length IT15 gene				
	R6/1	R6/2	YAC	HD	Hdh50	Hdh94	Hdh150
Number of CAG repeats	115	145	18-72	16-89	48	94	150
Age of death (weeks)	32-40	10-13	>52	50-100	Not reporter	Normal lifespan	>52
Behavior	15-21 weeks (clasping)	5-6 weeks (motor deficits)	7 months hyperkinesia otherwise none	8 weeks hyperkinesia	None (up to 6 months)	2 months hyperkinesia, 4 months hypokinesia	4–10 months: gait and rotarod deficits, clasping, hypoactivity
Neuropathology	No morphological changes, NII at 5 months	Brain atrophy, NII at 4 weeks	Some striatal neuronal detah at 12 months otherwise none	Gliosis and neuronal loss in some models	None (up to 6 months)	Early cell dysfunction, no gliosis/neuronal death (at 6 months)	Gliosis, no neuronal death (up to 1 year)
Abbreviations: NII: neuronal intranuclear inclusions							

Table 2.0 Summary: Table depicting the various behavioral and morphological characteristics of the most commonly used HD mouse models. (L. B. Menalled & Chesselet, 2002) (L. B. Menalled, 2005) (Carter et al., 1999; L. B. Menalled & Chesselet, 2002)

The earliest model, the knock-out, showed that homozygous knock-out of htt was lethal in utero, suggesting an essential embryonic role for the protein (M. P. Duyao et al., 1995). It was subsequently shown that mutant hungintin protein can rescue these homozygous knock-out

mice (i.e. no wild-type huntingtin protein), hence suggesting the gain-of-function hypothesis. (Dragatsis, Levine, & Zeitlin, 2000; L. B. Menalled & Chesselet, 2002). However, further experiments with conditional knock-out mice revealed that there was also a loss-of-function component (L. B. Menalled & Chesselet, 2002).

Another model developed was the knock-in. In theory, this should have reproduced human HD more faithfully, since it is a more accurate reproduction of the disease genotype. The human mutation responsible for HD is carried in the appropriate protein context under the endogenous mHtt promoter (L. B. Menalled, 2005). However, initial experiments were disappointing given that there was no gross behavioral phenotype typical of HD (L. B. Menalled & Chesselet, 2002). Further research discovered subtle motor abnormalities using more refined testing. For example, a dark cycle quantification of movements showed increased rearing in a knock-in mouse model. Similarly, pathological findings were unexpected. There was no overt neurodegeneration or gliosis (L. B. Menalled & Chesselet, 2002). On the other hand, nuclear staining revealed microaggregates of htt in younger mice and nuclear inclusions in older mice. Neurochemical abnormalities involving the direct and indirect pathways were observed. Knock-in mice have a decrease in messenger RNA (mRNA) encoding enkephalin (ENK). As well, they have increase sensitivity to N-methyl-D-aspartate (NMDA) (L. B. Menalled & Chesselet, 2002). This could suggest that neuronal dysfunction precedes neurodegeneration which would be responsible for subtle clinical findings in HD patients before they become overtly symptomatic. Interestingly, in knock-in mice, there is a specific regional CAG repeat instability. CAG repeats

become larger, especially in the areas classically affected by HD, the striatum and the cortex (L. B. Menalled & Chesselet, 2002). Therefore, although they have fallen out of favor, knock-in mouse model are still a good model for HD research. They can be used to assess the mHtt aggregates and their response to novel therapies. Furthermore, given their slow progression over months, they allow a unique possibility to dissect thoroughly the pathophysiology of HD (L. B. Menalled, 2005).

A few transgenic mouse models have been engineered. The most commonly mentioned are the R6 mouse and the YAC mouse. Yac mice have a complete human IT15 gene inserted with CAG repeats. They express the disease very slowly overtime likely attributed to their smaller amount of repeats and low level of transgene expression (i.e. 30-50%). However, they have a selective striatal neurodegeneration correlating well the human HD. At around 6 months of age they will show hyperactivity with subsequent motor dysfunction correlating with striatal neuron loss around 9 months of age (Beal & Ferrante, 2004). They also have a lifespan of over a year. As well, they present neurochemical abnormalities involving glutamatergic receptors. On the other hand they do not show overt microaggregates of mHtt (L. B. Menalled & Chesselet, 2002). Given that the whole huntingtin gene is present in their genome, even though the timeframe for appearance of clinical manifestations is slow, researchers have been rarely using this model to test new therapies (Gharami et al., 2008).

Another approach to generating transgenic mouse model is to only use the fragment of the human IT15 gene containing the expanded CAG repeats; these mice were named the R6 mice (L. B. Menalled & Chesselet, 2002). Several factors have contributed to the popular use of the R6 mice in the study of HD (Figure 2.4) (Li, Popovic, & Brundin, 2005). It showed characteristics behavior and morphological changes of HD and more importantly, it was the first model to be readily accessible with rapid disease progression allowing rapid commercialization. Depending on the number of CAG repeats inserted, many subtypes of the R6 group were created. For example, the R6/1 mice have around 115 repeats and the R6/2 mice around 145 repeats. The main advantage of those mice is the short time frame for appearance of clinical manifestations as well as a short natural history of the disease. Although many R6 lines have been generated, the R6/2 mice have been the most extensively studied (L. B. Menalled & Chesselet, 2002). Clinical manifestations are quick to appear, around 4-6 weeks of age. Initially, a decreased in balance and coordination and grip strength is observed. This decrease performance in motor function becomes increasingly severe correlating with the increased dystonic movements observed overtime (Stack et al., 2005). R6/2 mice also show marked weight loss around 9 weeks of age. There is a more prominent weight loss in male mice. Similarly, male mice showed reduced survival compared to their female counterpart (Wood, Glynn, & Morton, 2011). Significant brain atrophy also occurs as early as 4 weeks which correlates with initial neuron atrophy followed by later neuronal loss (Stack et al., 2005). There does not seem to be gliosis in R6/2 mouse brains (Mangiarini et al., 1996) (Ma, Morton, & Nicholson, 2003). The lifespan of the R6/2 mice is on average 12-14 weeks (Stack et al., 2005). The R6/2 mice given that they

reproduce behavioral and pathological findings of human HD over a short life span have been the focus of many therapeutic trials focusing on diverse physiopathological targets such as mHtt aggregates, transcriptional dysregulations and mitochondrial dysfunction (Beal & Ferrante, 2004) (Li et al., 2005).

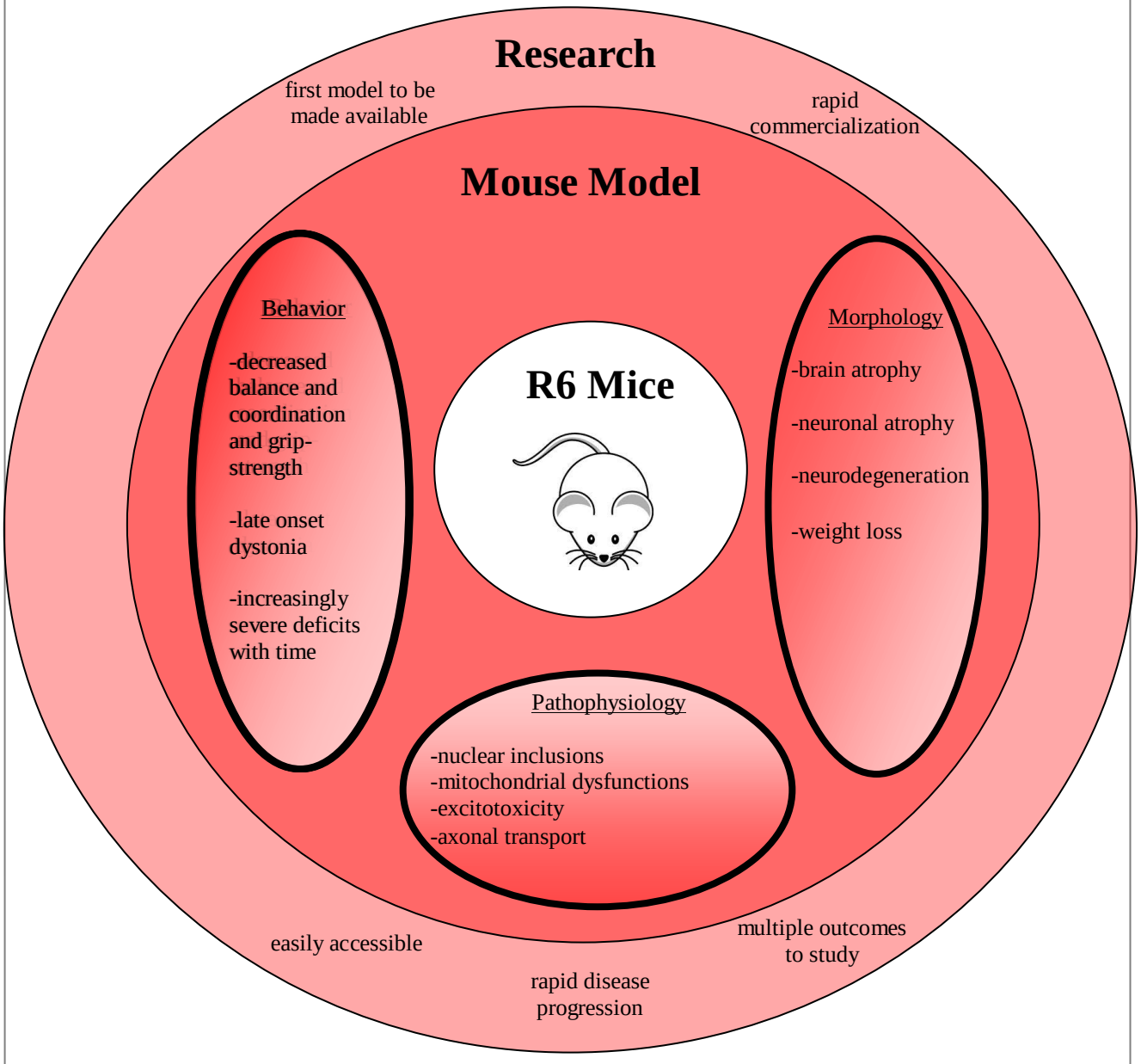

Figure 2.4: Main factors that contributed to the popularity of R6 mice in the study of HD. *Adapted from Li et al., 2005; L.B. Menalled and Chesselet, 2012; Carter et al.; 1999, Stack et al., 2005.*

2.10 Research Questions and Hypotheses-specific

We hypothesize that the R6/2 mouse model is an appropriate behavioral and anatomical model.

CHAPTER 3

THE RESEARCH QUESTIONS: AN APPROPRIATE MOUSE MODEL

(SAMADI ET AL., 2013)

3.1 Introduction

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder primarily affecting basal ganglia function. The latter consist of several functionally related subcortical nuclei, which interact with the cerebral cortex, thalamus and brainstem to modulate motor, cognitive and emotional behaviors (Tepper, Abercrombie, & Bolam, 2007). HD gene located on the chromosome 4 contains an expansion of the normal number of cytosine-adenine-guanine (CAG; glutamine) triplet repeats (generally >35) resulting in production of a mutant form of the huntingtin protein (htt) ("A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group," 1993; Reiner, Dragatsis, & Dietrich, 2011). Increased CAG repeat size is associated with more severe disease and earlier age of onset (M. Duyao et al., 1993). Motor disturbances are characterized by uncontrolled hyperkinetic movements, which can evolve to severe hypokinesia at terminal stages. Cognitive and emotional manifestations such as deficits in frontostriatal executive function, anxiety and depression may precede overt manifestations (Paulsen, 2011). Although mutant htt protein is expressed ubiquitously throughout the brain, the most striking neurodegenerative changes are preferentially observed in the medium spiny projection neurons of the striatum (Reiner et al., 1988; Rosas et al., 2003; Stack et al., 2005; Vonsattel et al., 1985). The cause of this predilection for striatal medium spiny neurons is unknown, but excitotoxic and anterograde neurotrophic mechanisms are

proposed (Kim, Bordiuk, & Ferrante, 2011; Raymond et al., 2011; Zuccato, Valenza, & Cattaneo, 2010).

The neurotrophic hypothesis of HD proposes impaired production of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, contributes to degeneration of neurons in the striatum (Saudou & Humbert, 2008; Zuccato et al., 2010). The striatum contains few if any BDNF mRNA-expressing neurons, but contains high levels of BDNF protein (Altar et al., 1997; Conner, Lauterborn, Yan, Gall, & Varon, 1997; Hofer, Pagliusi, Hohn, Leibrock, & Barde, 1990). In addition to corticostriatal glutamatergic afferents, the striatum also receives massive glutamatergic inputs from the parafascicular (Pf) thalamic nucleus (Berendse & Groenewegen, 1990; Sadikot, Parent, & Francois, 1992; Sadikot, Parent, Smith, & Bolam, 1992; Y. Smith et al., 2009) and dopaminergic (DA) inputs from the substantia nigra pars compacta (SNc) (A. Parent & Hazrati, 1995; M. Parent & Parent, 2010; Y. Smith, Bennett, Bolam, Parent, & Sadikot, 1994). Interestingly, the cerebral cortex, Pf and SNc all express high levels of BDNF mRNA (Conner et al., 1997). Lesions in cortical, nigral or thalamic afferents reduce BDNF protein concentrations in the striatum, suggesting anterograde transport of BDNF in striatal afferents (Altar et al., 1997; Conner et al., 1997; Sadikot et al., 2005). BDNF protein content in the striatum is reduced in both HD (Ferrer, Goutan, Marin, Rey, & Ribalta, 2000; Gauthier et al., 2004) and in animal models of HD (DeMarch, Giampa, Patassini, Bernardi, & Fusco, 2008; Giralt, Carreton, Lao-Peregrin, Martin, & Alberch, 2011; Peng et al., 2008; Spires et al., 2004). Reports of BDNF levels in cerebral cortex of HD patients vary from no

significant loss in sampled temporal or parietal cortices to significant reduction in both mRNA and protein (Ferrer et al., 2000; Gauthier et al., 2004; Zuccato et al., 2008; Zuccato et al., 2010). A reduction in cortical BDNF mRNA is detected in transgenic mouse models of HD (Luthi-Carter et al., 2002; "A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group," 1993; Zuccato & Cattaneo, 2007; Zuccato et al., 2001), although a systematic regional assessment in motor- and limbic-related areas has not been performed. Furthermore, little information is available regarding the levels of BDNF mRNA expression in other major sources of striatal afferents in HD or in animal models. Exploring BDNF expression in striatal afferents, motor behavior and changes in striatal morphology in the same animal model of HD would allow for better understanding of how neurotrophins may participate in mechanisms of neuronal degeneration, further insight into the relationship between morphology and behavior, and provide important normative data for preclinical therapeutic strategies.

The objective of this study is to characterize in detail the time course of neostriatal neuronal degeneration in the R6/2 mouse model of HD, and determine its relationship to motor impairment and reduced BDNF synthesis in major striatal afferents. The R6/2 mouse is the most widely used animal model for studies of the biology and treatment of HD (Gil & Rego, 2009; Kim et al., 2011). Initially produced to include exon 1 of the human HD gene with ~150 CAG repeats (Mangiarini et al., 1996), several lines with higher or lower numbers of CAG repeats are now in

use (Cummings et al., 2012; L. Menalled et al., 2009). Here, we take advantage of a widely available R6/2 line with ~110 CAG repeats.

We show a close temporal relationship between onset of loss of BDNF expression in motor cortices and the Pf, striatal degeneration and deterioration in motor behavior. Loss of BDNF mRNA in limbic cortices and the SNc precedes decreases seen in motor areas, and may correspond to early deficits in learning and memory. We suggest that the R6/2 mouse models many of the cardinal motor manifestations and morphological features of HD. Our results also indicate that in addition to corticostriatal afferents, thalamostriatal and nigrostriatal projections may play an important role in maintaining trophic support to striatal neurons in HD, and contribute to the pathophysiology of progressive motor abnormalities.

3.2 Materials and Methods:

3.2.1 Animals

The experiments were performed using male carrier (R6/2) and noncarrier (WT) mice from the same breeding colony (B6CBA-Tg(HDexon1)62Gpb/3J) maintained at the Montreal Neurological Institute facility in accordance with the standards of the Canadian Council on Animal Care. The breeders, males and ovary-transplanted females, were from the same strain, obtained from a line maintained at The Jackson Laboratory (Bar Harbor, ME, USA) involving a C57BL/6 and CBA background (stock number 006494). Offspring were genotyped by Laragen (Culver City, CA, USA), CAG repeat lengths of 105–112 were confirmed and cohorts of R6/2 and WT littermates

were evaluated. All testing was performed during the light phase of a 12-h light–dark cycle. Weight gain and survival of female R6/2 mice are significantly less than male R6/2 mice (Stack et al., 2005; Wood et al., 2011). Only males were used in order to reduce variability related to sex (L. Menalled et al., 2009). Animal numbers used for different behavioral tests, history of reuse after another behavioral test and the number of animals used in morphological analysis are summarized in Tables S1 and S2, Supporting Information.

3.2.2 Evaluation of motor behavior

3.2.2.1 Spontaneous activity in an open field

Spontaneous locomotor activity was measured in an open field using a video tracking system (Videotrack, Viewpoint Life Sciences, Montreal, Canada) with infrared backlighting (Bailoo, Bohlen, & Wahlsten, 2010). Locomotor activity measured was categorized as follows: inactivity or nonambulatory movements (<5 cm/second); moderate movement speed (between 5 and 20 cm/second) or fast movements (>20 cm/second). Distance refers to the total distance traveled by the animal during the testing period, and was measured separately for moderate and fast activity periods. Duration is the total duration spent in inactivity or nonambulatory movements, moderate activity or fast activity. Locomotor activities were monitored continuously during the 2-h testing period, with output intervals of 3 min.

3.2.2.2 Grip strength

Grip strength (Columbus Instruments, Columbus, OH, USA) determines the peak tension (T-PK) and peak compression (C-PK) developed by a rodent as it instinctively grips a wire mesh grid. The examiner attempts to overcome the grip by gently pulling at the base of the tail (Hockly et al., 2002). The device provides a digital readout of maximal force generated, expressed in grams (g) or Newton. Seven consecutive trials were performed with each mouse, separated by a rest period on the table of 5 seconds. The observer was blind to genotype. The mean of at least five successful trials was taken for analysis. Peak tension forces (T-PK) generated by the forelimbs, or all limbs as mice pulled on the wire mesh, or peak compression forces (C-PK) generated by the hindlimbs were assessed.

3.2.2.3 Clasping score

In this test, mice were suspended by the tail at a height of at least 30 cm, for two 20-second intervals, and limb movements were videotaped. Clasping was defined as a retraction of a limb toward the body, and rated by an observer blind to genotype in order to provide a semiquantitative index of abnormal involuntary movements. Clasping was rated as *mild* when the forelimb or hindlimb retracted toward the midline but did not reaching the midline, and the contraction was not sustained. *Moderate* clasping was a high-amplitude limb retraction to or beyond the midline, but not sustained. Severe clasping was a high-amplitude limb retraction sustained for more than 15 seconds. Quality of clasping at each limb was graded as: none = 0, mild = 0.25, moderate = 0.5, severe and constant = 0.75, for a maximal clasping score of 3. The score from the two tail suspension trials was averaged and recorded.

3.2.2.4 Rotarod test

A rotarod apparatus (Columbus Instruments) was used to measure limb coordination and balance using an incremental fixed speed protocol (Carter et al., 1999; Monville, Torres, & Dunnett, 2006). At the beginning of the experiments, mice were trained on the rotarod during 3 consecutive days. Each day mice had four trials at a constant speed (24 r.p.m.) for a maximum of 60 seconds, to obtain a training baseline. On the 4th day mice received three to four trials at nine increasing speeds (5–44 r.p.m.) for a maximum of 5 min each, with a 20-min rest period between each trial (Carter et al., 1999). The mean latency to fall at each speed, at different ages, was used in the analysis. The observer was blind to genotype.

3.2.3 Tissue preparation and stereology

Animals at different ages were deeply anesthetized and then perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains were removed and immersed for 24 h in the same fixative and then passed through a series of graded concentrations of phosphate-buffered sucrose solutions to a final concentration of 30%. Brains were cut in the coronal plane at 40 µm using a freezing microtome and free-floating sections were collected serially in six vials containing phosphate-buffered saline (PBS, 0.1 M, pH 7.4). One of the six sets of sections was mounted out of distilled water onto slides, stained with 0.1% Cresyl Violet (Nissl stain) and coverslipped using Permount (Fisher, Fairlawn, NJ, USA).

Unbiased stereology was carried out on Nissl-stained coronal sections using the Stereo Investigator program (Microbrightfield, Willston, VT, USA) and an Olympus BX-40 microscope equipped with a motorized XYZ stage. The optical fractionator and nucleator were used as stereology probes to obtain unbiased estimates of the total number of neurons and estimates of cell size, respectively, as detailed in our previous work (Luk, Kennedy, & Sadikot, 2003; Rymar, Sasseville, Luk, & Sadikot, 2004). The neostriatum was delineated according to defined boundaries (Sadikot & Sasseville, 1997) using a stereotaxic atlas of the mouse brain (Franklin & Paxinos, 2008). Briefly, the selection includes levels throughout the neostriatum including regularly spaced sections caudal and rostral to the decussation of the anterior commissure. The dorsal, medial and lateral limits of the neostriatum are well defined (Franklin & Paxinos, 2008). Ventrally, the neostriatum interfaces with the amygdala and substantia innominata in its postcommissural part and with the nucleus accumbens in its precommissural division. The ventral limit of the striatum at the postcommissural part is well delineated on Nissl stains. However, the ventral limit of the neostriatum at its precommissural part is an arbitrary interface. At the two precommissural levels analyzed, we therefore delimit the dorsal striatum from the nucleus accumbens with a line that extends from above the ventralmost part of the lateral ventricle medially, to the tapered external capsule laterally, at an angle of 25–30° below the axial plane. Section thickness was assessed and neuronal counts were performed under oil immersion using a 100× objective with a high aperture lens (NA 1.4). The systematic random sampling grid size was 500 × 500 μm, and mean section thickness was measured at every fifth sampling site. The Cavalieri estimator was used to measure the striatal reference volume. The

optical fractionator brick size was 60 × 60 µm, with a mean section thickness of 12 µm, and guard zones of 1 µm at the top and bottom surface, resulting in a dissector height of 10 µm. Neurons were defined as Nissl-stained profiles measuring at least 7 µm in diameter, with a lighter cytoplasm containing organelles. The four-ray nucleator was used to estimate neuronal size (Gundersen et al., 1988). The observer was blind to genotype.

3.2.4 *In situ* hybridization

A second set of 1/6 sections from each animal was processed for *in situ* hybridization under RNAase-free conditions. [^{35}S]UTP-labeled cRNA probes with either sense or antisense orientation were synthesized by *in vitro* transcription from a full-length cDNA clone encoding rat BDNF (MGC105254). The single-stranded cRNA probe was synthesized and labeled using Riboprobe Kit of Promega (Promega, Madison, WI, USA) with T7 RNA polymerase for antisense and [^{33}S]UTP (Perkin Elmer Inc., Woodbridge, Ontario, Canada). The sense single-stranded RNA probe was synthesized with SP6 RNA polymerase and [^{33}S]UTP. After short fixation of 5 min in 4% paraformaldehyde (PFA)/PBS, free-floating sections were washed in PBS and incubated for 10 min in proteinase K/PBS, followed by second fixation in 4% PFA/PBS during 10 min. After washing with PBS and ddH_2O, sections were incubated for 10 min in 0.25% acetic anhydride in 2% triethanolamine and then washed in saline-sodium citrate for 5 min. *In situ* hybridization was performed at 58°C overnight in a standard hybridization buffer containing 50% formamide as previously described (Beaudry, Langlois, Weppe, Rouillard, & Levesque, 2000). Following stringency washes, sections were mounted onto superfrost plus slides, air-dried and

dehydrated during 2 min in 30%, 60% and 100% ethanol. The slide-mounted tissue sections were then air-dried and exposed to radioactive-sensitive films (Kodak, Biomax MR, New Haven, CT, USA) for 5 days at room temperature. Films were scanned, and analysis of cerebral cortex, thalamus and midbrain sections in R6/2 and WT sections was performed using public domain National Institutes of Health image analysis software (NIH ImageJ, v1.45s, Bethesda, MD), normalizing optical density using the corpus callosum as a reference for background activity.

Statistical comparisons for differences in behavior and morphology between R6/2 carrier mice and WT mice at different ages were performed using analysis of variance (ANOVA) followed by *post hoc* analysis using the Fisher PLSD test (Stat-View, Abacus Corporation, Baltimore MD, USA). Data are expressed as a mean ± standard error of the mean (SEM), and $P < 0.05$ is considered significant.

3.3 Results

3.3.1 Motor behavior

3.3.1.1 Locomotor activity

3.3.1.1.1 Total distance traveled

When the total distance traveled during the 2-h test interval by R6/2 ($n = 197$) and WT ($n = 135$) mice was compared at different ages, significant differences were noted with respect to main effects and their interaction [$F(\text{genotype})_{1,310} = 49.19$, $P < 0.0001$; $F(\text{age})_{10,310} = 3.04$, $P < 0.0001$; $F(\text{genotype} \times \text{age})_{10,310} = 2.185$, $P = 0.01$]. Further analysis showed that the total distance

traveled by R6/2 mice was comparable to WTs at 4 and 6 weeks, and R6/2 mice showed a trend to reduced activity of marginal significance at 7 weeks ($F_{1,33}$ =3.82, P = 0.05; Fig. 3.0a). Motor activity in R6/2 mice declined progressively at 8–15 weeks (Fig. 3.0a) compared with age-matched WT controls, deteriorating to 58% by 15 weeks ($F_{1,15}$ =18.32, P < 0.001).

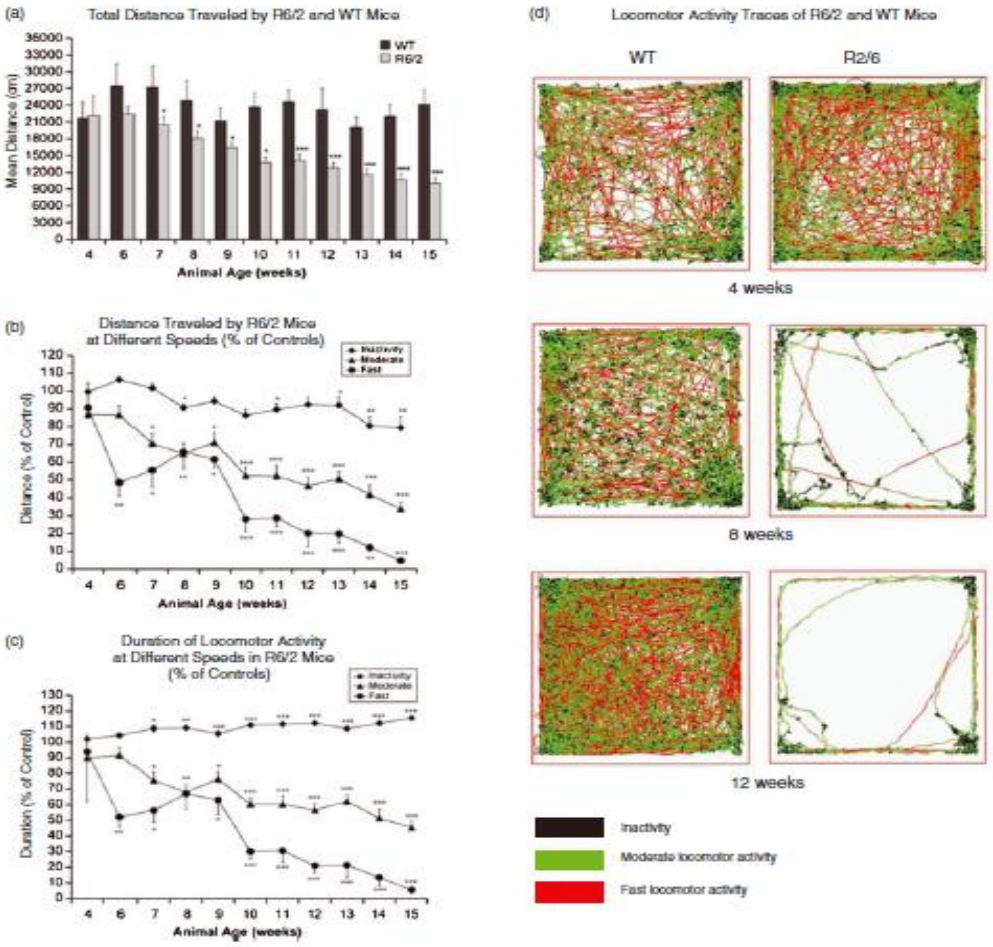

Figure 3.0. Spontaneous locomotor activity of R6/2 mice compared with WT controls at different ages in a video-tracked open field. (a) Total distance traveled by R6/2 mice compared with WTs. (b) Distance traveled at different movement speeds. (c) Duration of locomotor activities at different movement speeds or during nonambulatory behavior. (d) Sample video traces generated during 2-h sessions at 4, 8 and 12 weeks illustrate patterns of spontaneous locomotor activity in the open field. Trace colors code for different movement speeds. Although activity traces of 4-week-old R6/2 mice are virtually identical to age-matched WT controls, marked deterioration is seen by 8 and 12 weeks, with mice favoring movement close to the edges of the open field. *P < 0.05, **P < 0.01 or P < 0.005, ***P < 0.001, R6/2 vs. WT. (Samadi et al., 2013).

3.3.1.1.2 Distance traveled at different movement speeds

The contribution of distances traveled at different movement speeds to the observed differences in total distance traveled was analyzed in R6/2 and WT mice. Results in R6/2 mice are expressed as a percentage of controls (Fig. 3.0b). There was no difference in distances traveled at different movement speeds in 4-week-old R6/2 mice ($n = 6$) compared with WT controls ($n = 7$) [$F(\text{genotype})_{1,11} = 0.004$, $P = 0.95$; $F(\text{genotype} \times \text{distance at different speeds})_{2,22} = 0.12$, $P = 0.88$]. Six-week-old R6/2 mice ($n = 38$) showed no difference compared with WTs ($n = 11$) in total distance traveled at different movement speeds [$F(\text{genotype})_{1,47} = 2.15$]; however, there was a significant interaction effect between genotype and distance traveled at different movement speeds [$F(\text{genotype} \times \text{distance at different speeds})_{2,94} = 5.93$, $P = 0.003$]. *Post hoc* analysis showed a significant reduction of fast activity at 6 weeks [50% of control ($F_{1,47} = 8.28$, $P = 0.006$)] (Fig. 3.0b), but there was no significant difference at other speeds. Seven-week-old R6/2 mice ($n = 23$) showed marginal significance for distance traveled compared with WT ($n = 12$) mice, but there was a significant interaction effect [$F(\text{genotype})_{1,33} = 3.82$, $P = 0.05$; $F(\text{genotype} \times \text{distance at different speeds})_{2,66} = 5.78$, $P = 0.005$]. At this age, R6/2 mice show significant reduction in distance traveled during both fast and moderate locomotor activity [70% of control ($F_{1,33} = 4.28$, $P = 0.04$)] (Fig. 3.0b). Distances traveled by R6/2 mice during both moderate and fast activity declined progressively at each week from 9 ($n + 26$) to 15 ($n = 7$) weeks. For example, 9-week-old R6/2 mice ($n = 26$) showed a significant reduction in distance traveled during both moderate [72% of control ($F_{1,35} = 5.27$, $P = 0.02$)] and fast activity [63% of control ($F_{1,35} = 4.35$, $P = 0.04$)] compared with WT mice

(n = 11). By 15 weeks (Fig. 1b), R6/2 mice (n = 7) showed severe reduction in distance traveled during both moderate activity [32% of control ($F_{1,15}$ = 19.32, P = 0.0005)] and fast activity [5% of control ($F_{1,15}$ = 11.28, P = 0.004)] compared with WT mice (n = 10).

3.3.1.1.3 Duration of locomotor activity at different movement speeds

The time spent in movement at fast, moderate and nonambulatory activities was compared in R6/2 and WT mice at different ages. There was no significant difference in time spent at different movement speeds in 4-week-old R6/2 (n = 6) compared with WT (n = 7) mice [F(genotype)$_{1,11}$ = 0.85, P = 0.38; F(genotype × duration at different speeds)$_{2,22}$ = 0.92, P = 0.42]. Although total distance traveled remained unchanged compared with controls (Fig. 1a), 6-week-old R6/2 mice (n = 38) showed a significant difference in time spent at moderate and fast movements speeds compared with WT controls (n = 11) [F(genotype)$_{1,47}$ = 5.31, P = 0.02]. Interaction between genotype and duration of locomotor activity was not significant at this age [F(genotype × duration at different speeds)$_{2,94}$ = 1.27, P = 0.28]. Reduced duration of fast activity was apparent in 6-week-old R6/2 mice (n = 38) compared with WT (n = 11) controls [50% of control ($F_{1,47}$ = 7.18, P = 0.01)] (Fig. 3.0c). At 7 weeks, there was a significant interaction between genotype and duration of locomotor activity at different speeds [F(genotype × duration at different speeds)$_{2,35}$ = 4.21, P = 0.01], an effect also noted at older ages up to 15 weeks [F(genotype × duration at different speeds)$_{2,15}$ = 18.42, P < 0.0001]. *Post hoc* analysis at 7 weeks showed a marginal reduction in duration of moderate activity [75% ($F_{1,33}$ = 4.08, P = 0.05)] in R6/2 mice (n = 23) compared with WT controls (n = 12; Fig. 3.0c), with

concomitant reduction in total distance traveled (Fig. 3.0a). R6/2 mice older than 9 weeks showed a progressive reduction in duration of moderate and fast activity compared with WT controls. By 15 weeks, R6/2 mice ($n = 7$) spent significantly reduced times in moderate [47% of control ($F_{1,15} = 18.39$, $P = 0.0006$)] and fast activity [4% of control ($F_{1,15} = 12.63$, $P = 0.002$)] compared with WT mice ($n = 10$; Fig. 3.0c). The duration of time spent in nonambulatory movements is mildly increased in R6/2 mice ($n = 23$) from 7 weeks [110% of control: $n = 12$ ($F_{1,33} = 4.29$, $P = 0.04$)] onward, possibly suggesting hypokinesia, and increase in grooming or stereotyped motor behaviors (Fig. 3.0c). To illustrate the pattern of locomotor activity in the open field, video traces generated during sample 2-h sessions are shown at 4, 8 and 12 weeks (Fig. 3.0d). Although activity traces of 4-week-old R6/2 mice are virtually identical to age-matched WT controls, marked deterioration is seen by 8 and 12 weeks, with mice favoring movement close to the edges of the open field (Fig. 3.0d).

3.3.1.2 Grip strength

R6/2 mice showed progressive loss of grip strength compared with WT controls. When mice held the wire mesh grid using all limbs, a significant difference is detected between R6/2 ($n = 129$) and WT mice ($n = 54$) at different ages [$F(\text{genotype})_{1,163} = 180.79$, $P < 0.0001$; $F(\text{age})_{9,163} = 3.68$, $P = 0.0003$; $F(\text{genotype} \times \text{age})_{9,163} = 10.68$, $P < 0.0001$]. Impaired grip strength was first detected at 9 weeks of age in R6/2 mice ($n = 12$) compared with WT ($n = 5$) controls ($F_{1,15} = 16.45$, $P = 0.001$; Fig. 3.1a). Much of this loss is accounted for by impaired forelimb strength [$F(\text{genotype})_{1,163} = 76.97$, $P < 0.0001$; $F(\text{age})_{9,163} = 6.14$, $P < 0.0001$;

$F(\text{genotype} \times \text{age})_{9,163} = 3.02$, $P = 0.002$], which was progressively lost beginning at 9 weeks ($F_{1,15} = 7.254$, $P = 0.01$; Fig. 3.1b). Hindlimb grip strength was also impaired [$F(\text{genotype})_{1,163} = 42.32$, $P < 0.0001$; $F(\text{age})_{9,163} = 7.27$, $P < 0.0001$; $F(\text{genotype} \times \text{age})_{9,163} = 2.06$, $P = 0.03$]. However, hindlimb grip strength was lost later at 10 weeks (R6/2: $n = 14$ and WT: $n = 5$; $F_{1,17} = 9.14$, $P = 0.007$; Fig. 3.1c).

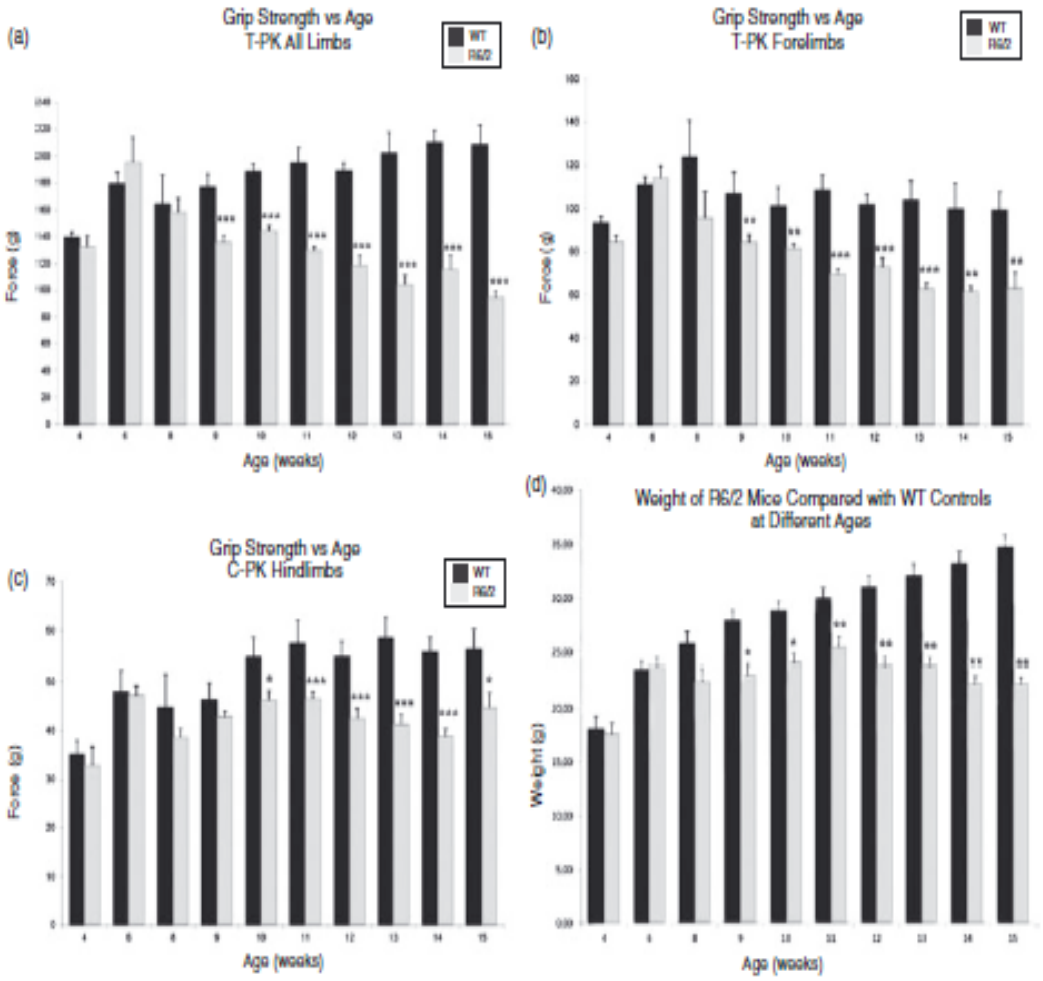

Figure 3.1. Grip strength of R6/2 mice compared with WT controls at different ages. Grip strength generated when mice held the wire mesh grid using all limbs (a) or using only the forelimbs (b). Grip strength generated when mice held the wire mesh grid using the hindlimbs (c). (d) Body mass of male R6/2 compared with WT mice at different ages. The reduction of weight in male R6/2 mice was evident by age 9 weeks. When normalized for weight, muscular force reduction declined significantly after 10 weeks, when grip strength was measured with all limbs touching the grid (data not shown). Muscular force assessed by grip strength is presented as the mean of T-PK or C-PK for the force of forelimbs, hindlimbs or all limbs ± SEM. $*P < 0.05$, $**P < 0.01$ R6/2 vs. WT. (Samadi et al., 2013).

R6/2 mice ($n = 12$) also showed significantly impaired weight gain [$F(\text{genotype})_{1,163} = 161.59$, $P < 0.0001$; $F(\text{age})_{9,163} = 19.26$, $P < 0.0001$; $F(\text{genotype} \times \text{age})_{9,163} = 9.15$, $P < 0.0001$] beginning at 9 weeks compared with WT controls ($n = 5$; $F_{1,15} = 10.08$, $P = 0.006$; Fig. 3.1d). A significant reduction of weight-normalized grip strength was first evident in 11-week-old R6/2 mice ($n = 14$) compared with WT controls ($n = 5$) [86% of control ($F_{1,17} = 8.43$, $P = 0.009$)], with all limbs pulling on the grid, and remained impaired until 15 weeks (data not shown). Weight-normalized forelimb grip strength of R6/2 mice was also virtually identical to WT controls at 10 weeks and significantly reduced at 11 weeks (R6/2: $n = 14$ and WT: $n = 5$) [84% of control ($F_{1,17} = 4.18$, $P = 0.05$)] and 13 weeks (R6/2: $n = 15$ and WT: $n = 6$) [85% of control ($F_{1,19} = 6.54$, $P = 0.01$)]. No significant difference was observed in weight-normalized hindlimb grip strength at any age in R6/2 mice compared with WT controls (4–15 weeks, $P > 0.05$). If normalized for weight, pulling using hindlimbs alone therefore appears to be a less sensitive indicator in R6/2 mice, but pulling using the forelimbs or all four limbs remain sensitive measures of grip strength abnormality.

3.3.1.3 Involuntary clasping movements

Involuntary clasping movement scores were significantly different in R6/2 mice ($n = 77$) compared with WT controls ($n = 64$) at different ages [$F(\text{genotype})_{1,119} = 1320.75$, $P < 0.0001$; $F(\text{age})_{10,119} = 22.79$, $P < 0.0001$; $F(\text{genotype} \times \text{age})_{10,119} = 28.43$, $P < 0.0001$]. Clasping movements were rare or absent in WT controls (Fig. 3.2). Mild dystonic movements (clasping score = 0.5)

were detectable in R6/2 mice as early as age 4 weeks (R6/2: $n = 7$ and WT: $n = 7$; $F_{1,12} = 15.00$, $P = 0.002$) and at 6 weeks (R6/2: $n = 22$ and WT: $n = 14$; $F_{1,34} = 3.82$, $P = 0.05$). The severity of these dystonic movements increased progressively with each week in R6/2 mice, and by 9 weeks clasping involved both forelimbs and hindlimbs (clasping score = 2; R6/2: $n = 28$ and WT: $n = 13$; $F_{1,39} = 110.84$, $P < 0.0001$). Maximum clasping was detected by age 14 (R6/2: $n = 7$ and WT: $n = 13$; $F_{1,18} = 13433.55$, $P < 0.0001$) and 15 weeks (R6/2: $n = 4$ and WT: $n = 13$; $F_{1,15} = 1.16E17$, $P < 0.0001$), with severe retraction of all four limbs upon tail suspension (clasping score = 3).

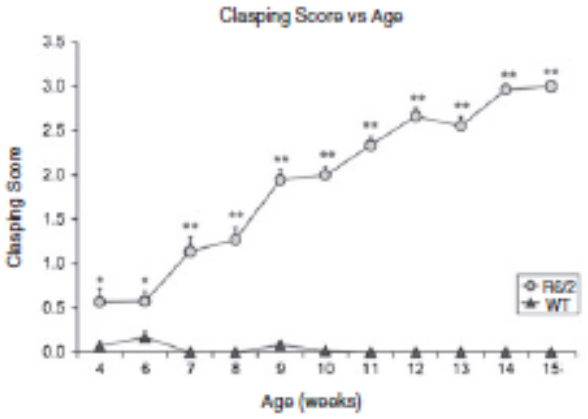

Figure 3.2. Clasping score in R6/2 mice at different ages. Clasping score ± SEM, *$P < 0.05$, **$P < 0.0001$, R6/2 vs. WT.(Samadi et al., 2013).

3.3.1.4 Rotarod performance

A significant decline in rotarod performance was observed at all rotation speeds in 7- to 13-week-old R6/2 mice compared with their respective age-matched WT controls ($P < 0.005$–<0.0001; Fig. 3.3), except at 5 r.p.m. and 8 r.p.m. in 7- to 8-week-old mice. Thus, a significant decline in rotarod performance was observed at speeds as low as 5 r.p.m. [$F(\text{genotype})_{1,101} = 44.30$, $P < 0.0001$; $F(\text{age})_{5,101} = 4.09$, $P = 0.002$; $F(\text{genotype} \times \text{age})_{5,101} = 4.09$, $P = 0.002$] and as high as 44 r.p.m. [$F(\text{genotype})_{1,101} = 74.74$, $P < 0.0001$; $F(\text{age})_{5,101} = 0.68$,

$P = 0.64$; $F(\text{genotype} \times \text{age})_{5,101} = 0.92$, $P = 0.47$] in R6/2 mice ($n = 63$) compared with their respective age-matched WT controls ($n = 50$; Fig. 3.3). However, *post hoc* analysis did not detect any difference between 7-week-old R6/2 mice ($n = 20$) and WT controls ($n = 6$) at 5 and 8 r.p.m. (5 r.p.m.: $F_{1,24} = 1.82$, $P = 0.19$; 8 r.p.m.: $F_{1,24} = 3.81$, $P = 0.063$).

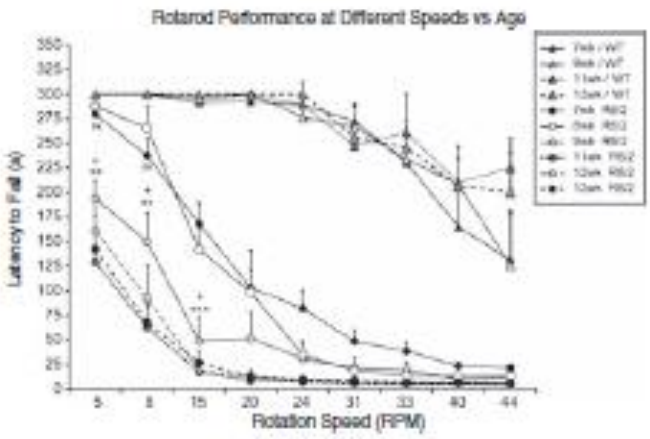

Figure 3.3. Incremental fixed speed rotarod performance in R6/2 mice and WT controls at different ages. Data are presented as the mean of latency to fall ± SEM. $^{**}P < 0.005$, $^{***}P < 0.0005$, +<0.05 for 9-week-old animals and ns refers to 7- and 8-week-old animals at 5 and 8 r.p.m.(Samadi et al., 2013).

When latency to fall of 9-week-old R6/2 mice ($n = 14$) is compared with 7- to 8-week-old mice (Fig. 3.3), progressive loss of performance is noted at lower rotation speeds (7 weeks: 5, 8, 15 and 24 r.p.m.; $P < 0.05$ to $P < 0.001$). At higher rotation speeds, curves of impaired latency of R6/2 mice are not significantly different at 7 ($n = 20$) and 9 ($n = 14$) weeks. No significant difference is observed in latency to fall at any speed when comparisons are made between 9- ($n = 14$), 11- ($n = 13$), 12- ($n = 5$) and 13- ($n = 5$) week-old R6/2 mice. Indeed, there is virtual overlap of latency to fall curves of R6/2 mice at 11, 12 and 13 weeks, especially at speeds greater than 8 r.p.m., indicating that performance deterioration on this test had reached a plateau. Therefore, a rotation speed of 15 r.p.m. appears to be the most useful in resolving progressive differences in motor performance in 7- to 13-week-old R6/2 mice during a 5-min

testing session. Rotation speeds greater than 24 r.p.m. do not provide useful additional information regarding progressive motor loss in R6/2 mice.

3.3.2 Morphology of striatum quantified using unbiased stereology over the R6/2 mouse lifespan

3.3.2.1 Volume of neostriatum

A significant difference in striatal volume is detected in R6/2 mice ($n = 19$) compared with controls ($n = 22$) [$F(\text{genotype})_{1,31} = 31.41$, $P < 0.0001$; $F(\text{age})_{4,31} = 3.79$, $P = 0.01$; $F(\text{genotype} \times \text{age})_{4,31} = 2.96$, $P = 0.03$]. *Post hoc* analysis indicates that neostriatal volume was virtually identical in 4-week-old R6/2 ($n = 4$) and WT ($n = 4$) mice ($F_{1,6} = 0.28$, $P = 0.62$). However, striatal volume was significantly reduced in 6-week-old R6/2 mice ($n = 3$) compared with WT controls ($n = 4$; 88% of control, $F_{1,5} = 10.69$, $P = 0.02$), and decreased progressively as R6/2 animals aged. By 13 weeks, striatal volume in R6/2 mice ($n = 4$) was 71% of WT ($n = 4$) controls ($F_{1,6} = 31.69$, $P = 0.001$; Fig. 3.4a,d).

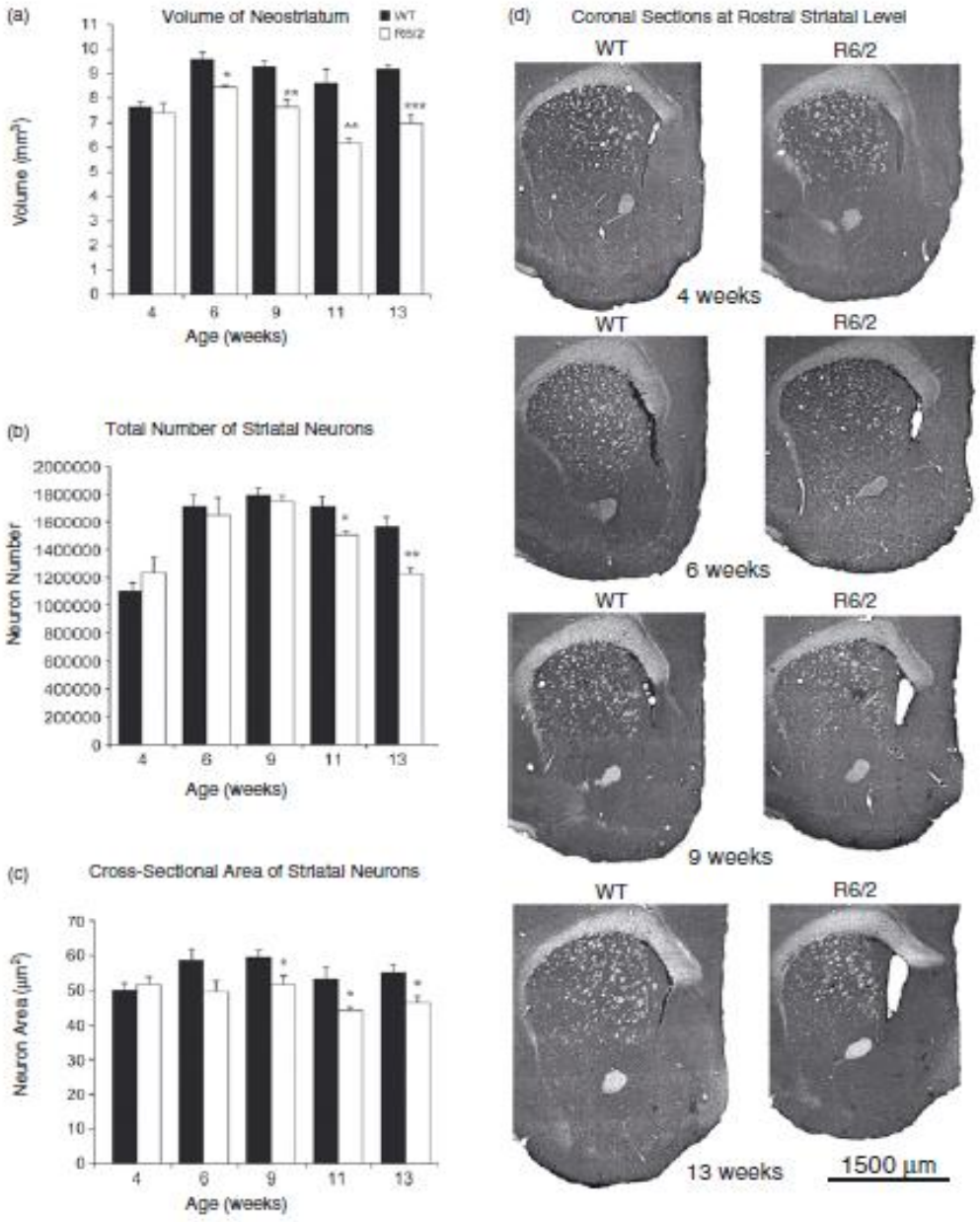

Figure 3.4. Unbiased stereology for quantification of morphological changes in the neostriatum at different ages in R6/2 mice and WT controls. (a) Striatal volume at different ages. (b) Total number of neostriatal neurons. (c) Cross-sectional area of striatal neurons. (d) Photomicrographs of coronal sections of striatum from R6/2 mice and age-matched WT controls at 4, 6, 9 and 13 weeks. Note progressive enlargement of the lateral ventricle with reduced striatal volume. Scale bar: 1500 µm. Data are presented as the mean of values ± SEM. *$P < 0.05$, **$P < 0.01$ or $P < 0.005$, ***$P < 0.001$ vs. WT.(Samadi et al., 2013).

The Gundersen coefficients of error (CEs) for the Cavalieri volume estimates ($m = 1$) at different ages in WT and R6/2 mice, respectively, were as follows: 0.009 ± 0 (mean ± SEM) vs. 0.014 ± 0.0033 at 4 weeks; 0.009 ± 0.0005 vs. 0.009 ± 0.0009 at 6 weeks; 0.008 ± 0.0005 vs. 0.009 ± 0.0003 at 9 weeks; 0.005 ± 0.0004 vs. 0.011 ± 0.0014 at 11 weeks and 0.009 ± 0.0007 vs. 0.009 ± 0.0006 at 13 weeks.

3.3.2.2 Number of neostriatal neurons

Quantification of the total number of neostriatal neurons in R6/2 mice ($n = 19$) using unbiased stereology (Fig. 3.4b) showed no significant difference compared with age-matched WT controls ($n = 22$) in 4- to 9-week-old animals, but significant differences were noted in older mice. On *post hoc* analysis a significant decrease in neuron number was first detected at 11 weeks in R6/2 mice ($n = 4$) compared with age-matched WT ($n = 4$) mice [87% of control; WT: 1 711 615 ± 71 470 (mean ± SEM) vs. R6/2: 1 502 640 ± 29 308; $F_{1,6} = 7.31$, $P = 0.03$]. The number of neostriatal neurons was further reduced in 13-week-old R6/2 mice ($n = 4$) compared with WT ($n = 4$) mice [78% of control; WT: 1 564 184 ± 72 986 vs. R6/2: 1 225 461 ± 41 816; $F_{1,6} = 16.21$, $P = 0.006$].

The Gundersen CEs for neuronal counts ($m = 1$) at different ages in WT and R6/2 mice, respectively, were as follows: 0.03 ± 0 (mean ± SEM) vs. 0.03 ± 0.003 at 4 weeks; 0.03 ± 0 vs.

0.03 ± 0 at 6 weeks; 0.02 ± 0.002 vs. 0.02 ± 0 at 9 weeks; 0.02 ± 0 vs. 0.03 ± 0.002 at 11 weeks and 0.03 ± 0 vs. 0.03 ± 0 at 13 weeks.

When WT mice were compared at 11 ($n = 4$) and 13 ($n = 4$) weeks, no significant difference in the number of striatal neurons is detected ($F_{1,6} = 2.08$, $P = 0.20$). However, a significant difference in the number of striatal neurons was detected between R6/2 mice at 11 ($n = 4$) and 13 ($n = 4$) weeks ($F_{1,6} = 29.46$, $P = 0.001$). In young WT mice, an increase in the number ($F_{1,6} = 36.00$, $P = 0.001$) of striatal neurons is noted between 4 ($n = 4$) and 6 ($n = 4$) weeks. A marginal increase in the number of striatal neurons is also noted when comparing 4- ($n = 4$) and 6- ($n = 3$) week-old R6/2 mice ($F_{1,5} = 6.41$, $P = 0.05$). Glial profiles were also counted at each age. There was no significant difference in glial number at earlier ages (data not shown), but 13-week-old animals showed a 26% increase (434 970 ± 58 505 in WTs vs. 587 292 ± 32 396 in R6/2 mice; $F_{1,6} = 5.19$, $P = 0.041$).

3.3.2.3 Cross-sectional area of neostriatal neurons

Interestingly, reduction in cross-sectional area of neostriatal neurons [$F(\text{genotype})_{1,31} = 23.20$, $P < 0.0001$; $F(\text{age})_{4,31} = 14.15$, $P < 0.0001$; $F(\text{genotype} \times \text{age})_{4,31} = 2.85$, $P = 0.04$] preceded neuronal loss (R6/2: $n = 19$ and WT: $n = 22$; Fig. 3.4b,c). The cross-sectional area of striatal neurons was not changed in R6/2 mice at 4 weeks ($n = 4$; $F_{1,6} = 0.32$, $P = 0.59$) and 6 weeks ($n = 3$; $F_{1,5} = 5.28$, $P = 0.07$) compared with WT controls ($n = 4$; Fig. 3.4c). Reduction in neuronal area was significant in 9- (R6/2: $n = 4$ and WT: $n = 6$; 80% of control; $F_{1,8} = 7.69$, $P = 0.02$), 11-

(R6/2: $n = 4$ and WT: $n = 4$; 76% of control; $F_{1,6} = 7.22$, $P = 0.03$) and 13-week-old R6/2 mice (R6/2: $n = 4$ and WT: $n = 4$; 77% of control; $F_{1,6} = 11.14$, $P = 0.01$). As a caveat, nucleator-based estimates of cell size assume isotropic shape of the object measured. The results can therefore be biased if striatal neurons have an anisotropic orientation, unless randomly oriented sections are used (Gundersen et al., 1988). In the nervous system, analysis on independent uniform random sections may be difficult, especially when delineating an irregular reference space with complex boundaries, such as the striatum (Schmitz, Schuster, Niessen, & Korr, 1999). As we performed measurements of cell size in conjunction with the optical fractionator, neurons were sampled in an unbiased manner. However, a bias could be introduced in estimating absolute size of medium spiny neurons if they were anisotropically oriented. As the presence of mutant huntingtin protein (mhtt) is not expected to alter the orientation of striatal neurons, we would expect that size comparisons between WT and R6/2 mice are still valid, although the absolute value may not be 'unbiased'.

3.3.3 BDNF mRNA expression in majors afferents to the neostriatum

To determine whether a neurotrophin with known anterograde action (Fawcett et al., 2000; von Bartheld, Byers, Williams, & Bothwell, 1996) in mammals could help account for impaired motor behavior and morphological changes in the striatum of R6/2 mice, we analyzed the time course of expression of BDNF in the major sources of afferents to the neostriatum. These include glutamatergic inputs from limbic and motor areas of the cerebral cortex, glutamatergic inputs from the thalamus and dopaminergic (DA) inputs from the midbrain (Y. Smith et al.,

1994). Interestingly, these sources of major striatal afferents also show high levels of BDNF expression (Conner et al., 1997). Representative autoradiograms of hybridization to antisense BDNF probe (Fig. 3.5) show the anatomical distribution of BDNF mRNA expression in the forebrain (Bregma 1.70 and Bregma 1.18), thalamus (Bregma −2.18) and midbrain (Bregma −3.16) of 11-week-old R6/2 and WT mice (Franklin & Paxinos, 2008). There is virtual absence of BDNF mRNA expression in the striatum (Str). In contrast, WT mice show moderate to dense expression in sources of major striatal afferents, including the cerebral cortex, the thalamic Pf nucleus and the SNc. R6/2 mice show reduced BDNF expression in the cerebral cortex, thalamus and midbrain (Fig. 3.5).The nonspecific control represents hybridization to sense BDNF probe (Fig. 3.5).

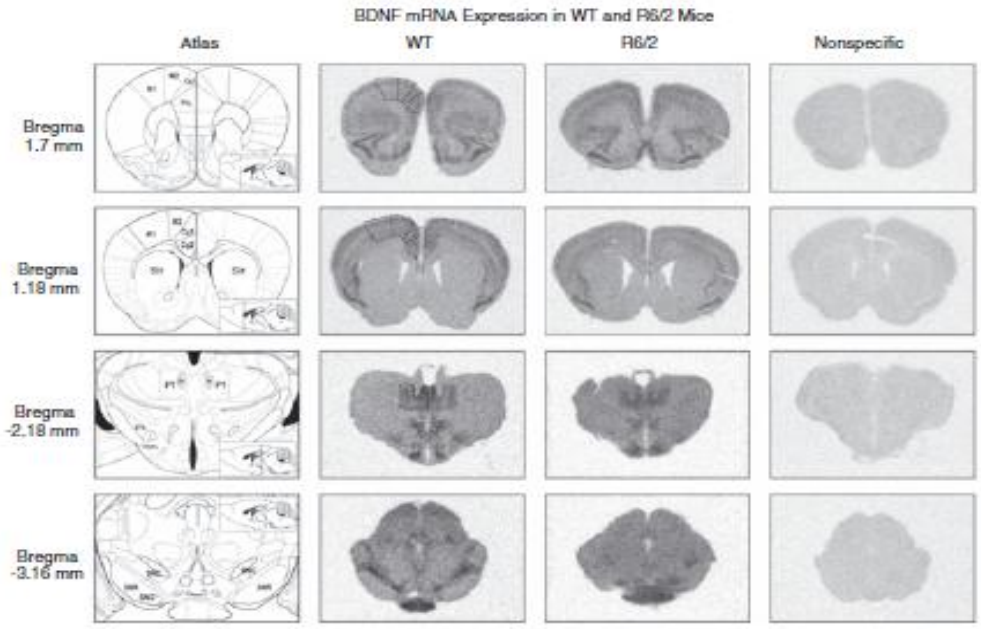

Figure 3.5. Representative autoradiograms of BDNF mRNA showed by *in situ* hybridization. BDNF mRNA expression in different brain regions following hybridization to antisense probe is shown in paired coronal sections from R6/2 and WT mice. Nonspecific negative control represents hybridization to sense probe. Nuclear outlines are adapted from a stereotactic mouse brain atlas (Franklin & Paxinos, 2008). PrL, prelimbic cortex; Cg1 and Cg2, cingulate cortices; M1, motor cortex; M2, premotor cortex; Pf, parafascicular nucleus of thalamus; fr, fasciculus retroflexus; STh, subthalamic nucleus; SNc and SNr, substantia nigra compacta and reticulata.(Samadi et al., 2013).

3.3.3.1 Cerebral cortex

To compare changes in BDNF mRNA expression in motor and associational/limbic areas of the frontal lobe at different ages in R6/2 mice and WT controls, prelimbic, cingulate, primary motor (M1) and accessory motor (M2) areas were analyzed (Fig. 3.5). Two-way ANOVA detected significant differences between R6/2 mice ($n = 27$) and their age-matched controls ($n = 28$) in concentrations of BDNF mRNA in prelimbic cortex [$F(\text{genotype})_{1,47} = 76.97$, $P < 0.0001$; $F(\text{age})_{3,47} = 1.67$, $P = 0.18$; $F(\text{genotype} \times \text{age})_{3,44} = 0.63$, $P = 0.60$]; cingulate cortex [$F(\text{genotype})_{1,47} = 55.97$, $P < 0.0001$; $F(\text{age})_{3,47} = 1.05$, $P = 0.38$; $F(\text{genotype} \times \text{age})_{3,47} = 3.62$, $P = 0.02$]; M1 cortex [$F(\text{genotype})_{1,47} = 52.59$, $P < 0.0001$; $F(\text{age})_{3,47} = 0.62$, $P = 0.61$; $F(\text{genotype} \times \text{age})_{3,47} = 3.44$, $P = 0.02$] and M2 cortex [$F(\text{genotype})_{1,47} = 52.59$, $P < 0.0001$; $F(\text{age})_{3,47} = 0.62$, $P = 0.61$; $F(\text{genotype} \times \text{age})_{3,47} = 2.38$, $P = 0.08$]. *Post hoc* analysis indicated that BDNF mRNA expression was significantly reduced as early as 4 weeks in R6/2 mice ($n = 8$) compared with WT controls ($n = 8$) in prelimbic [49% of control ($F_{1,14} = 8.55$, $P = 0.01$)] and cingulate cortices [71% of control ($F_{1,14} = 4.38$, $P = 0.05$)] (Fig. 7a,b). By 13 weeks, BDNF mRNA in markedly reduced in R6/2 ($n = 6$) compared with WT ($n = 6$) mice in both prelimbic [34% of control ($F_{1,10} = 119.10$, $P < 0.0001$)] and cingulate cortices [40% of control ($F_{1,10} = 37.05$, $P < 0.0001$)] (Fig. 3.6a,b).

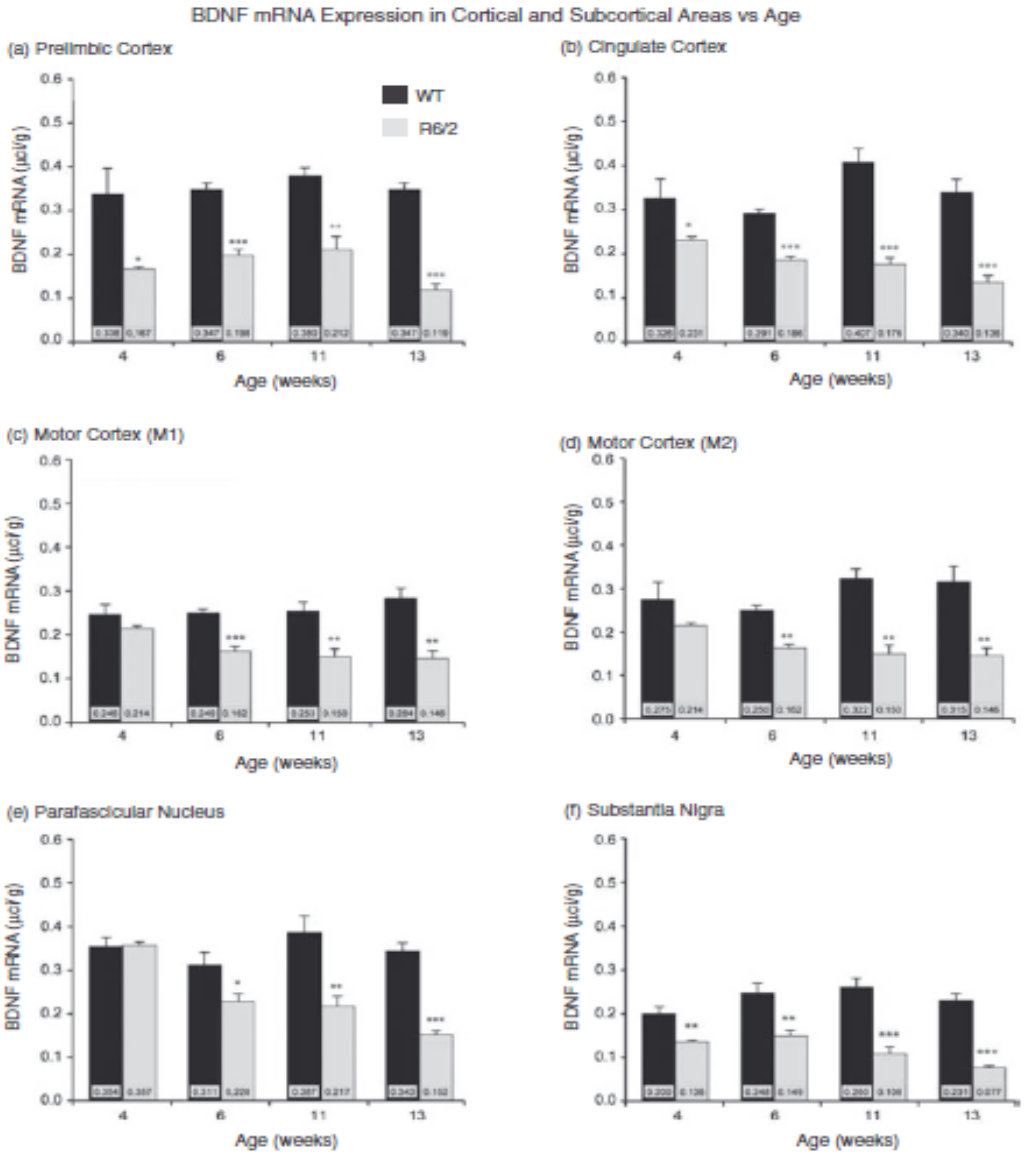

Figure 3.6. BDNFmRNA expression in cortical and subcortical areas of R6/2 mice and WT controls. BDNF mRNA expression in (a) prelimbic cortex, (b) cingulate cortex, (c) M1 motor cortex, (d) M2 motor cortex, (e) Pf nucleus of thalamus and (f) SNc in R6/2 mice compared with WT controls is compared at different ages. Values for [^{35}S] BDNF mRNA are presented in µci/g of tissue as the mean for each region ± SEM. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, R6/2 vs. WT.(Samadi et al., 2013).

BDNF mRNA expression is not significantly altered in motor cortical areas (M1 and M2) in 4-week-old R6/2 ($n = 8$) mice compared with WT ($n = 8$) controls (Fig. 3.6c,d). However, by

6 weeks, R6/2 mice ($n = 7$) show significantly reduced levels of BDNF mRNA compared with WT ($n = 6$) controls in both M1 [65% of control ($F_{1,11} = 32.84$, $P = 0.0001$)] and M2 [64% of control ($F_{1,11} = 40.64$, $P < 0.0001$)] (Fig. 3.6c,d). There is a progressive reduction in BDNF mRNA expression in primary and accessory motor cortices during the remainder of the R6/2 mouse lifespan. At 11 weeks, BDNF mRNA expression in R6/2 mice ($n = 6$) is reduced to 59% of WT controls ($n = 8$) in M1 ($F_{1,12} = 14.39$, $P = 0.002$) and to 47% of controls in M2 ($F_{1,12} = 28.02$, $P = 0.0002$). Finally, by 13 weeks, BDNF mRNA expression in R6/2 mice ($n = 6$) is reduced to 51% of WT mice ($n = 6$) in M1 ($F_{1,10} = 22.93$, $P = 0.0007$) and to 46% of control in M2 ($F_{1,10} = 18.48$, $P = 0.001$).

To determine whether the observed early changes in BDNF mRNA expression are related to neuronal loss, we performed a stereological analysis of the number of neurons in the frontal lobe in 6-week-old R6/2 and WT mice. There was no significant difference in the number of neurons in different areas of the frontal cortex. Specifically, the number of neurons in different cortical regions delineated according to the atlas of Franklin and Paxinos (Franklin & Paxinos, 2008) in R6/2 and WT mice, respectively, was as follows: primary motor cortex (M1): 480 170 ± 40 317 (mean ± SEM) vs. 447 861 ± 22 840 ($P = 0.51$); secondary motor cortex (M2): 482 158 + 47 617 vs. 468 083 + 10 164 ($P = 0.78$); cingulate cortex: 350 440 + 30 537 vs. 354 771 + 16 008 ($P = 0.90$) and prelimbic/infralimbic cortex: 136 187 ± 13 893 vs. 121 732 ± 17 444 ($P = 0.54$).

3.3.3.2 Parafascicular nucleus of the thalamus

BDNF mRNA levels in Pf were significantly reduced in R6/2 ($n = 27$) mice compared with WT ($n = 28$) controls [$F(\text{genotype})_{1,48} = 43.85$, $P < 0.0001$; $F(\text{age})_{3,48} = 8.31$, $P = 0.0001$; $F(\text{genotype} \times \text{age})_{3,48} = 7.38$, $P = 0.0004$]. As in the motor cortex, *post hoc* analysis indicated no significant difference in the level of BDNF mRNA in the Pf nucleus of 4-week-old R6/2 mice ($n = 8$) compared with WT controls ($n = 8$; $F_{1,14} = 0.15$, $P = 0.90$). However, by 6 weeks, BDNF mRNA expression in Pf was significantly reduced in R6/2 ($n = 7$) compared with WT ($n = 6$) mice [73% of control ($F_{1,12} = 5.76$, $P = 0.03$)]. There was progressive decrease in BDNF expression during the remainder of the R6/2 lifespan. By 11 weeks, BDNF mRNA levels in R6/2 mice ($n = 6$) were reduced to 56% of WT ($n = 8$) controls ($F_{1,12} = 12.76$, $P = 0.004$); BDNF mRNA levels in 13-week-old R6/2 mice ($n = 6$) were reduced to 44% of age-matched WT ($n = 6$) controls ($F_{1,10} = 84.25$, $P < 0.0001$; Fig. 3.6e).

3.3.3.3 Substantia nigra pars compacta

BDNF mRNA levels in the SNc were significantly reduced in R6/2 ($n = 27$) mice compared with WT ($n = 28$) controls [$F(\text{genotype})_{1,49} = 110.48$, $P < 0.0001$; $F(\text{age})_{3,47} = 3.03$, $P = 0.03$; $F(\text{genotype} \times \text{age})_{3,49} = 3.78$, $P = 0.01$]. *Post hoc* analysis shows a robust reduction of BDNF mRNA expression in the SNc as early as 4 weeks in R6/2 mice ($n = 8$) compared with WT ($n = 8$) controls [68% of controls ($F_{1,13} = 17.51$, $P = 0.001$)]. BDNF mRNA expression in DA neurons is reduced progressively during the remainder of the R6/2 mouse lifespan. By 11 weeks, R6/2 mice ($n = 6$) show a marked reduction in BDNF mRNA levels compared with WT ($n = 8$) mice

[42% of control ($F_{1,12} = 30.43$, $P = 0.0001$)]. By 13 weeks, R6/2 mice ($n = 6$) show further reduction in BDNF mRNA levels in the SNc [33% of control ($F_{1,10} = 91.57$, $P < 0.0001$)] (Fig. 3.6f).

3.4 Discussion

Our results confirm and extend previous work demonstrating striatal neuronal atrophy and neuronal loss are associated with impaired motor coordination in the R6/2 mouse, a widely used model of morphological and behavioral features of HD (Carter et al., 1999; Cummings et al., 2012; Hickey, Gallant, Gross, Levine, & Chesselet, 2005; Kim et al., 2011; Mangiarini et al., 1996; Stack et al., 2005; Sun, Del Mar, Meade, Goldowitz, & Reiner, 2002; Zuccato et al., 2010). R6/2 mice also show progressive loss of the neurotrophin BDNF in sources of afferents to the striatum. Progressive reduction of BDNF expression is noted not only in motor and limbic cortices but also in neurons of origin of the massive nigrostriatal and thalamostriatal tracts. Severity and time course of phenotypic changes in R6/2 mice can vary with gender (Cowin et al., 2012; Cummings et al., 2012; L. Menalled et al., 2009; Stack et al., 2005; Wood et al., 2011), with the number of CAG repeats expressed in a given strain (Cowin et al., 2012; Cummings et al., 2012; L. Menalled et al., 2009; Stack et al., 2005) and with genetic background (Cowin et al., 2012). Our analysis in strain and sex-matched animals with a uniform number of CAG repeats, therefore, provides a unique opportunity for rational interpretation of the relationship between motor behavior, morphology and gene expression.

3.4.1 Evolution of motor behavior in R6/2 mice

Amongst a wide variety of HD models described in different host species, R6 mouse lines are the most widely used (Kim et al., 2011). R6/2 mice carry about 1 kb of the human mutant htt promoter, driving expression of exon 1 of the htt protein gene with ∼110 to ∼320 CAG triplet repeats, resulting in abnormally long polyglutamine tracts (Cowin et al., 2012; Cummings et al., 2012; Mangiarini et al., 1996; L. B. Menalled et al., 2010). R6/2 mice show more rapidly evolving behavioral changes than other commonly used mouse models, and may represent a juvenile form of HD (Mangiarini et al., 1996; Nance & Myers, 2001; Robitaille, Lopes-Cendes, Becher, Rouleau, & Clark, 1997).

Progressive motor abnormalities are detected in R6/2 mice (Carter et al., 1999; Hickey et al., 2008; Mangiarini et al., 1996; Stack et al., 2005). Abnormal limb clasping, a measure of dystonia, is an early motor sign. R6/2 mice in this study show a clear weekly progression in clasping score, with mild limb dystonia (score: 0.5–1.5) at 4–8 weeks, moderate dystonia by 9–11 weeks (score: 1.5–2.5) and severe dystonia (score >2.5) involving all limbs in 12- to 15-week-old mice. Reports in mice with larger CAG repeat lengths in the 125–150 range show some variability, with onset of clasping either at a later age (8 weeks) without clear progression or at 4–6 weeks with progression (Carter et al., 1999; Cummings et al., 2012; Hickey et al., 2008; Mangiarini et al., 1996; Stack et al., 2005). This dystonic phenomenon is reduced or absent in R6/2 mouse lines with high CAG repeat lengths (∼200–300 CAG), which show milder and more variable phenotypes, limiting utility of these mice in therapeutic trials (Cummings et al., 2012; Kim et al., 2011; Stack et al., 2005).

CAG repeat size, sex and light–dark conditions can influence locomotor activity (Cummings et al., 2012; Dunnett et al., 1998; Hickey et al., 2008; L. Menalled et al., 2009). In R6/2 mice, spontaneous locomotor activity varies from hyperactivity detected at 3 weeks, followed by several weeks of activity similar to WT controls, and reduced activity after 8 weeks (Luesse et al., 2001). Male R6/2 mice with CAG repeat sizes in the 125–150 range show mild reduction in total distance traveled by 4–5 weeks during the dark phase of the diurnal cycle and by 6 weeks during the light phase (L. Menalled et al., 2009). Reduced locomotion is more apparent throughout the diurnal cycle in both sexes by 8 weeks (Carter et al., 1999; Hickey et al., 2008; L. Menalled et al., 2009; L. B. Menalled et al., 2010).

In this study using male R6/2 mice with ~110 CAG repeats, locomotor activity was measured over a prolonged period to reduce effects related to initial measures of novelty, anxiety and habituation (Bolivar, Manley, & Messer, 2003; File, Mahal, Mangiarini, & Bates, 1998; O'Keefe, Nadel, & Willner, 1979). A trend to reduced travel distance is first noted at 6 weeks, followed by significant and progressive reduction after 7 weeks. As significant reduction in fast activity is noted by 6 weeks, movement speed appears to be a more sensitive measure of early decline in locomotor activity compared with distance traveled alone. As mice were not tested earlier than 4 weeks, we cannot exclude the period of hyperactivity detected at 3 weeks in previous studies (Luesse et al., 2001).

Latency to fall at different speeds on a rotarod apparatus is a measure of balance and coordination (Dunham & Miya, 1957; Jones & Roberts, 1968; Monville et al., 2006; Rustay, Wahlsten, & Crabbe, 2003). Mild impairment in performance is seen as early as 6–7 weeks in R6/2 mice (Carter et al., 1999; Cummings et al., 2012; Hickey et al., 2008; Stack et al., 2005), and more significant declines are noted by age 9–11 weeks, followed by a plateau. We obtained similar results using an incremental fixed speed rotarod protocol (Carter et al., 1999; Cummings et al., 2012; Monville et al., 2006). Optimal rotation speeds for evaluating motor performance using this protocol in R6/2 mice are in the 5–24 r.p.m. range, with little advantage at higher speeds. Abnormal grip strength is a later motor sign. Previous studies of R6/2 mice have indicated variable loss of grip strength ranging from 7 to 12 weeks (Hickey et al., 2005; L. Menalled et al., 2009; Stack et al., 2005). In this study, loss of grip strength is first detected at 9 weeks when mice pull on a wire mesh either with their forelimbs alone or with all four limbs. Loss of strength in the hindlimbs is a later sign.

3.4.2 Evolution of morphological changes in the neostriatum in R6/2 mice

A major hallmark of HD is loss of projection neurons in the neostriatum, with relative sparing of interneuron subpopulations (Ferrante et al., 1985; Reiner et al., 1988; Vonsattel et al., 1985). Amongst striatal projection neurons, there is a predilection for loss of enkephalin-expressing cells of the indirect pathway at early stages of HD, with relative sparing of projection neurons of the direct pathway, which express substance P (Reiner et al., 1988; Richfield, Maguire-Zeiss, Vonkeman, & Voorn, 1995). Initial studies in R6/2 mice detected striatal atrophy (Gil & Rego,

2009; Mangiarini et al., 1996; Sun et al., 2002; Turmaine et al., 2000). Gene expression studies suggest a decrease in the number of enkephalin-positive neurons (Luthi-Carter et al., 2000; L. Menalled et al., 2000; Sun et al., 2002). Unbiased stereological analysis of the precommissural striatum in an R6/2 line with ~150 CAG repeats documents atrophy and progressive loss of projection neurons (Stack et al., 2005), and Golgi analysis shows loss of dendritic spines (Klapstein et al., 2001).

In our analysis, neostriatal volume loss is first detected in R6/2 mice at age 6 weeks, prior to loss of striatal neuronal volume. This time point corresponds to mild early dystonic movements, decreased latency to fall on a rotarod especially at higher speeds and mild reduction in spontaneous locomotor activity. Neuronal atrophy does not occur until 9 weeks and precedes neuronal loss, which is first detected at 11 weeks. The early phase of abnormal motor behavior with striatal volume loss may therefore correspond to loss or atrophy of striatal afferents or efferents, and these degenerative changes in neuropil may progress throughout the R6/2 lifespan.

Moderate limb clasping abnormalities, increasingly poor balance on a rotarod, further loss of spontaneous activity and onset of grip strength abnormalities correlate best with striatal neuronal atrophy without cell loss at 9 weeks. Finally, severe abnormalities on motor tests are noted after 11 weeks, corresponding to the onset of progressive neuronal loss and further neuronal atrophy.

3.4.3 BDNF expression in frontal cortices and their relationship to striatal morphology and behavior

Corticostriatal abnormalities may precede striatal dysfunction and morphological abnormalities in HD (Coyle & Schwarcz, 1976; Laforet et al., 2001; Sapp et al., 1999; Stack et al., 2007; Zuccato et al., 2001). BDNF expression can be regulated by htt, which has an important role in intracellular transport, synaptic function, neurogenesis and neuronal gene transcription (Baquet, Gorski, & Jones, 2004; Gauthier et al., 2004; Raymond et al., 2011; L. L. Wu, Fan, Li, Li, & Zhou, 2010; Zuccato et al., 2001; Zuccato et al., 2010). Wild-type but not mutant htt increases BDNF production by cytosolic sequestration of the transcriptional repressor, RE1-Silencing Transcription factor/Neuron-Restrictive Silencer Factor (REST/NSRF), preventing binding to its nuclear target neuron-restrictive silencer element (NSRE), which has a negative regulatory effect on the BDNF gene (Zuccato et al., 2003). Loss of normal htt function in HD may lead to reduced BDNF gene transcription (Cattaneo, Zuccato, & Tartari, 2005; Zuccato et al., 2003). Polyglutamine expansion in HD also alters the interaction between huntingtin-associated protein 1 (HAP1) and BDNF leading to impaired fast axonal anterograde transport of the neurotrophin along microtubules (Gauthier et al., 2004; L. L. Wu et al., 2010).

The striatum expresses abundant BDNF protein and trkB receptors, but contains virtually no BDNF mRNA, except for sparse expression in a few interneuron subpopulations (Conner et al., 1997; Fryer et al., 1996; Radka, Holst, Fritsche, & Altar, 1996). BDNF expression in the

corticostriatal pathway exerts an anterograde trophic effect on GABAergic medium spiny neurons and interneurons (Altar et al., 1997; Baquet et al., 2004; Xie et al., 2010). Overexpression of BDNF in forebrain of HD mouse models at least partially rescues striatal neurons and improves motor function (Gharami et al., 2008; Xie et al., 2010). Conversely, reduced striatal BDNF expression accelerates motor dysfunction and loss of enkephalin-containing striatal neurons in R6/1 mice (Canals et al., 2004).

Studies of BNDF in cerebral cortex from HD patients vary from no mRNA loss (Ferrer et al., 2000; Gauthier et al., 2004) to a decrease of both message and protein in samples of parietal cortex without a correlation with disease state (Zuccato et al., 2008). It is proposed that reduced BDNF protein consistently noted in HD striatum (Gauthier et al., 2004; Seo, Sonntag, & Isacson, 2004), and in animal models (DeMarch et al., 2008; Giralt et al., 2011; Peng et al., 2008; Simmons, Mehta, Lauterborn, Gall, & Lynch, 2011) may reflect reduced cortical BDNF production (Zuccato & Cattaneo, 2009). Mutant htt may impair kinesin motors resulting in reduced axonal transport or terminal release of BDNF in striatal afferents and contribute to loss of neurotrophic support (Borrell-Pages, Zala, Humbert, & Saudou, 2006; Gauthier et al., 2004). In contrast to limited studies possible on human tissue, reverse transcriptase-polymerase chain reaction shows normal cortical BDNF mRNA levels in R6/2 mice in presymptomatic 4-week-old animals, but progressive reduction after 6 weeks (Apostol et al., 2008; Zuccato et al., 2005). A systematic evaluation of regional cortical loss of BDNF gene expression has not been performed in HD or in animal models.

On the basis of regional anatomical analysis using *in situ* hybridization, we provide evidence for progressive reduction in BDNF mRNA expression in different frontal areas in R6/2 mice. BDNF expression is downregulated in limbic frontal cortices in 4-week-old R6/2 mice, preceding loss in motor cortices. Interestingly, time course analysis of a vast array of genes in the R6/2 mouse cortex and striatum shows first alterations at 4 weeks (Luthi-Carter et al., 2002). BDNF gene expression changes in limbic frontal cortices may therefore reflect an early pathological change resulting in loss of corticostriatal integrity and correlating with early learning and memory deficits (Lione et al., 1999; Ruskin et al., 2011). If documented in HD patients, this early reduction in limbic cortical BDNF may contribute to psychiatric and cognitive manifestations, which can precede overt motor signs (Stout et al., 2011).

Reduction of BDNF mRNA expression in premotor and motor cortices follows early loss in limbic cortices and best correlates with the onset of motor dysfunction in 6-week-old R6/2 mice. Interestingly, the number of cortical neurons remains unaltered in both limbic and motor cortices at 6 weeks, indicating that the early loss of BDNF mRNA expression in these areas is not a reflection of neuronal loss. As striatal volume loss noted at 6 weeks precedes striatal neuronal atrophy, atrophy of corticostriatal afferents may occur as cortical BDNF expression is reduced. Further declines in BDNF in cortical and subcortical afferents, especially when combined, may contribute to striatal neuronal loss in older R6/2 mice.

Altered performance on the open field and rotarod tests, and worsened clasping scores at 6–9 weeks correlate with onset of BDNF gene expression changes in the motor cortex and the Pf, which are major sources of glutamatergic afferents to the striatum. Conditional knockout of BDNF mainly in the cerebral cortex of Emx-BDNF mice results in mild clasping abnormalities, unaltered rotarod performance and loss of striatal dendritic spines but only mild neuronal loss in aging animals (Baquet et al., 2004). It is, therefore, possible that loss of anterograde delivery of BDNF from other sources, such as the thalamus and midbrain, may also be important to generation of the striatal HD phenotype. Numerous other factors related to striatal expression of mutant htt, including increased neuronal vulnerability and excitotoxicity, likely also contribute to neuronal degeneration (Kim et al., 2011; Raymond et al., 2011; Saudou & Humbert, 2008; Zuccato & Cattaneo, 2009).

3.4.4 Time course of changes in BDNF expression in striatal afferents from the thalamus and midbrain and their relationship to behavior

In addition to forebrain cortical areas, R6/2 mice show progressive downregulation of BDNF mRNA expression in two other major sources of striatal afferents, the thalamus and the ventral midbrain. Dopaminergic neurons localized in the SNc (Bjorklund & Dunnett, 2007; Hillarp, Fuxe, & Dahlstrom, 1966; Sourkes & Poirier, 1965) are the source of a massive nigrostriatal projection, express high levels of BDNF (Conner et al., 1997; Seroogy et al., 1994) and depend on the neurotrophin for survival (Alonso-Vanegas, Fawcett, Causing, Miller, & Sadikot, 1999; Baquet, Bickford, & Jones, 2005; Baquet et al., 2004; Hyman et al., 1991). Changes in BDNF

expression in the SNc of R6/2 mice are detectable by 4 weeks of age and correlate with onset of dystonic clasping and with discriminant learning deficits sensitive to frontostriatal dysfunction (Lione et al., 1999). Interestingly, WNT-BDNF conditional knockout mice with reduced brainstem BDNF expression also exhibit hindlimb clasping and poor rotarod performance (Baquet et al., 2005; Pineda et al., 2005), suggesting a potential functional role for BDNF in the nigrostriatal system. R6/1 HD mice may show reduced anterograde transport of BDNF in the nigrostriatal system (Pineda et al., 2005) Previous work indicates no significant loss of DA neurons in HD except at advanced stages (Robitaille et al., 1997). Our results suggest that reduced nigrostriatal BDNF anterograde support may contribute to motor disturbance and striatal neuronal degeneration in HD.

Besides cortical inputs, the neostriatum also receives massive glutamatergic afferents from the intralaminar thalamic nuclei. In primates, the caudal intralaminar complex comprises the centromedian (CM) and Pf nuclei, which provide massive inputs to the sensorimotor and associative striatal territories, respectively (Berendse & Groenewegen, 1990; Sadikot, Parent, & Francois, 1992; Y. Smith et al., 2009). In HD, the thalamus appears normal at initial stages, but neuronal loss is detected in the CM at later stages (Sadikot & Rymar, 2009; Vonsattel, Keller, & Cortes Ramirez, 2011). In rodents, the CM and PF are not clearly distinct on Nissl stain, and the lateral part of the Pf is considered the homolog of the primate CM. The Pf expresses one of the highest levels of BDNF mRNA in the brain (Conner et al., 1997), and BDNF in the thalamostriatal system may play an important role in survival of vulnerable striatal neurons during

developmental apoptosis (Sadikot et al., 2005). Our results show an important reduction of BDNF mRNA expression in Pf of R6/2 mice at intermediate and later stages of the disease, corresponding to significant atrophy and reduced number of striatal neurons. In addition to corticostriatal afferents, anterograde thalamostriatal BDNF trophic support may therefore also play an important role in survival of striatal neurons in HD.

In conclusion, increased vulnerability of striatal projection neurons in HD results from multiple cell autonomous and extracellular factors. Current theories implicate excitotoxic and neurotrophic mechanisms (Raymond et al., 2011; Saudou & Humbert, 2008; Zuccato et al., 2010). We provide evidence for an age-dependent decrease in BDNF expression in major sources of afferents to the striatum in the R6/2 mouse model of HD. BDNF mRNA is progressively reduced not only in the cerebral cortex but also in subcortical sources of striatal afferents, including inputs from the thalamus and the midbrain. Analysis of behavior and morphology during the R6/2 mouse lifespan suggests that loss of BDNF plays an important role in motor and nonmotor abnormalities in HD, and contributes to striatal neurodegeneration. Restoring neurotrophic support to striatal neurons may therefore be a potential therapeutic strategy to be explored for patients with HD.

CHAPTER 4

DISCUSSION

4.1 Literature Review: neurotrophin hypothesis

4.1.1 Brain-derived neurotrophic factor hypothesis

In this chapter, I further interpret the results obtained based on the animal model presented in Chapter 3, in light of the neurotrophin hypothesis of HD. I will review the literature of BDNF and its implications in HD, then review the main striatal afferents and finally explain the neurotrophin hypothesis.

4.1.1.1 Brain-derived neurotrophic factor

The neurotrophins history started in the mid-twentieth century with the discovery of the nerve growth factor. Trophic effects include survival and growth-promoting effects on neurons and these were attributed to the neurotrophins. In 1982, BDNF was shown to also have trophic effects (Binder & Scharfman, 2004). Essentially, BDNF promotes survival and growth of neurons. It is also involved in brain development (Binder & Scharfman, 2004). Further research led to the discovery of other neurotrophins including neurotrophin-3 and neurotrophin-4/5.

The BDNF gene, located on chromosome 11 in humans, essentially contains 5 exons, each with their own promoter. Exon V code for the mature protein (Binder & Scharfman, 2004). Exons I-III transcriptional initiation sites encode 8 mRNAs found predominantly within the brain whereas exon IV promoter variant is mainly found within the lung and the heart. However, new research emphasizes the need to refine BDNF structure nomenclature (Aid, Kazantseva, Piirsoo, Palm, &

Timmusk, 2007). Interestingly, BDNF and the other neurotrophins have approximately 50% of shared amino acid sequences. Of note, neurotrophins including BDNF are initially produced as proneurotrophins with different biologic activity in comparison to their mature forms. Prohormone convertases usually cleave the neurotrophins to their active forms after it is shuttled from the trans-Golgi system (Binder & Scharfman, 2004) (Farhadi et al., 2000).

BDNF binds to tropomyosin-related kinase-B (trk) receptors, a subgroup of the family of receptors tyrosine kinase. Binding of the receptors leads to dimerization and kinase activation with subsequent intracellular interactions with proteins such as PLC and Shc, after which Ras-MAP and cAMP cascade activations follows (Binder & Scharfman, 2004). Surprisingly, it is thought that trk receptors may exist in truncated form associated with different functions. For example, after a traumatic injury to the central nervous system, the truncated form of trk receptors is upregulated and may modulate neuronal vulnerability (Binder & Scharfman, 2004). Neurotrophins also bind to a different class of receptors related to the tumor necrosis factor proteins, the p75 receptors. Although unclear, their action is related to cellular apoptosis. Previous studies showed that often trk and p75 receptors are co-expressed on the same cell and lead respectively to an anti- and pro-apoptotic cascade. It seems that the proneurotrophins may have a higher affinity for the p75 receptors therefore promoting cell apoptosis whereas the mature neurotrophins have an opposite effect on trk receptors (Dechant & Barde, 2002).

Initial studies showed that different neurotransmitters regulated BDNF production in different manners. Glutamate and acetylcholine increase BDNF production whereas gamma-amino-butyric acid (GABA) decreases it (Lindholm, Castren, Berzaghi, Blochl, & Thoenen, 1994). In addition, more subtle physiologic regulation takes place depending on the location of BDNF. For example, BDNF production in the visual cortex increases with light and electrical stimulation while long-term potentiation causes the same effect in the hippocampus (Lindholm et al., 1994). More recent literature confirms these findings. Calcium influx via voltage-gated calcium channel as well as NMDA receptors upregulate BDNF production (Zheng, Zhou, Moon, & Wang, 2012). Exon-III containing BDNF mRNA has been the most widely studied given its high level within cortical neurons. There are 3 calcium-responsive elements at the regulatory region of exon III. These are regulated by upstream calcium-regulated kinas such as Ras/ERK, cAMP/PKA and CaM (Zheng et al., 2012). As well as transcriptional factors, epigenetics also seem to play an important role in BDNF production regulation. For example, chromatin remodeling influences BDNF production: histone deacetylase 2 deficient (HDAC2) mice versus mice that over expressed HADC2 had opposite findings on memory formation (Guan et al., 2009). DNA methylation may also play a key role as DNA hypermethylation globally decrease BDNF production (Zheng et al., 2012). BDNF production regulation is multimodal allowing neurons to cope with environmental changes.

The BDNF protein in the CNS is widely spread but benefits a discrete localization (Conner et al., 1997), specifically, BDNF rich areas including the frontal cortex, hippocampus, anterior

thalamus and the substantia nigra (Conner et al., 1997). Interestingly, initial localization of BDNF mRNA with in situ hybridization differed from the localization of BDNF proteins with immunohistochemistry. Indeed, many areas, including the striatum, revealed extensive fiber and terminal labeling but contained no BDNF mRNA. This led to the hypothesis of anterograde transport of BDNF and was supported by various other findings. Destruction of afferents neurons or inhibition of axonal transport decreased BDNF proteins level in afferent area (Altar et al., 1997) (Conner et al., 1997). Also, pro-BDNF is stored within dense-core vesicles and synaptosomes are strongly implicated in neuropeptide storage and release at the synaptic junction (Altar & DiStefano, 1998).

BDNF has been implicated in various pathologies such as visual cortex development and social defeat stress hypothesis (Huang et al., 1999) (Berton et al., 2006). Given the high hippocampal BDNF concentration, previous studies have shown an association with learning and memory (Hall, Thomas, & Everitt, 2000). As mentioned previously, glutamate is implicated within the regulation of BDNF production; previous experiments have studied the role of BDNF in epilepsy. Not surprisingly, BDNF is increased during a seizure event. It seems also that BDNF have pro-epileptogenic effects (Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991). In fact, although not reflective of a causal link, temporal cortex specimens from patients with temporal lobe epilepsy showed increased BDNF levels (Takahashi et al., 1999). Of more importance, classically BDNF has been associated with HD given the relationship between htt and the latter (Roze et al., 2008) (Zuccato et al., 2001).

4.1.1.2 Huntington's disease and BDNF

Initially, what led the scientific community to implicate BDNF in HD was the decreased BDNF level found in HD animal models and patients (Gharami et al., 2008) (Zuccato et al., 2001). Similarly, a BDNF heterozygous R6/1 mouse model showed accelerated HD pathology (Canals et al., 2004). Interestingly, TrkB receptors are preferentially expressed on medium spiny neurons of the indirect pathway which were known to be the most susceptible in HD (Baydyuk et al., 2011). Zuccato et al. illustrated the close relationship between htt and BDNF. Initially, in vitro experiments showed that mHtt decreases BDNF protein production whereas htt increases BDNF protein production. Interestingly, Htt mainly exerts its effects on BDNF via the promoter of exon II (Zuccato et al., 2001). This seems to be an effect at the transcriptional level; the htt inhibits the silencing activity of the neuron restrictive silencer element therefore upregulating BDNF transcription whereas mHtt is less capable of retaining this silencer element (Zuccato et al., 2003). Of note, Htt effect on BDNF production seems to be specific as it does not influence the expression of other neurotrophins (Zuccato et al., 2003). A previous study measured serum BDNF in HD patients and showed that increased number of CAG repeats, and thus disease severity, was associated with lower serum BDNF levels (Ciammola et al., 2007). Similarly, in vivo experiments with transgenic mice containing mHtt revealed decreased BDNF mRNA in areas known to produce BDNF such as the cortex. Similar finding was found in post-mortem HD human brains (Zuccato et al., 2001). Therefore, these studies indicate that Htt likely regulates BDNF protein. Additionally in HD, mHtt downregulates the production of BDNF. Further

experiments by our group revealed that BDNF mRNA levels in the cortex start to decrease around 6 weeks of age in R6/2 mice, which correlates, as previously mentioned, with early onset of behavioral deficits.

The relationship between Htt and BDNF is more complex and may involve axonal transport (Gauthier et al., 2004). Htt is found in the nucleus but even more so in the cytoplasm where it interacts with huntingtin-associated protein-1 (HAP-1). HAP-1 is involved in axonal transport given its interaction with dynactin, an essential component of the microtubule system (Engelender et al., 1997; Gauthier et al., 2004). Interestingly, both hypotheses of HD, gain of function and loss-of-function, seem to be at play in the axonal transport. Previous studies showed that Htt facilitates the transport of BDNf along microtubules whereas mHtt and its long associated polyQ repeats interacting with HAP-1 prevent efficient BDNF transport. Therefore, mHtt may contribute to HD pathology by both decreasing BDNF levels and also impairing its delivery to critical areas including the striatum.

mHtt may also exert a negative effect on BDNF by intervening in BDNF protein maturation. Even though previously reported immunohistochemistry assays consistently reported a decreased striatal BDNF level (Gauthier et al., 2004; Gharami et al., 2008; Samadi et al., 2013), studies using enzyme-linked immunosorbent assay (ELISA) assays did not always report similar findings (Canals et al., 2004). Of note, currently available ELISA assays do not differentiate between pro-BDNF and mature BDNF which could explain the discrepancies. It could be

hypothesized that there is pro-BDNF accumulation in the striatum which could be detrimental to neuron survival. Interestingly, mHtt may impair BDNF maturation since, as discussed previously, it impairs axonal transport. mHtt impair the formation of the HAP-1, sortilin (a co-binder of P75) and pro-BDNF which is thought to facilitate BDNF maturation process (Yang, Lim, Li, Zhong, & Zhou, 2011).

Restoring BDNF level has therefore been put forward as a rescue strategy (Zuccato et al., 2001). In fact, experiments using a HD excitotoxic model showed that overexpressing BDNF in the striatum was neuroprotective: the volume and number of neurons in the striatum were greater in the mice with overexpressed BDNF (Bemelmans et al., 1999). This represented one of the first morphologic rescues in a model of HD. Similarly, experiments comparing transgenic mouse model of HD overexpressing cortical BDNF versus their counterparts having received no treatment essentially showed a behavioral and morphological rescue (Gharami et al., 2008) (Xie et al., 2010).

4.1.2 Striatal neuroanatomy

The striatum has 3 major afferents. There are the glutamatergic excitatory inputs (i.e. sensorimotor cortical areas and the intralaminar nuclei of the thalamus) and the dopaminergic inputs from the midbrain (Y. Smith et al., 1994). It is thought that the inputs from the sensorimotor cortex are modulated by the midbrain inputs due to their convergent synaptic

inputs. On the other hand, the thalamic inputs seem to be independent from the dopaminergic inputs (Y. Smith et al., 1994).

The thalamic inputs come mainly from the intralaminar nuclei, more specifically the centromedian and PF complex. The CM-PF complex projections to the striatum have highly focal convergent sites of termination in the striatum whereas the other thalamic nuclei have diffuse and less convergent innervations (Y. Smith et al., 2009). This complex is most developed in primates and distinguishable on Nissl stain. The latter does not distinguish a clear CM on rodent brain. The lateral part of the PF is usually considered to be the homologue of the primate CM (Sadikot & Rymar, 2009). The CM-PF complex projects mainly to the striatal patches rather than the striatal matrices. The CM and PF are thought to have slightly different functions. The PF afferents are from the associative-limbic part of the GPi and its efferents project to the rostral striatum, and is therefore linked with associative areas. The CM afferents are motor cortices and motor thalamic nuclei and its efferents project to the post commissural striatum, and is therefore associated with motor areas (Sadikot & Rymar, 2009) (Y. Smith et al., 2009).

The cortex is a large structures representing an important afferent to the striatum (i.e. the striatal dendritic spines). The thalamostriatal system, although a more discrete structure, may have a more privileged access to the proximal striatal dendrites. As mentioned, the midbrain

also has striatal afferent inputs. These structures could be amenable to focus therapies such as stereotaxic injections.

4.1.3 Conceptualized Neurotrophin Hypothesis

Knowing that striatal BDNF in HD is decreased and that overexpressing BDNF may help rescue neurons in HD, it can be postulated that the anterograde transport hypothesis could supply BDNF to the degenerating neurons in the striatum if the latter could be overexpressed in the striatal afferents (figure 4.1).

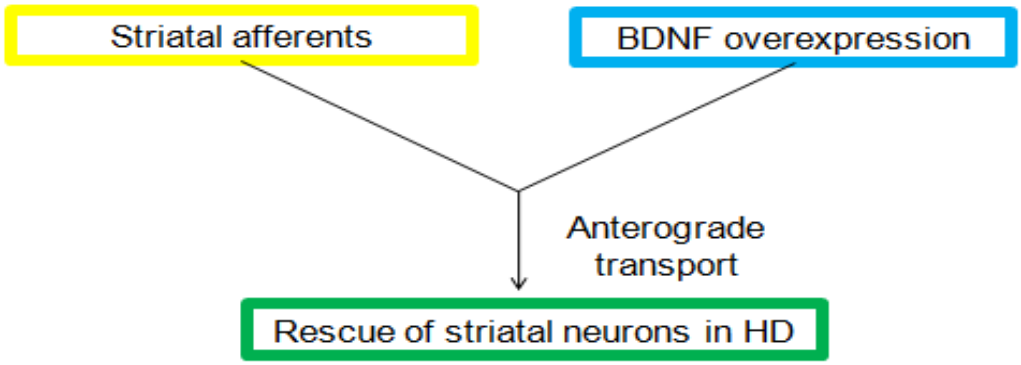

Figure 4.0 Experiment paradigm: therapeuthic strategy using the anterograde transport hypothesis by overexpressing BDNF in the thalamostriatal systems to rescue striatal neurons in HD.

There are multiple strategies that have been used to overexpress BDNF in the central nervous system (Figure 4.1) (Nagahara & Tuszynski, 2011). Many systemic strategies have been tried to overexpress BDNF in the central nervous system (CNS); stimulation of BDNF production with various agents such as antidepressants, modulators of AMPA receptors or by enriching the environments have been previously used with satisfying results. (Peng et al., 2008) (Simmons et al., 2009) (Spires et al., 2004). For example, sertraline improved behavior and morphological

outcomes in R6/2 mice. Other drug delivery routes have been used in the hope of achieving higher drug concentration in the central nervous system while minimizing systemic effect. These routes include intrathecal and intranasal injections as well as transient blood-brain-barrier opening (Coureuil et al., 2010; Dhuria, Hanson, & Frey, 2010; R. A. Smith et al., 2006). A small study showed beneficial effects of systemically administered BDNF, although primary outcomes such as mortality were not assessed (Giampa et al., 2013). The goal of the current hypothesis is to overexpress BDNF in a specific location of the brain, the PF, given its important relationship with the degenerative striatum in HD.

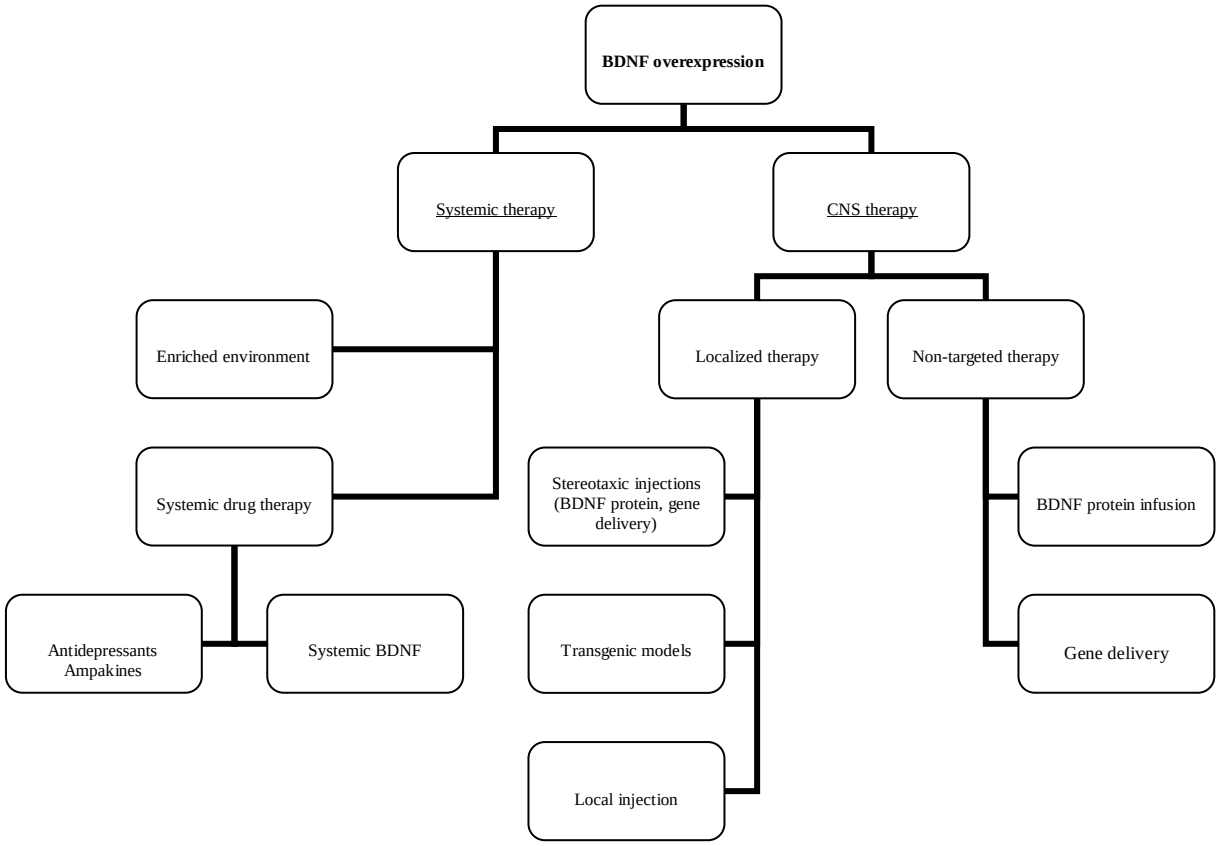

Figure 4.1 Summary of reported therapies using BDNF overexpression. *Adapted from Nagahara and Tuszynki, 2011.*

Strategies for localized BDNF overexpression include local (Nawa, Pelleymounter, & Carnahan, 1994) and stereotaxic injections and engineered mouse models. To our knowledge, although previous studies have used an engineered mouse models allowing specific cortical BDNF overexpression (Huang et al., 1999), there is no engineered mouse model that offers specific BDNF overexpression in the PF. Stereotaxic injections of stem cells have previously been used to overexpress BDNF in a specific brain location (Blurton-Jones et al., 2009). However, our experimental paradigm, as described previously, uses an existing neural pathway. Injecting stem cells in a specific location would increase BDNF level locally but it is unlikely that the injected stem cells would develop projection in the pre-existing pathway to deliver BDNF to the downstream target. Overexpression of BDNF using a transgene expressed in specific brain areas has been described (Gharami et al., 2008) (Xie et al., 2010). However, we are not aware of a current transgene model that reliably and specifically locates in other striatal afferents, such as the PF. Lastly, BDNF can be expressed in a specific brain areas using stereotaxic injection of, for example, viral vectors.

The most common viral vectors used are the adeno-associated virus (AAV) and herpes simplex virus (HSV). The latter infects neurons efficiently, but the immunogenicity and potential adverse effects remain to be studied, whereas the former has been most widely studied. AAV is a small, single-stranded 4.7-kb DNA genome, with a simple non enveloped icosahedral protein coat (Peel & Klein, 2000). Approximately 80% of the human population has positive serotype for AAV. There has been no disease associated to its presence (Goncalves, 2005). Modern AAV

vector only contain 96% of the initial viral vectors, sufficient for packaging and integrating into the target DNA. This absence of viral sequences translates into the absence of de novo viral protein synthesis, limiting inflammatory reaction. Limitations include the small size of the vector, limiting DNA packaging ability to make recombinant AAV (rAAV) (Peel & Klein, 2000). AAV uses the ubiquitous glycosaminoglycan receptors as primary attachment and internalization is aided by co-receptors including integrins and fibroblast growth factor receptors (Goncalves, 2005). In humans, AAV integrates onto chromosome 19 and follows a lysogenic pathway. This means AAV gene expression is auto-repressed and latency ensues. This targeted integration allows the virus to be perpetuated though the host cells division (Goncalves, 2005). In summary, AAV lacks pathogenicity, has a wide range of infectivity and has a long-term transgene expression.

There are multiple rAAV serotypes. The oldest and most studied one was the rAAV serotype 2 (rAAV2). Most of the work done on CNS transgene expression has been done with AAV-2. It efficiently transduces into neurons. The newer different serotypes differ in their properties including transduction and trophism (Z. Wu, Asokan, & Samulski, 2006). Serotypes 1,2,4 and 5 have been reported to have the highest transduction efficiency in the CNS (Burger et al., 2004). For example, rAAV serotype 5 (rAAV5) has a high transduction rate in the CNS while rAAV serotype 4 (rAAV4) seems to target specific cell population in the CNS such as ependyma and astrocyte in the subventricular zone. The trophism of rAAV5 is also broad, usually targeting

mainly neurons without discrimination (Burger et al., 2004). Transgene expression peaks between one to two weeks post-injection (Peel & Klein, 2000).

Overexpression of BDNF in the CNS using rAAV has been previously studied with good results. It has been mostly studied in the retina to rescue neurons in glaucoma and age-related macular degeneration (Martin et al., 2003) (Di Polo, Aigner, Dunn, Bray, & Aguayo, 1998). It is interesting to integrate the new literature to conceptualize the neurotrophin hypothesis. Repletion of the striatal BDNF using naturally occurring neuroanatomy (i.e. overexpressed BDNF in striatal afferents) may become a sound physiologic therapy in HD.

4.2 Conclusion

In the early 1980s, scientists were hopeful that HD would soon have a cure. However, even though the etiology of this monogenic disorder was identified more than 30 years ago, there is no treatment for this lethal disease. Scientific advances have been made: there are now accepted mouse models to study the pathophysiology of HD as well as pharmaceutical trials. As further research ensues, we have come to realize that HD is far more complex than a mutation on chromosome 4. It is a multimodal interplay between the mutated protein and the physiologic regulation of neurons involving, amongst all, neurotrophins.

We have discussed the R6/2 mouse as an appropriate anatomical and behavioral model for understanding the mechanisms of neuronal degeneration in HD. The BDNF hypothesis in HD

was reviewed with the most recent literature. Future work is promising regarding therapeutic trials in HD.

We hypothesize that using the neurotrophin hypothesis we can improve behavioral outcomes in the R6/2 mouse model. However, future work needs to use a refined neurotrophin hypothesis. Physiologic overexpression of BDNF should be used to replicate as close as possible physiologic neuronal regulations. As well, as shown with our previous work (Samadi et al., 2013), striatal BDNF decrease correlates well with poor behavior performances in R6/2 mice. We therefore hypothesize that overexpressing BDNF within the CM-PF complex could represent a novel therapeutic approach to HD.

The obvious striatal changes in HD may not be the primary event, but they could be secondary to earlier striatal afferents degeneration. Even though, the neurotrophin hypothesis is based on longstanding neuroanatomical and pharmacological research, a recently published study may cause the neurotrophin hypothesis to be re-postulated; while BDNF in striatal afferents may be decreased, unimpaired anterograde transport was observed, rather, dysregulation of TrkB downstream cascade was found.

Appendix I: Instrumental Analysis

1.0 Behavior assessment

1.1 Open-field test

The open-field test consists in observing a mouse placed in an enclosed space, usually square. It is a common and simple experiment to measure of exploratory behavior and general activity (i.e. spontaneous locomotor activity) (Brooks & Dunnett, 2009). Observation may be done using various medias, from the observer own eye to a video-tracking system. Quality and quantity of movements may be assessed. For example, distanced moved at different speeds, time spent inactive, grooming, freezing, or preferential space location may be measured (Brooks & Dunnett, 2009). The open-field test may also be used to test pharmaceutical venues. Many parameters can influence the reliability of this test including mouse handling, housing conditions, behavior history and equipment used for the test (Brooks & Dunnett, 2009).

1.2 Grip Strength

The grip strength test consists in using a dynamometer linked with a metal grid recording the force required to pull the mouse off the grid (Brooks & Dunnett, 2009). This specifically assesses strength and depending on mouse handling, forelimbs, hind limbs or all limbs strength may be assessed. The grip strength test may also be used to test pharmaceutical venues. However, limitations include unwillingness of the mice to cooperate (Brooks & Dunnett, 2009).

1.3 Clasping score test

The clasping score test consist in a short tail suspension test observing for paws pressed against the body, otherwise known as clasping (Lin et al., 2001). Clasping has been observed in various mouse models of neurological disease including HD (Lin et al., 2001) (Mangiarini et al., 1996). It is thought to represent a part of a cumulatively predictive set of behavioral symptoms. The clasping score test may also be used to test pharmaceutical venues. There does not seem to be defined parameters for this test. We designed our own clasping score test using a modified scale previously used by our laboratory. Limitations include inter-rater reliability and test-retest reliability.

1.4 Rotarod Test

The rotarod test consists in placing a mouse on a rotating rod and record latency to fall (Brooks & Dunnett, 2009). It is the most common test used to measure balance and coordination. Different protocols may be used depending on the goals of the experiments. For example, a fixed-speed rotarod protocol should be used to detect small changes in balance and coordination as it is more sensitive and an accelerating-speed rotarod protocol should be used to characterize the magnitude of behavior changes as it is quicker and easier to perform (Monville et al., 2006). The rotarod test may also be used to test pharmaceutical venues. Overall, the rotarod test remains one of the most common use behavioral tests due to its ease of use and sensitivity (Brooks & Dunnett, 2009).

1.5 Cylinder Test

The cylinder test consists in placing a mouse in a see-through cylinder for a short period of time and observation may be done using various medias, from the observer own eye to a video-recording system, and from the side or from below (Brooks & Dunnett, 2009). It assesses the use of forelimbs in the context of the naturally occurring drive in rodents to explore the environment by standing on their hind limbs and lean on walls using forelimbs. Limb-use asymmetry may therefore be assessed (LeDoux, 2005). This test is therefore useful in the case of expected limb-use discrepancy and therefore is less sensitive for most genetic mutations with bilateral limb dysfunctions (Brooks & Dunnett, 2009).

1.6 Fisher LSD Test

The Fisher's least significant difference test is used as a post-hoc analysis to essentially analyze specific comparisons after an analysis of variance. It does pair wise comparisons between to discover which means were rejected in the stage 1 analysis of variance. It protects from type I error for analysis with up to 3 populations (Seaman, Levin, & Serlin, 1991) (Hayter, 1986).

2.0 Post-Mortem brain assessment

2.1 Stereology

Stereology consists in an unbiased way to quantify histology. The method essentially relies on systematic random sampling assuming all counted objects have an equal probability of being sampled (West, 1993). It allows quantification of various morphological parameters such as

brain volume as well as cell numbers and volume. It represents an accurate and reliable method for morphological analysis (Howard & Reed, 1998)

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2.2 In-situ hybridization

In-situ hybridization consists in using an hybridization reaction between a labeled nucleotide probe and a complimentary RNA or DNA sequence thus allowing assessment of gene expression in histological sections or cytological preparations (Jin & Lloyd, 1997). These probes may be detected with chromogen development allowing for qualitative analysis or with audioradiographic emulsions allowing quantitative analysis. This method preserves cell integrity within the tissues and thus allowing anatomic correlations (Jin & Lloyd, 1997). Multiple protocols have been tried to optimize sensitivity and specificity. In the current experiments, we chose a modified free-floating protocol in order to increase sensitivity due to the relatively high thickness of the histological sections used (Levsky & Singer, 2003).

2.3 Immunohistochemistry

Immunohistochemistry consists in using monoclonal or polyclonal antibodies to detect specific antigens (Duraiyan, Govindarajan, Kaliyappan, & Palanisamy, 2012). These may be detected with chromogen development or with audioradiographic emulsions. This allows localization of antigens within histological sections or cytological preparations used for a variety of applications (Matos, Trufelli, de Matos, & da Silva Pinhal, 2010). Multiple protocols have been

engineered to reduce tissue damage and reduce the amount of antibody needed to optimize sensitivity and specificity (Duraiyan et al., 2012).

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