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Huntington's Disease

Molecular Pathogenesis and Current Models

Edited by Nagehan Ersoy Tunalı

HUNTINGTON'S DISEASE - MOLECULAR PATHOGENESIS AND CURRENT MODELS

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http://dx.doi.org/10.5772/62999
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Notice

First published in Croatia, 2017 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

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Huntington's Disease - Molecular Pathogenesis and Current Models
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p. cm.

Print ISBN 978-953-51-3049-9

Online ISBN 978-953-51-3050-5

eBook (PDF) ISBN 978-953-51-4897-5

Meet the editor

Nagehan Ersoy Tunalı received her PhD, MSc, and BSc degrees in Molecular Biology and Genetics at Boğaziçi University, Istanbul (TR). Her PhD work involved "Molecular Analysis of Polyglutamine Diseases and Investigation of the Interaction between Huntingtin and Nuclear Receptor Corepressor." She had the opportunity to get experienced in Huntington's disease (HD) research at University of Manchester (UK), CNR-Istituto di Medicina Sperimentale e Biotecnologie (IT), and University of Wales College of Medicine (UK). She served as the editor in chief of *Journal of Cell and Molecular Biology* between years 2006 and 2016. Nagehan Ersoy Tunalı currently works as an assistant professor Dr at İstanbul Medeniyet University, Department of Molecular Biology and Genetics, Istanbul (TR). Her research interests cover molecular diagnosis of polyglutamine repeat diseases, genetic modifiers of HD, localization and interactions of huntingtin, and the molecular mechanisms of excitotoxicity in HD. Her specializations are also extended to genetic susceptibility to kidney stone disease and urological cancers, biomarker discovery for bladder and breast cancers, genetic analysis of childhood metabolic disorders, and pharmacogenetics in personalized medicine.

Contents

Preface

Nearly 145 years have passed since Dr. George Huntington described Huntington's chorea in his fascinating clinical report, titled "On Chorea" [1]. His observations and still appreciated detailed descriptions of the condition opened a new perspective in medical science. The collaborative research efforts devoted to explore the molecular mechanisms of the disease serve as an outstanding example for the scientific communities. Moreover, the established guidelines for genetic counseling of the affected Huntington's disease (HD) families are regarded as a fundamental source for almost all neurodegenerative and dominantly inherited conditions.

The gene for Huntington's disease, IT-15, was mapped to the short arm of chromosome 4 in 1983 [2]. Despite intensive efforts, the causative mutation could be identified ten years later by Huntington's Disease Collaborative Research Group [3], and HD came forward in scientific literature for being the first hereditary disease where DNA markers were utilized for the localization of the disease gene.

HD is described as a late onset progressive neurodegenerative disorder of the brain. The underlying pathogenic mutation is the expansion of the repeating CAG trinuclotides in the first exon of the IT-15 gene. More than 40 CAG repeats in the gene definitely cause the disease. However, repeats between 36 and 39 CAGs are considered to have reduced penetrance. The extended CAG repeat tract is translated into a toxic polyglutamine stretch in the huntingtin protein (HTT). The fact that there is only one gene and one mutation involved in disease pathogenesis certainly does not simplify molecular research. Rather, there's a huge complexity regarding genotype-phenotype correlations and the cellular mechanisms at the molecular level. There's a wide variation in both ages of onset of HD patients and disease severity, pointing out some genetic and environmental modulators of disease progression [4]. The mutant HTT gains a toxic function due to the expanded CAG repeat tract in the gene. As the repeat expands, HTT becomes more prone to cleavage by proteases, and the toxic fragment bearing the CAG repeats is separated from the rest of the protein. In addition to causing cytoplasmic distress including formation of aggregates, clogging of proteasomes, and mitochondrial dysfunction, the toxic fragment may also enter into the nucleus and interfere with transcription, and thus the whole molecular network. The toxic gain of function eventually leads to loss of function of HTT, which further complicates the matters [5]. The underlying complex mechanisms and molecular players of this cellular cascade still need to be deciphered in detail despite considerable advances. Once solved, the related molecular mechanisms will not only enlighten the HD story but will also shed light on other polyglutamine diseases and similar brain disorders. For the time being, the missing pieces of this neurodegeneration puzzle leave the patients with only symptomatic relief, but no cure. HD is still known as the most debilitating condition. In this respect, we, as researchers working in

this field, should put extraordinary efforts to work hard in our labs and share our experiences and knowledge with HD families to support them on genetic, ethical, and legal issues.

This book, *Huntington's Disease - Molecular Pathogenesis and Current Models*, is planned to cover recent scientific achievements in understanding the cellular mechanisms of HD. The chapters provide comprehensive description of the key issues in HD research. The book will serve as a source to help the clinicians and researchers in the field and also life science readers to increase their understanding and awareness of the clinical correlates, genetic aspects, neuropathological findings, and potential therapeutic interventions involved in HD.

In the first chapter of the book, "Genetic Modifiers of CAG.CTG Repeat Instability in Huntington's Disease Mouse Models" by E. Dandelot and S. Tomé, the authors provide an overview of the data recently published about CAG repeat instability. Expansions of repetitive sequences on DNA, including trinucleotide repeats, are responsible for a very wide range of disorders. Understanding the nature of somatic and meiotic instability of the trinucleotide repeats is at the heart of solving the molecular mysteries of triplet repeat diseases. In addition to that, the authors provide information about the roles of genetic modifiers of trinucleotide repeat dynamics in mouse models and their use as a possible therapeutic intervention. The data presented in this chapter point out that variations in DNA replication and repair genes can modulate somatic mosaism and intergenerational instability and thus the progression of the disease. Despite great advances in understanding of repeat instability, further studies are needed to assess how the various DNA repair and replication proteins act collectively in germline and somatic tissues to modulate CAG repeat expansions. Identification of such modulators is valuable in terms of their use in the reversion of repeat expansions on the way to a cure for all repeat expansion–related disorders.

In Chapter 2, titled "NR1 Receptor Gene Variation Is a Modifier of Age at Onset in Turkish Huntington's Disease Patients" by A. Açar Hazer and N. Ersoy Tunalı, the authors discuss the role of the NR1 receptor gene polymorphisms in the age of onset of HD. The major determinant of age of onset is the length of the CAG repeats. There's an inverse correlation between the age of onset and repeat size. However, the CAG repeat size alone cannot be used to predict the onset age. Even HD patients with the same number of repeats may have significant variations in their ages of onset. These findings led the researchers to investigate potential genetic modifers of disease progression. The N-methyl-D-aspartate receptors (NMDAR) have been proposed as an important putative modifier, since NMDAR-mediated excitotoxic death is involved in HD pathogenesis. The authors aimed to determine whether NMDAR gene polymorphisms contribute to the variation in ages of onset. According to their findings, rs6293 polymorphism of the GRIN1 gene can be considered as an AO modifier for Turkish HD patients with 50 or higher CAG repeats. Testing of candidate genetic modifiers in different populations is useful since their effects may exist only in specific groups. Moreover, defining such modifiers will help in the understanding of HD pathogenesis.

Chapter 3, "Pathogenesis of Huntington's Disease: How to Fight Excitotoxicity and Transcriptional Dysregulation" by Anglada-Huguet *et al.*, gives an extensive summary of the molecular pathogenesis of the disease and stresses out the importance of transcriptional dysregulation and related therapeutic potentials. It has been shown by many research groups that HTT interactions are altered by the polyglutamine expansion in the HTT protein. The mutant HTT loses some of its interactions and certainly gains new ones. Moreover, some of the transcription factors were shown to be upregulated or downregulated in the

presence of mutant HTT. Especially the HTT interactors involved in transcriptional processes attract most of the attention, since any alteration in the level and/or localization of a transcriptional regulatory protein may have destructive outcomes in the cell. Excitotoxicity is an equally important mechanism in all neurodegenerative diseases. Glutamate excitotoxicity is one of the main mechanisms involved in neuronal dysfunction and death. In this chapter, the need to continue the research on antiglutamatergic drugs to overcome excitotoxicity and the development of therapies targeting altered transcription are discussed in detail.

Chapter 4, "Porcine Model of Huntington's Disease" by Rausova *et al.*, introduces a rather unusual as well as a very useful animal model of HD. Currently, many different HD animal models are available, which predominantly are rodent models. They are very useful in understanding genotype-phenotype relationships, complex progression of the disease, and molecular pathogenesis. However, they come with limitations, such as small brain size, neuroanatomical differences to humans, and short life span. In order to overcome these drawbacks and perform safety and tolerability tests for potential therapeutic agents, upscaled models are needed. Here the authors describe the only viable transgenic HD minipigs available, which express the N-terminal part of human mutant *HTT* with 124Q under the control of human huntingtin promoter. In this chapter, unique experimental approaches utilized for the establishment of these HD minipigs are explained in detail, and phenotype progression of the minipigs is discussed. The advantages of using pigs, such as similar body weight, neuroanatomical patterns, physiological and metabolic similarities, and cognitive and motor abilities, make them preferable for longitudinal studies and studies related to disease progression. The authors provide detailed information regarding the usefulness of these transgenic minipigs, which were already used in preclinical testing of therapeutics, in fulfilling the missing link between rodent models and patients.

Chapter 5, "Pluripotent Stem Cells to Model and Treat Huntington's Disease" by Wenceslau *et al.*, addresses the importance of stem cell therapies for the treatment of neurodegenerative diseases. Pluripotent stem cells (PSCs) have been of great interest for the treatment of neurodegenerative disorders including HD, owing to their capacity to become neuronal cells. Besides, isolated PSCs that carry HD genotype have been used in many studies to produce neural progenitor cells (NPCs) *in vitro.* In this chapter, the authors discuss the current situation with HD-PSC lines, advantages and limitations, advances in their use in preclinical HD models, and their potential use in drug screening.

Chapter 6, "Transplantation in HD: Are We Transplanting the Right Cells?" by Precious *et al.*, addresses one of the most tingling issues in cell transplantation. The characteristics of the donor cells, efficacy of transplantation, and functionality of the transplanted cells are the major concerns of the cellular transplantation protocols. In this chapter, the authors discuss the important parameters of cell transplantation and requirements for striatal differentiation of the transplanted cells. In addition to that, clinical trial data are reviewed with critiques involving the improvement of functional and reproducible transplants.

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Chapter 1

Genetic Modifiers of CAG.CTG Repeat Instability in Huntington's Disease Mouse Models

Elodie Dandelot and Stéphanie Tomé

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66438

Abstract

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder whose characterstics were first described by George Huntington in 1872. Several decades later, in 1993, the mutation behind this disease was found to be an unstable expanded CAG repeat within exon 1 of the *HTT* gene localized on the short arm of chromosome 4. The majority of HD patients carry more than 40 CAG repeats, which become unstable and usually increase in size in successive generations and in tissues. In order to dissect the molecular mechanisms underlying CAG repeat instability, several HD mouse models have been created in the 1990s. Significant data have revealed that the absence of proteins from the mismatch repair (MMR) or the base and nucleotide excision repair decreased the pathogenic expansion-biased somatic mosaicism and/or intergenerational expansions. Some polymorphic variants of MMR genes have also been associated with reduced somatic expansions. Since expansion-biased somatic mosaicism likely contributes to disease manifestations, these results suggest that genetic modifiers of instability may also affect disease severity. In this chapter, we provide an overview of the data recently published about DNA instability; the roles of genetic modifiers of trinucleotide repeat dynamics in mouse models; and the possible therapeutic interventions.

Keywords: Huntington disease, DNA instability, mouse models, genetic modifiers, MMR

1. Introduction

Expansions of repetitive DNA sequences, including trinucleotide repeats, are associated with a large number of neurological and neuromuscular disorders, such as fragile X syndrome,

myotonic dystrophy type 1 and Huntington's disease (HD) [1, 2]. In the healthy population, the triplet repeat tract size varies between 5 and 30 repeats and is stable. In HD patients, the pathogenic allele contains more than 40 repeats and becomes highly unstable and usually increases in size in successive generations (intergenerational instability) and in somatic tissues (somatic instability). Longer expanded alleles are associated with more severe forms of disease and result in a decreasing age of onset from one generation to the next [1, 3, 4]. Among trinucleotide repeat disorders, HD disease is the fourth reported.

1.1. Clinical picture of HD

Huntington's disease is an autosomal dominant neurodegenerative disorder with a worldwide incidence varying from 0.1 to 10 per 100,000 people depending on the country. The estimation of prevalence varies according to haplogroups studied: it is estimated from 2 to 7 per 100,000 in the Caucasians and only 0.1–1 per 100,000 in Asians and Africans [5, 6]. Adult-onset Huntington disease is the most common form of HD and usually presents in early middle life. HD symptoms include uncontrolled movements such as chorea, progressive cognitive impairment and neuropsychiatric manifestations. The rare early-onset form of the disease also called juvenile form presents more severe symptoms with rigidity and motor dysfunctions [7]. HD symptoms and severity vary greatly among family patients and between juvenile and adult onset forms. Currently, no treatment is suitable to stop or reverse any form of HD.

1.2. Genetic of HD

HD is caused by an unstable expanded CAG repeat within exon 1 of the *huntingtin* (*HTT*) gene also called *HD* or *IT15* that localizes on the short arm of chromosome four, 4p16.3 [8]. The normal *HTT* gene contains from 5 to 35 stable CAG repeats, while the majority of HD patients have expanded repeats of above 40 CAG units that are fully penetrant. In rare cases, HD symptoms are associated with small CAG repeats from 36 to 39 CAG, which show low penetrance [9, 10]. Abnormal CAG repeat tracts become unstable in the germline, with a striking tendency toward expansions. Because longer alleles are associated with more severe form of HD, expansion-biased intergenerational instability results in a decreasing age of onset from one generation to the next, a phenomenon known as anticipation. Typically, 40–50 CAG repeats correlate with later-onset of HD, whereas a mutation greater than 50 CAG repeats results in a juvenile form. Two large analyses in HD patients (360 and 440 individuals, respectively) have reported a high negative correlation between the disease age of onset and the inherited CAG repeat length [11, 12]. Intergenerational instability biased toward expansions provides the molecular basis for clinical anticipation observed in HD (**Figure 1**).

1.2.1. Intergenerational instability

The frequencies of expanded, unchanged and contracted alleles have been investigated by directly comparing the length of the repeat tract in each parent with that is observed in their progeny to estimate the degree of intergenerational instability in each set of HD cohort. Small normal alleles with CAG repeat size ranging from 10 to 28 CAG are genetically stable with germline mutation rates <1% per generation [13]. However, the mutation frequency rises

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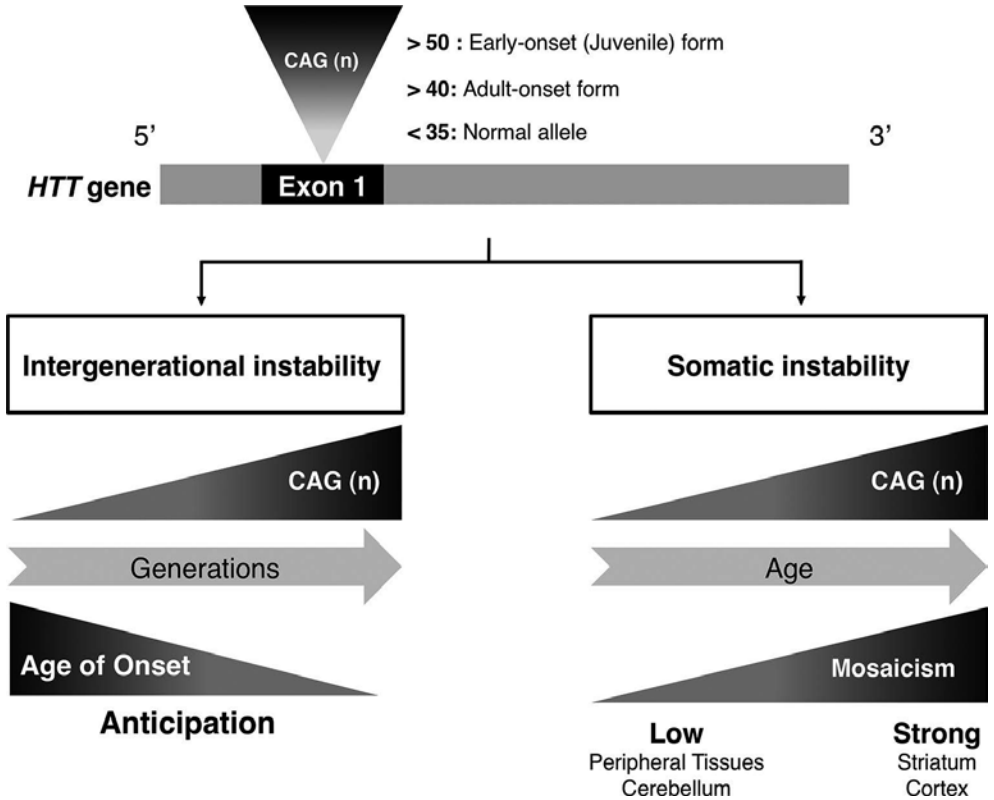

Figure 1. CAG repeat dynamics in HD: features and implications of intergenerational and somatic instabilities.

dramatically with the increasing size of the allele. Indeed, a CAG repeat size change on expanded allele in the range of 36–49 repeats occurs in >70% of transmissions from affected parents to HD children. A similar rate of expansion was found between multiethnic cohorts [13–19]. In the two largest cohorts (>250 parent-offspring pairs), the frequency of expansions was estimated to be 52.1% in a multiethnic HD population and 67.3% in the Dutch cohort, whereas only 18.1% and 25.2% contractions were observed, respectively [13, 18]. For individuals carrying more than 49 CAG repeats, the mutation rates go up to >95% per generation [14, 20]. In all cases, the frequency of expansions always exceeds the frequency of contractions in HD populations. The instability of the CAG repeat between generations depends on the sex of the transmitting parent and the length of the repeat itself. Studies of the two cohorts of HD individuals with the mean size of ~43 CAG repeats have shown that 61–68% of paternal transmissions resulted in expansions, whereas the majority (>60%) of maternal transmissions resulted in contractions or CAG stabilization [13, 18]. The largest expansions, associated with the juvenile form of HD, are almost observed in male transmissions and are influenced by the CAG repeat length of the transmitting parent [13]. The largest HD cohort study (337 transmissions) has shown that the age of the transmitting parents and the sex of offspring do not affect the intergenerational instability, suggesting that the gender of affected parents is the

major modifier of intergenerational instability [18]. Repeat size variability has been investigated in spermatogonia, postmitotic spermatid and matura spermatozoa collected by laser capture microdissection of testis from two HD patients in order to determine the timing of repeat instability. Interestingly, CAG repeat expansions were already present before the end of the first meiotic division and the frequency continues to increase in postmeiotic cell population suggesting that the primary source of instability occurs in spermatogonia [21].

1.2.2. Somatic instability

Several studies have reported that the expanded CAG repeat allele is also unstable in somatic tissues and increases in length over time [22–25]. Somatic CAG repeat size variation was analyzed by bulk PCR in each tissue whereas the degree of somatic mosaicism was quantified by a more sensitive PCR-based approach called Small-Pool PCR [26]. This method allows to accurately assess the variation of CAG repeat length of each HD expanded allele in tissues, using successive DNA dilutions in order to amplify few template molecules per reaction (**Figure 2A**). The dynamics of somatic CAG repeat instability varies between and within tissues with the highest instability observed in the striatum and cortex, two tissues that show the most pronounced neuropathological abnormalities [22, 23, 25]. In a large Venezuelan HD cohort, a positive correlation was reported between the size of progenitor alleles (inherited alleles) and the expansion-biased somatic mosaicism in buccal cells from individuals at the same age. This observation suggests that the size of the inherited CAG repeat is an important modulator of somatic instability [27]. Furthermore, it has been reported that CAG repeat expansion length in the cortex is associated with an earlier age of disease onset suggesting that somatic instability is a significant predictor of the age of onset [28]. Interestingly, somatic instability was not observed in two fetuses at 12–13 weeks suggesting that the somatic expansion event occurs later in the stages of fetal development or from birth throughout the patient's life [29].

Together, these data have clearly demonstrated the contribution of the sex of the transmitting parent and the inherited length of the CAG repeat in the dynamics of intergenerational and somatic instability in HD patients. Moreover, both germline and somatic mosaicism level seems to be linked to the disease onset and to the progression of HD symptoms. Thus, aiming at decreasing the size of expanded alleles or the level of somatic mosaicism would be an attractive therapeutic strategy. In the majority of analyses, the degree of expansion length variability between tissues and individuals cannot be explained only by the age, sex of the transmitting parent and the progenitor allele size, therefore implying that genetic factors might influence either germline or somatic instability. In 2012, the study of a large Portuguese HD cohort has reported some HD families with extreme repeat length changes from parents to offspring suggesting the existence of modifiers that may be heritable [19]. Hence, the understanding of CAG repeat instability is crucial to improve the therapeutic possibilities. Analyses of genetic modifiers of instability and dissection of mechanisms involved in this process are compromised by the limited accessibility of human samples and clinical information. Then, knockout, transgenic and knock-in HD mouse models have been generated to dissect the molecular mechanisms of instability and the pathogenesis of HD disease [30, 31].

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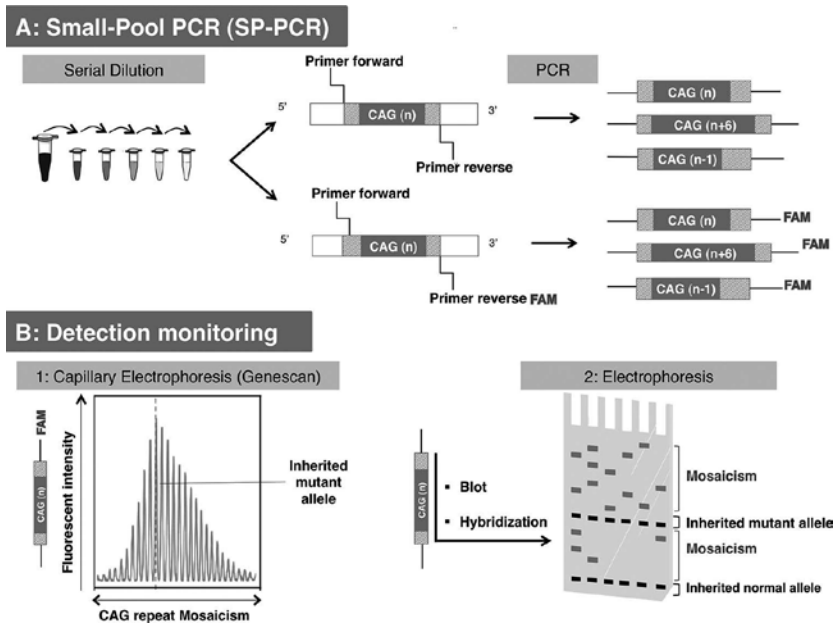

Figure 2. Methods to analyze CAG repeat length in germline and somatic tissues.

2. Mouse models of CAG repeat instability

The dynamics of expanded CAG repeat has already been analyzed in different simple organism strains such as bacteria and yeast by inserting a plasmid with a pathogenic CAG repeat. Analyses in *E. coli* and *S. cerevisiae* have provided valuable insight into factors affecting the CAG repeat instability. However, these organisms displayed a CAG repeat instability biased toward contractions in clear contrast to HD patients. Furthermore, both these organisms differ from mammals by cellular processes such as DNA repair and replication pathways. Therefore, mouse models have been generated to identify genetic modifiers of instability and to specify the mechanisms by which they act in HD. These mouse models including two HD transgenic mice with short gene fragment or BAC (R6 and BACHD), eight knock-in (the HdhQ20, HdhQ50, HdhQ92, HdhQ111, Hdh4/80, or Hdh6/72 lines, HdhQ150 and HdhQ80), have been created to analyze the dynamic of CAG repeat instability in germline and somatic tissues by different methods [24, 32–36] (**Table 1**). The first method determines CAG repeat size by using unlabeled primers flanking CAG repeat. PCR and SP-PCR products can be resolved on agarose gel with internal size standards and detected with radioactive probes [37]. The second method measures the length of CAG tracts by using primers flanking CAG repeat expansions, labeled with the 5-carboxyfluoroscein fluorochrome. PCR products are electrophoresed/separated in an automated sequencer together with internal size standards. In this case, the sizing of the PCR fragment is determined using GeneMapper software that represents the PCR fragments by peaks with single repeat unit resolution (**Figure 2**).

	Mouse models	Genetic background	Transgene	CAG repeat length	Mutation rate	Intergenerational instability (CAG length variation)	Somatic instability (partial list)	References
Transgenic mice	**BACHD**	FVB	Human *HTT* locus	97	None	None	None	[34]
	R6 (Excluded R6/T)	CBA/C57BL/6	Human *HTT* exon 1	>110	65–84%		Striatum> kidney> cerebellum	[32, 45, 46, 51, 52]
Knock-in mice	**Hdh^{Q80}**	C57BL6/J		80	~20%	↑ expansions (male transmissions)		[35]
	Hdh4/^{Q80}	129Svter/ C57BL6		80	~20%	↓ contractions (female transmissions)	Striatum> cerebellum> liver	[25, 36]
	Hdh6/^{Q72}			72	~20%			
	Hdh^{Q50}			48	4%	Low	None	[54]
	Hdh^{Q20}		CAG repeat locus	18	None	None		[24]
	Hdh^{Q92}	129SvEv/CD1		90	49%	↑ expansions (male transmissions)	Striatum> kidney> cerebellum	[24, 56, 57]
	Hdh^{Q111}			109	73%	↓ contractions (female transmissions)		[24, 56, 57, 58]
	Hdh^{Q150}	C57BL6/129Ola		150	16%	ND	Striatum> olfactory bulb> cerebellum	[25, 33, 38, 39]

Table 1. HD mouse models of CAG repeat instability.

BACHD mouse model was established by the introduction of a full-length human *htt* locus containing exon 1 with 97 mixed CAA-CAG repeats in the *FVB* background. These mice do not exhibit any repeat instability or contraction in germline and in brain tissues at 12 month of age [34]. The stability of CAG triplet repeat results from the CAA interruption within the CAG repeat tract, which probably modifies the DNA structure and then the repeat dynamics [1]. Compared to BACHD mice, HdhQ150 knock-in mice were generated by replacement of the murine short CAG repeat in exon 1 with a 150 CAG repeat expansion in a mixed C57BL/6/129Ola genetic background [33]. HdhQ150 animals reproduce somatic mosaicism in different brain regions, most particularly in the striatum like HD patients [25, 38]. HdhQ150 mice displayed some HD symptoms that seem more severe in homozygous mice [33, 39, 40]. Recently, heterozygous hdhQ250 mice have been generated from hdhQ150 by selective breeding and shown more severe neurological symptoms than heterozygous hdhQ150 mice [41]. Hdh4/Q80 and Hdh6/Q72 mice have also been obtained by replacement of short CAG repeat with 72 or 80 CAG repeat expansions in *htt* murine gene context. Both these lines have shown intergenerational instability biased toward expansions in paternal transmissions and contractions in maternal transmissions like in HD patients. However, the mutation frequency is only 20% across generations compared to 70% in HD individuals [36]. Hdh4/Q80 and Hdh6/

Q72 have displayed somatic mosaicism that is tissue-specific, age-dependent and CAG repeat length dependent [25, 36, 42, 43]. Some neurological and motor impairment have also been described in these mice and might be correlated to the somatic mosaicism level [25, 36, 44]. A different HdhQ80 mouse model has been created by replacement of the murin exon 1 with the human exon 1 carrying ~80 CAG repeat using C57BL6/J mice. Small expansions upon paternal transmissions and CAG repeat contractions across maternal transmissions have been reported in about 20% of cases. As observed in HdhQ150, Hdh4/Q80 and Hdh6/Q72, HdhQ80 mice have shown somatic mosaicism that is age-dependent and biased toward expansions with the highest levels in the striatum and liver [35]. Compared to BACHD and knock-in mice described above, R6 and HdhQ111 mice are the most commonly used to identify the genetic modifiers of CAG repeat instability [24, 32, 45]. Therefore, we will review the somatic and intergenerational instability features for both these animal models in the next section.

2.1. R6 transgenic mouse lines

The first successful HD transgenic mouse model was created in 1996 and called R6 lines of HD transgenic mice [32]. These mice were obtained by random integration of a short 5′ fragment of human *HTT* gene containing 1000 bp of 5′UTR, exon 1 with ~130 CAG repeat tracts and the beginning of intron 1 in a CBA/C57BL6 genetic background. Five lines of mice were obtained with different insertion sites and CAG repeat lengths. The R6/T line carries a truncated HD transgene without CAG repeat expansions, the R6/0 line carries 142 CAG repeats, R6/1 carries 116 repeats, the R6/2 carries 144 CAG and the R6/5 line carries multiple copies of transgene with 128, 132, 135, 137 and 156 CAG repeats, respectively. R6/0 mice have shown no transgene expression and no phenotype compared to R6/1, R6/2 and R6/5. These three mouse lines develop progressively neurological abnormalities and show a variable age of onset that depends on the CAG repeat length and on the transgene expression levels. R6/1 and R6/2 are the most studied of these lines to assess both HD pathogenesis and CAG repeat instability.

To evaluate intergenerational CAG repeat lengths, fluorescent PCR using DNA from tail biopsy at 3 weeks of age was performed in R6/0, R6/1, R6/2 parents and offspring. The comparison of CAG repeat lengths between parents and their progeny is limited in R6/5 mice due to the integration of multiple transgene copies in the genome of this line. Compared to R6/1 and R6/2, R6/0 mice do not show any evidence of CAG repeat instability and any transgene expression. As observed in HD patients, R6/1 and R6/2 mice mimic intergenerational instability biased toward expansions across paternal transmissions and toward contractions during R6/1 maternal transmissions (R6/2 female mice are infertile) with a mutation rate from 65 to 84% [45, 46]. Interestingly, the CAG repeat size changes depend on the gender of R6/1 embryos with a high expansion rate in males and high contraction rate in females from the same fathers suggesting that offspring sex-dependent genes modulate intergenerational instability in R6/1 mice [46]. In R6/2 mice, the size of transmitted CAG expansion increases with the age of transmitting males [45]. A selective R6/2 breeding enabled to obtain numerous R6/2 colonies with inheriting CAG repeat ranging from ~110 to 450 [47–49]. The size of CAG repeat is positively correlated with the severity of symptoms up to ~160 CAG repeats [47]. Surprisingly, some neurological symptoms and a lifespan are greatly ameliorated in R6/2 mice

carrying more than 200 CAG repeat expansions [47–49]. These unexpected results can be explained by transgene expression decrease observed in these mice [48]. A spontaneous contraction from 116 to ~89 CAG repeat was described in R6/1 mice [50]. These mice showed a decreased age of onset and a HD phenotypic improvement compared to R6/1 mice with 116 CAG repeat supporting the relationship between the CAG repeat size and the progression of symptoms.

Somatic instability of the CAG repeat tracts has been also reported in R6 lines carrying CAG repeat expansions excepted for the R6/0 line [45]. R6/1 and R6/2 recreated expansion-biased, age-dependent and tissue-specific somatic mosaicism as observed in HD patients [38, 51, 52]. Liver and striatum have shown the highest levels of instability biased toward expansions compared to other tissues that have shown low or no instability in both lines. Two distinct modes of somatic expansion have been described in tissues from R6/1 mice. Striatum and cortex have shown a periodic expansion, whereas the other tissues reproduce a short continuous expansion overtime suggesting different mechanisms of instability in these tissues [51]. Large spontaneous expansions (>200 CAG) have been described in striatum and cortex from R6/2 mice [52] consistent with the observations done in brain from HD patients [25, 43]. In R6/2 mice, the somatic mosaicism is correlated with the transmitted CAG repeat size but the somatic variation is not linear, particularly in striatum [52]. Interestingly, the frequency of CAG contractions increases in brain tissues and liver from mice with more than 500 CAG repeats [52] and could also explain the progressive reduction of neurological symptoms and prolonged lifespan in R6/2 mice with >200 CAG repeats [47–49]. Somatic instability has been noticed in dividing cells suggesting a role of DNA replication in the dynamic of triplet repeat instability. However, an increase of CAG repeat length has also been reported in terminally differentiated neurons from R6/1 mice suggesting the role of cellular processes independent of DNA replication in the somatic mosaicism [38]. Recently, an effect of mouse genetic backgrounds on the dynamics of CAG expansions has been reported in tissues from R6/1 mice with high CAG somatic mosaicism on a B6 background and low level in BALB/cBy backgrounds suggesting the existence of genetic modifiers of instability [53].

2.2. HdhQ92-111 mouse models

The first knock-in mice called HdhQ50 have been generated in 1997 using homologous recombination in ES cells to replace short murine CAG repeat by 48 CAG repeats in 129SvEv/CD1 mice [54]. In 1999, three other knock-in mouse models (HdhQ20, HdhQ92 and HdhQ111) using the same strategy have been generated with 18, 90 and 109 CAG repeat tracts, respectively [24]. These four knock-in mice share the identical murine genomic environment (91% of similarities with *HTT* human) and differ only by the size of CAG repeat length. Knock-in mice with >50 CAG repeats reproduce the pattern of intergenerational instability observed in HD patients. The mutation rate is only 4% in HdhQ50, 49% in HdhQ92 and 73% in the HdhQ111 supporting that intergenerational instability depends on CAG repeat length as described in R6 mice [24]. However, no age effect has been observed in these knock-in mice compared to R6/2 [24]. These divergent results could be explained by the CAG repeat genomic context and the genetic background. Interestingly, an effect of mouse genetic backgrounds on mutation

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rate and range of CAG repeat length changes upon male transmission was reported in HdhQ111 supporting the role of genetic modifiers on CAG instability process [55].

Somatic CAG repeat variations have been observed in HdhQ92 and HdhQ111 mice in brain and some peripheral tissues with the highest accumulation of expansions in striatum and liver [24, 56, 57]. Both these tissues showed a bimodal distribution of repeat lengths compared to spleen and tail that showed a unimodal distribution [56]. CAG expanded alleles were broadly distributed in striatum compared to liver that showed distinct populations of CAG repeat expansions [56]. Somatic instability depends on the CAG repeat size and the age of animals and is tissue-specific as reported in R6 mice [24, 56, 57]. The relationship between somatic mosaicism and HD phenotype remains unclear but some data have reported that somatic mosaicism is not correlated with the initiation of disease but may be correlated with the progression of HD phenotypes [57, 58].

In conclusion, HD mouse models closely reproduced the dynamic of instability observed in HD patients. Intergenerational instability is biased toward expansions and depends on the CAG repeat length and the sex of transmitting parent. HD transgenic and knock-in mouse models also mimic the somatic instability of HD patients, with the highest somatic mosaicism in the striatum that is the most affected tissue in HD. Some differences in the dynamics of intergenerational instability between HD patients and HD mouse models can be noticed. Despite a high level of instability biased toward expansions in paternal transmissions and contractions in maternal transmissions in both species, the critical CAG repeat threshold length differs between human and mice corresponding to 35 CAG repeats in human and more than 80 CAG repeats in mice. Moreover, no spontaneous large CAG repeat expansion has been observed in HD mouse models during paternal transmissions, in contrast to HD patients. These differences may be explained by genetic and environmental factors. Despite these divergences, the development of HD mouse models provided a powerful tool to explore trinucleotide repeat dynamics. Several data have suggested that the size, sex and the age factors are not sufficient to explain the level of meiotic and mitotic instabilities observed in HD patients and mice supporting the contribution of genetic modifiers in CAG repeat instability processes. Among described mouse models, R6 and HdhQ111 were commonly used to investigate the role of genetic modifiers on the level of intergenerational and somatic instability in HD.

3. Genetic modifiers of CAG repeat instability

The absence of correlation between CAG repeat somatic mosaicisms and the corresponding tissue proliferative rates and the destabilization of CAG repeat in murine mature neurons support the involvement of DNA repair pathways in the CAG repeat instability processes (**Table 2**). To identify the DNA repair pathways involved in the germline and somatic CAG repeat instability, R6/1 or HdhQ111 mice were crossed with mouse lines deficient for individual DNA repair genes. CAG repeat length changes upon transmissions were determined by comparing the CAG repeat size in the HD transmitting mice with CAG repeat length in the HD progeny for each DNA repair genotype (+/+ to +/+ and -/- to -/- and/or +/- to +/-). Further-

more, different methods have been described to quantify the degree of somatic instability and have made it possible to compare the level of somatic mosaicism between HD mice mutated and not for DNA repair genes [53, 57, 59–61].

Gene modifiers	DNA repair systems	Gene status	Mouse models	Effect on CAG repeat length		References
				Intergenerational instability	Somatic instability	
Msh2	MMR	KO	Hdh^{Q111}	↓ expansions ↑ contractions (male transmissions)	CAG repeat stabilization	[58, 67]
				No change (female transmissions)		
			R6/1	No expansion (male transmissions) ND (female transmissions)	↓ expansions	[59, 63]
Msh3			Hdh^{Q111}	No significant change	CAG repeat stabilization	[64]
		C57BL/6J	R6/1	ND		[53]
		BALB/cByJ				
Msh6		KO	Hdh^{Q111}	No change (*Msh6*-/-)	No change	[64]
Mlh1				ND	↓ expansions	[69]
Mlh3						
Ogg1	BER		R6/1	No change	↓ expansions	[65, 70]
Neil1				↓ expansions (male transmissions)		[61]
Fen1				ND (female transmissions)	No change	[66]
Csb	NER			↑ expansions ↓ contractions		[65]
Xpc				No change		[64]

ND, not determined.

Table 2. DNA repair genetic modifiers involved in CAG repeat instability in mouse models.

3.1. Genetic modifiers of intergenerational instability

Despite some controversial results, the analyses in *E. coli* and *S. cerevisiae* suggested an effect of mismatch repair (MMR) proteins on the dynamics of CAG repeat instability. MMR proteins preserve genome integrity by repairing erroneous insertion, deletion and misincorporation of bases that occur during replication and escape proofreading. Two MutS heterodimeres, MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3) recognize replication errors and recruit MutLα (MLH1-PMS1/2) and MutLγ (MLH1-MLH3) to activate the repair pathway [62]. The breeding of HD mouse models in MMR-deficient genetic backgrounds has provided insight into the mechanisms of CAG repeat instability. In R6/1 mice, *Msh2* deficiency abolishes CAG

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repeat expansion in the male germline suggesting that MSH2 promotes CAG repeat expansion (no data for maternal transmissions) [59, 63]. A further study in HdhQ111 knock-in mice revealed that the effects of *Msh2* mutation on the intergenerational dynamics seem to be more complex. The absence of two *Msh2* alleles suppresses the expansions in favor of contractions without changing the mutation rate (corresponding to expansion and contraction frequencies) in paternal transmissions [58, 64]. In contrast, a majority of contractions and a few expansions were detected in female germline transmissions in both *Msh2*+/+ and *Msh2*-/- backgrounds [24, 58]. Therefore, although MSH2 appears to be required in paternal CAG repeat expansions, the CAG repeat gains in female germline and CAG repeat contractions seem to be generated by *Msh2*-independent processes [58]. MSH2 binding partners, MSH6 or MSH3, did not alter the frequency of maternal changes, which is consistent with the lack of involvement of MSH2 in female germline. The effects of MSH3 and MSH6 on paternal transmissions of the expanded CAG repeat are more complex. The loss of *Msh6* or *Msh3* did not significantly affect the paternal mutation frequencies and the frequency of expansions and contractions. However, a shift from expansion to unchanged and contracted CAG repeat length is observed in *Msh3*-/- or *Msh3*+/- transmissions compared to *Msh3*+/+ transmissions suggesting that some paternal expansions might depend on MSH3 protein. These results together suggest that the majority of paternal expansions occur via MSH2, independently of MSH3 and MSH6 partners in HdhQ111 mice and that other DNA repair proteins are involved in CAG repeat parental expansions and contractions observed in HD mice [64].

The involvement of base excision repair (BER) and nucleotide excision repair (NER) in CAG repeat instability have been tested in R6/1 mice bred in a BER gene (*Ogg1* or *Neil1*) and NER gene (*Csb* or *Xpc*) deficient backgrounds. The loss of 7,8 dihydroxy-8-oxoguanin-DNA-glycosylase, OGG1 did not affect the dynamic of instability in the germ cells [65]. However, NEIL1, another glycosylase of BER contributed to paternal expansions in R6/1 mice. In the absence of *Neil1*, the CAG repeat tracts were more stable with a tendency toward contraction in male germline compared to *Neil1*+/+ [61]. Interestingly, an increase of CAG repeat expansions and a decrease of contractions in paternal transmissions have been observed in *Csb*-deficient mice suggesting that CSB promotes CAG repeat contractions during paternal transmissions just like MSH2 promotes expansions in HD mouse models [65]. In contrast to *Cbs* results, *Xpc* did not affect the dynamic of CAG repeat instability in R6/1 mice. It has also been reported that FEN1, an endonuclease involved in the DNA replication but also in BER intermediates, may stabilize CAG repeat in the *Fen1*+/- male germline by preventing deletions and modestly increasing expansions but the effect seems to be low [66].

In conclusion, these data have shown that MSH2 and NEIL1 proteins are involved in the formation of intergenerational repeat expansions in HD mouse models with the highest effect of MSH2, suggesting that these genes are genetic modifiers of intergenerational instability in HD. Moreover, the shift toward contractions observed in the absence of *Msh2* and *Neil1* reveals that the repeat could be processed through a distinct pathway leading to contractions via CSB or other DNA repair proteins.

3.2. Genetic modifiers of somatic mosaicism

The analysis of CAG repeat instability has revealed a relationship between the severity of HD phenotypes and the level of expansion-biased somatic mosaicism in patients and mice. Thus, HD mouse models in DNA repair deficient background have also been used to identify genetic modifiers of somatic instability. In R6/1 and HdhQ111 mice, *Msh2* deficiency was initially reported to stabilize CAG repeat expansion in somatic tissues supporting that MSH2 also drives instability toward expansions, like in germline cells [58, 59, 67].

Compared to the results obtained in *Msh6*-deficient mice, the loss of both *Msh3* alleles stabilize CAG repeat tracts in somatic tissues suggesting that MSH3 acts as an enhancer of CAG expansions-biased somatic instability but not MSH6 [53, 64, 68]. Interestingly, the absence of one allele of *Msh3* is sufficient to decrease the somatic mosaicism in the striatum in contrast to *Msh2* supporting the idea that MSH3 levels modulate the degree of somatic instability and the progression of HD disease [64]. Various degree of repeat instability in different HdhQ111 and R6/1 mouse strains harboring the identical CAG repeat length suggest the existence of other candidate factors as a source for strain-specific variation in CAG repeat pattern [53, 55]. Interestingly, CAG repeat somatic mosaicism has been associated with *Msh3* polymorphisms and the level of MSH3 protein [53]. It has been reported that expansion changes were higher in striatum and liver from R6/1 mice carrying the homozygous B6 *Msh3* gene on a CBy genetic background than mice carrying the homozygous CBy *Msh3* gene on a B6 genetic background (mice obtained by selective breeding). The loss of one B6 *Msh3* allele in mice on a CBy genetic background was sufficient to decrease CAG repeat instability, consistent with the results obtained in *Msh3*-deficient mice [53]. Thus, naturally occurring MSH3 protein polymorphisms modify the dynamic of CAG repeat instability in mice and could modulate HD pathogenesis in humans. Together, these data have shown that MSH2 and MSH3 proteins are strongly required in the generation of somatic expansions.

To identify other genetic modifiers of CAG repeat instability, linkage analyses have been performed in different HdhQ111 strains that showed CAG repeat instability variation [69]. A single quantitative trait locus on chromosome 9 and particularly in MutL homolog *Mlh1* gene has been identified and associated with CAG repeat instability. Then, somatic instability has been quantified in B6 HDHQ111 mice in the absence of one or two *Mlh1* alleles. Although one functional *Mlh1* allele was still sufficient to generate high levels of repeat expansion, the loss of both *Mlh1* alleles abolished CAG repeat expansion in striatum suggesting that MLH1 was required in somatic expansion. A second MutL homolog has been shown to act as an enhancer of CAG repeat expansions. Indeed, expansion-biased somatic mosaicism is reduced in *Mlh3* heterozygous knockout mice and totally abolished in *Mlh3* homozygous knockout mice suggesting that MLH3 is a limiting factor on the process of expansion as reported for MSH3 protein [69].

Other DNA repair systems, such as BER and NER have also been investigated in R6/1 mice to understand the somatic expansion variation observed between and within tissues. A loss of *Ogg1* suppressed CAG somatic expansions in 70% of R6/1 mice. The same study has reported that OGG1 initiated age-dependent CAG repeat expansion mice, suggesting that age-dependent somatic expansion associated with HD occurs in the process of removing

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oxidized base lesions [70]. Deletion of *Neil1* also reduced somatic expansions in male and female R6/1 mice with a higher effect in different brain regions from male mice [61]. In contrast to the results obtained in male germline, the absence of *Csb* and *Fen1* did not affect the dynamic of somatic instability in tissues suggesting that the role of *Csb* is specific of paternal contractions [65] and that *Fen1* partially contributes to CAG repeat expansion upon parental transmissions [66].

In conclusion, MSH2 and MSH3, partner proteins in the MutSβ MMR complex and MutLγ (MLH1-MLH3) are essential to promote expansions in HD mouse models suggesting that MutSβ and MutLγ promote CAG expansion via the mismatch repair machinery. Furthermore, CAG repeat expansion depends only partially on OGG1, NEIL1 and FEN1 proteins suggesting that other DNA repair pathways are involved in the process of instability. Some genetic modifiers such as *Ogg1* and *Fen1* impact CAG repeat instability in either somatic or germline tissues, but not in both supporting that CAG repeat instability involves different genetic players between tissues and may occur via different mechanisms. It has also shown that the degree of somatic mosaicism appears to be modulated by *Msh3* and *Mlh1* variants in B6 mice where CAG repeat expansion levels are the highest suggesting that somatic instability variation observed in HD patients could be explained by DNA repair gene and/or protein variants. Different expression levels of MSH3 and MLH3 have been identified in mouse strains that exhibit different expansion frequencies supporting that the level of DNA repair proteins might be correlated with the degree of CAG repeat instability. Other studies also support a role for the stochiometries of DNA repair proteins in CAG repeat instability [4, 64, 71–73]. Few data have reported the role of genetic factors in CAG repeat contractions mainly observed in HD maternal transmissions and only *Csb* has been reported to promote contractions in paternal transmissions. CSB protein could act on CAG repeat contraction via BER, NER, or chromatin maintenance/remodeling activity independently of MSH2 protein.

4. Are genetic modifiers a therapeutic target?

The identification of genetic modifiers of underlying CAG repeat instability is important to uncover novel therapeutic targets to slow down somatic instability and to decrease the intergenerational expansions in favor of CAG repeat contractions to prevent the disease. It has been reported that *Msh2* alleles delay the accumulation of mutant protein and destruction of mutant huntingtin in striatum and in specific neuron type from knock-in HdhQ111 mice [58, 67]. Moreover, MLH1 also contributes to nuclear huntingtin and HD inclusion phenotypes [69]. Both data suggest that MSH2 and MLH1 may enhance the HD pathogenic process by modulating the somatic mosaicism in cooperation with MSH3 and MLH3 via the mismatch repair pathway. Among MMR proteins, MSH3 and MLH3 are currently the most promising targets to decrease CAG repeat expansions, thus delaying pathogenic process, given their minor roles in the initiation of human cancer. To date, no drug has been identified to decrease the expression of MLH3 and MSH3 protein and then the somatic instability. NEIL1 and OGG1, two glycosylases of the BER pathway partially contribute to CAG repeat expansions suggesting that oxidative base damage is responsible of some CAG repeat expansions. Antioxidants may

then decrease the expansion process. Mollersen and colleagues have suggested that several antioxidants like anthocyanin decrease CAG repeat expansion in the brain from R6/1 male mice [61]. The identification of new genetic factors involved in the formation of CAG repeat contractions and a better understanding of expansion mechanisms are essential. Novel therapies based on activating the DNA repair pathways promoting contractions might be expected to have lower risk of side effects than therapies based on inhibiting the DNA repair pathways that promote expansions.

5. Conclusion

The data summarized in this chapter have shown that *cis*-elements such as DNA sequence and transcription level, mismatch repair, base excision repair and nucleotide excision repair proteins can modulate the pathogenic expansion-biased somatic mosaicism and/or intergenerational expansions contributing to the progression of HD phenotype. Natural polymorphisms in *Msh3* and *Mlh1* genes have been associated with the degree of somatic expansions in HD mice suggesting that MMR variants are involved in the somatic mosaicism variation observed in HD patients and may modulate the disease severity and age of onset. Despite a great advance on the understanding of instability, the process remains complex. Then, further studies will be needed to assess how the various DNA repair and replication proteins collaborate all together in germline and/or somatic tissues to mediate CAG repeat expansions. Moreover, future studies will be essential to identify new factors that promote contractions in the germline and in somatic tissues, to reverse the HD expansion and to stop the disease.

Acknowledgements

The authors thank Geneviève Gourdon, Mario Gomes-Pereira and Diana Dinca for helpful comments and discussions. The authors also thank Christopher Pearson group, HD/CAG.CTG repeat colleagues, Canadian Institutes of Health Research, Imagine institute, INSERM, the Association Française contre les Myopathes (AFM) and the université Paris Descartes.

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http://dx.doi.org/10.5772/66438

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Chapter 2

NR1 Receptor Gene Variation is a Modifier of Age at Onset in Turkish Huntington's Disease Patients

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67766

Abstract

The length of the CAG repeat tract is the major determinant of age of onset (AO) of Huntington's Disease (HD) However, there remains a significant variance in AO when the expanded repeat size is ruled out. The search for genetic modifiers has revealed various candidate loci; however, many reports have been contradictory. The N-methyl-D-aspartate receptors (NMDAR) have been proposed as an important putative modifier. We aimed to determine whether polymorphisms in NMDAR-coding genes have an effect on the AO. We analyzed the association between GRIN1 (rs6293), GRIN2A (rs1969060), and GRIN2B (rs1806201, rs890) polymorphisms and AO of Turkish HD patients. According to our findings, expanded CAG repeat size explains 41.8% of the variance in AO. Upon classification of genotypes into CAG repeat length intervals, rs6293 can be considered as an AO modifier for Turkish HD patients with 50 or higher CAG repeats. In addition to that, we found a significant association of this polymorphism to HD, with the GG genotype constituting a risk factor. Candidate genetic modifiers should be tested in different populations since their effects may exist only in groups of specific ethnic origins. Defining such modifiers will help in complete understanding of HD pathogenesis and in designing therapeutic targets.

Keywords: Huntington's disease, polyglutamine repeats, age of onset, NMDA receptors, GRIN

1. Introduction

Huntington's disease (HD) is a progressive neurodegenerative disorder of the central nervous system characterized by involuntary movements, cognitive impairment, and emotional

disturbances [1]. The prevalence of the disease is about 1/10,000 among individuals of European descent [2]. The disease follows an autosomal dominant pattern of inheritance and is associated with an expanded block of CAG repeats in exon 1 of the IT15 gene [3]. The CAG repeats are translated into a polyglutamine (polyQ) tract near the N-terminus of the huntingtin (htt) protein, which acquires a deleterious gain of function in the mutant protein. This ubiquitously expressed protein is shown to be essential for development [4–6]. Analysis of htt-interactors revealed that htt may function in intracellular trafficking, neurotransmission, retrograde transport, cytoskeletal function, and transcriptional regulation [7–9].

Four CAG repeat size intervals associated with varying disease risks were defined. The alleles with up to 35 repeats are considered normal. However, repeats in the range of 29–35 have been shown to exhibit meiotic instability. Rare alleles with 36–39 repeats are in the reduced penetrance range, since some people with repeats in this range develop HD and others do not. On the other hand, alleles with 40 or more CAG repeats definitely cause HD in a normal life span [10].

HD usually strikes in the third to fourth decade of life and gradually worsens over a course of 10–20 years until death. The patients may differ in the type and severity of their symptoms, age of onset (AO), and disease duration. The number of CAG repeats in the IT-15 gene is the most important factor determining the AO. There is a significant inverse correlation between the AO and the CAG repeat length [11, 12]. However, the number of the CAG repeats does not allow an accurate prediction of AO, only 30–70% of the variance in AO can be explained by the repeat size alone [13–20]. The AO varies significantly among individuals with the same CAG size, and even monozygotic HD twins may show phenotypic discordance for the disease [21, 22]. This has led to a search for genetic modifiers and environmental factors that influence the AO. Diverse modifier candidate loci have been suggested to be associated with AO in HD such as GRIN, TP53, hCAD, UCHL1, BDNF, ASK1, and MTHFR [14, 17, 23–32]. However, many reports from different research groups have been contradictory.

The NMDA glutamate receptors (GRIN: glutamate receptor, ionotropic, N-methyl-ᴅ-aspartate) have been proposed as an important putative modifier, since glutamate (Glu) mediates fast excitatory neurotransmission in the brain. Neurodegeneration in HD is highly selective for striatal GABA-ergic medium-sized spiny neurons (MSNs) that project to the substantia nigra (SN) and globus pallidus [33]. The underlying mechanisms are poorly understood; nevertheless, models of neurodegeneration suggested excitotoxicity, oxidative stress, and impaired energy metabolism as relevant to selective pathogenesis in HD [34]. Excitotoxicity may play a major role in the pathogenesis of HD, because Glu is the principal excitatory neurotransmitter in the mammalian central nervous system (CNS) and its excessive extracellular levels for prolonged periods can lead to excitotoxicity [35]. Glutamate activates two classes of receptors in neurons: metabotropic glutamate receptors (mGluRs) coupled to G-proteins and ionotropic glutamate receptors (iGluRs) controlling ion channels. The most intensely studied subclass related to excitotoxicity is the NMDARs, since intrastriatal injections of an

http://dx.doi.org/10.5772/67766

NMDA receptor agonist, quinolinic acid, produce lesions similar to the HD neuropathology, selectively affecting the striatal projection neurons [36–38]. Upon activation, the NMDAR channel becomes permeable to Ca^{2+} and Na^{+}, and NMDAR overactivity results in excessive Ca^{2+} loads. As homeostatic Ca^{2+} mechanisms are overwhelmed, a number of intracellular mechanisms are induced, leading to cellular damage by degradative enzymes [39].

Individual NMDARs are multimeric assemblies of subunits and the combination of NR1 with different NR2 subunits alters the characteristics of the NMDAR ion channel. NR2A-containing receptors are generally expressed at synapses and they are associated with developmental regulation and synaptic maturation; NR2B-containing receptors seem to be predominant at extrasynaptic sites. Therefore, the expression patterns of NR2A- and NR2B-containing NMDARs may have differential roles in mediating excitotoxic neuronal death [40]. The striatum, the most vulnerable region of the brain in HD, appears to express higher levels of NR2B subunit relative to other NR2 subunits when compared to other brain regions [41]. Studies with postmortem HD brains showing losses of striatal NMDAR-binding sites indicate that MSNs with higher levels of NMDAR expression are at particular risk [42]. A number of studies have demonstrated enhancement of the NMDAR currents in several transgenic HD mouse models [42–46]. Furthermore, an NR2B-selective hypothesis of mutant htt-mediated enhancement has been suggested [47]. Mutant protein caused an increase in the responses of NMDARs composed of NR1/NR2B, whereas NR1/NR2A NMDARs were not differentially affected by normal or mutant htt [48]. YAC72 MSNs were shown to be more susceptible to NMDA-induced toxicity than wild-type MSNs, and additionally, the enhancement of apoptosis by mutant htt in YAC46 and YAC72 MSNs was proportional to the length of the polyQ repeat, arguing a relationship of altered NMDAR signaling and mutant htt polyQ length [49]. These findings strongly support the hypothesis of excitatory amino acid-mediated and particularly NMDAR-induced cell death in HD. Therefore, NMDAR activity and/or subunit composition may have effects not only in the downstream effector pathways but also on the AO in HD patients.

Recent analysis by Arning and coworkers [17] indicated that variations of the NR2A and NR2B genes could explain the variance in AO especially in HD patients with CAG repeat lengths in the high 30s to the low 40s. In a replication study with Venezuelan kindreds, no evidence was obtained for the association of NR2B single nucleotide polymorphisms (SNPs); however, they found evidence for association of an NR2A SNP [24]. In an expanded HD cohort, Arning and coworkers confirmed the results of the previous study [14]. In addition to that, gender stratification of patients revealed differences in the variability in AO attributable to the CAG repeat number and highly significant differences in the AO association with the NR2A and NR2B variations [14].

In this study, we aimed to investigate the modifier effects of GRIN1, GRIN2A, and GRIN2B SNPs (rs6293, rs1969060, rs1806201, rs1042339, and rs890) on AO in Turkish HD patients. Defining genetic modifiers of AO in different ethnic populations is of great importance, since they may provide further clues to explain disease pathology and to construct neuroprotective strategies.

2. Materials and methods

2.1. Subjects and patient diagnosis

DNA samples of 102 unrelated HD patients (46 men and 56 women) were included in the study. Clinical examination and determination of motor AO was performed by experienced neurologists. The CAG repeat sizes of the patients were ascertained by polymerase chain reaction (PCR) using primers 5′FAM-HD3: 5′-GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC-3′ and P3F: 5′-TCTGCTTTTACCTGCGGCC-3′, followed by capillary electrophoresis and fragment analysis using PeakScanner v.1.0 software (Iontek, Turkey). One hundred and two healthy age- and sex-matched controls were also genotyped for comparison. The control subjects and their families were free of neurological disorders. All subjects provided written informed consent for genotyping. The experiments performed comply with the current laws of Turkey, and the study was approved by the Halic University Human Researches Ethics Committee.

2.2. SNP genotyping

All studied SNPs were checked from the single nucleotide polymorphism database (dbSNP). Genotyping of GRIN1, GRIN2A, and GRIN2B polymorphisms was performed by PCR-RFLP analysis [17]. PCR was carried out in a final volume of 25 μl with 50 ng of DNA, 2.0 mM Mg^{2+}, 0.8mM dNTP, 10 pmol of each primer, and 1 U Taq polymerase. The cycling conditions for each polymorphism included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48–60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (**Table 1**). PCR products were digested with 2 U of the appropriate restriction enzymes under optimal reaction conditions (**Table 1**) for 2 h and visualized on 2% agarose gels stained with ethidium bromide. Genotypes were confirmed with Sanger sequencing (Iontek, Istanbul) sequence analysis of 25 randomly selected samples.

2.3. Statistical analysis

Hardy-Weinberg (HW) test statistics was computed for each SNP. In order to compare allele frequencies between cases and controls, a standard case-control association analysis was performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). The dependence of AO on the expanded CAG repeat size was assessed by linear regression. The effects of the GRIN1, GRIN2A, and GRIN2B genotypes on AO were analyzed by multiple linear regression approach. The CAG repeat number and the respective genotypes were used as independent variables, and AO was used as the dependent variable. The CAG repeat size was considered as numerical, modifying genotypes were considered as nominal variables and they were encoded as "0, 1, 2." The odds ratios (OR) and independent-samples *T*-tests for genotypes were computed for the association of genotypes with AO. All the statistical analyses were performed with Statistical Package for Social Sciences (SPSS) version 17.0 for Windows.

Gene/polymorphism	Primer sequences 5′ → 3′	Annealing temperature	Restriction enzyme	Allele sizes (bp)
GRIN1/rs6293	NR1F: CGTTCTTGCCGTTGATGA NR1R: GTAAGAGCCAGCAACGGAG	60.3°C	MspI	G allele: 138 + 113 + 59 + 114 A allele: 251 +173
GRIN2A/rs19690600	2Ars1969060F: GGTTTTAAGATTTGTGCCAGG 2Ars1969060R: CTTAGACCGAGTTGGCAACA	60.3°C	DdeI	T allele: 280 + 45 C allele: 166 + 114 + 45
GRIN2B/rs1806201	2Brs1806201F: AGACTATTCGCTTCATGC 2Brs1806201R: GTGTGTTGTTCATGGCTG	48.3°C	PstI	C allele: 210 T allele: 194 + 16
GRIN2B/rs890	2Brs890F: GCTGTCAGCCATTCCTGTT 2Brs890R: CATGAATTTAGCCAGAGCCTC	57.1°C	PsuI	G allele: 283 T allele: 194 + 89
GRIN2B/rs1042339	2Brs1042339F: GACCACAAGCGCTACTTCAG 2Brs1042339R: TGTCATACAGGTTGCCTGCT	53.7°C	PsyI	G allele: 263 + 146 A allele: 409

Table 1. PCR and RFLP conditions of the studied polymorphisms.

http://dx.doi.org/10.5772/67766

3. Results

The patients were given the clinical diagnosis of HD upon neurological examination and thereafter clinical diagnosis was ascertained with molecular test results. The clinical symptoms of the patients included choreic movements, intellectual and cognitive impairment, dementia, and depression.

In the studied patient cohort, 22 different mutant alleles carrying 39–75 CAGs were determined; the most frequent range was between 42 and 47 repeats. The disease AO ranges between 16 and 80 years, the mean AO (±SD) being 40.75 (±12.97) years (**Table 2**). There are wide variations in ages of onset for a given CAG repeat length in our patient population, which is a strong indicative of other genetic or environmental modifiers.

CAG repeat length	AO range (years)	AO mean (years)
39	69	69
40	42–73	55.25
41	20–75	43.71
42	35–60	50.27
43	40–80	52.25
44	28–48	41.58
45	24–57	38.88
46	37–52	42
47	30–62	40.23
48	26–36	32.8
49	28–45	37.6
50	27	27
51	25–39	30
52	25–36	31.17
53	25–32	28.5
54	23–35	29
55	23	23
56	19–23	21
58	22	22
59	16	16
67	22	22
75	16	16

Table 2. CAG repeat sizes of the patients and corresponding AO ranges and means.

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Allele and genotype frequencies were deduced in HD cases and controls according to HW principles and evaluated with chi-squared goodness-of-fit test. In patients and controls, allele and genotype frequencies were found in HW equilibrium. All genotypes except the 3501A variant (rs1042339) in the GRIN2B gene were observed in the patient and control groups; therefore, this polymorphism was excluded from the analyses. Genotype frequencies of studied SNPs among HD patients and controls are summarized in **Table 3**.

Linear regression analysis has shown that 41.8% of the variance in AO could be attributed to the expanded CAG repeat size in the studied population ($R^2 = 0.418$, $p < 0.0001$). In order to evaluate the contributions of the studied SNPs to the AO, R^2-values were determined for each SNP in conjunction with the effect of the expanded CAG repeat size. The R^2-values of the SNP genotypes and the CAG repeats alone were compared to determine the change in R^2 (ΔR^2) (**Table 4**). In our HD patient cohort, the studied genotype variations at GRIN1, GRIN2A, and GRIN2B did not show any significant increase in the R^2-values; therefore, they do not contribute to the variation in AO. Gender stratification of patients did not reveal any differences in the variability in AO attributable to the CAG repeat number and putative modifier polymorphisms. Upon classification of the CAG repeat sizes into four groups (40–45, >45, 40–50, and >50 CAGs) to expand the findings, a significant regression model was obtained only for GRIN1 rs6293 ($p = 0.016$) in the >50 CAGs group. The other groups did not reveal any significant regression models. The power of the analysis reached 99.9%, and at least 42

Gene polymorphism		**GRIN1 rs6293**	**GRIN2A rs1969060**	**GRIN2B rs1806201**	**GRIN2B rs890**	**GRIN2B rs1042339**
Genotypes		AA	CC	CC	GG	AA
		AG	CT	CT	GT	AG
		GG	TT	TT	TT	GG
Patients	%	42.16	1.96	44.12	26.47	0
		48.04	30.39	48.04	46.08	0
		9.8	67.65	7.84	27.45	100
	n	43	2	45	27	0
		49	31	49	47	0
		10	69	8	28	102
Controls	%	52.94	6.86	41.18	24.51	0
		43.14	27.45	49.02	54.90	0
		3.92	65.69	9.80	20.59	100
	n	54	7	42	25	0
		44	28	50	56	0
		4	67	10	21	102

Table 3. Genotype frequencies among patients and control subjects.

patients were essential to detect a significant effect with GRIN2A rs1969060, analysis of the other SNPs required 44 patients. With a sample population of 102 patients, we are beyond the minimum number of required patients.

Although there is no significant finding, the multiple linear regression data for GRIN2A rs1969060 could be regarded as a trend (p = 0.132) (**Table 4**). Moreover, HD patients with genotype CC had higher mean AO (50 years) than that of remaining HD patients (40 and 57 years) (**Figure 1**). The risk estimation analysis have shown that, compared to combined genotypes CT and TT, genotype CC can be regarded as protective (OR = 0.271). However, the observed differences in AO did not prove to be significant according to the results of independent samples t-test (p = 0.31).

	R^2	ΔR^2	% additional variance	p-value
HD CAG	0.418	–	–	0.000
HD CAG + GRIN1 (rs6293)	0.418	0.000	0	0.917
HD CAG + GRIN2A (rs1969060)	0.431	0.013	2.22	0.132
HD CAG + GRIN2B (rs1806201)	0.422	0.004	0.007	0.406
HD CAG + GRIN2B (rs890)	0.419	0.001	0.002	0.695

Table 4. Multiple linear regression analysis of the SNPs.

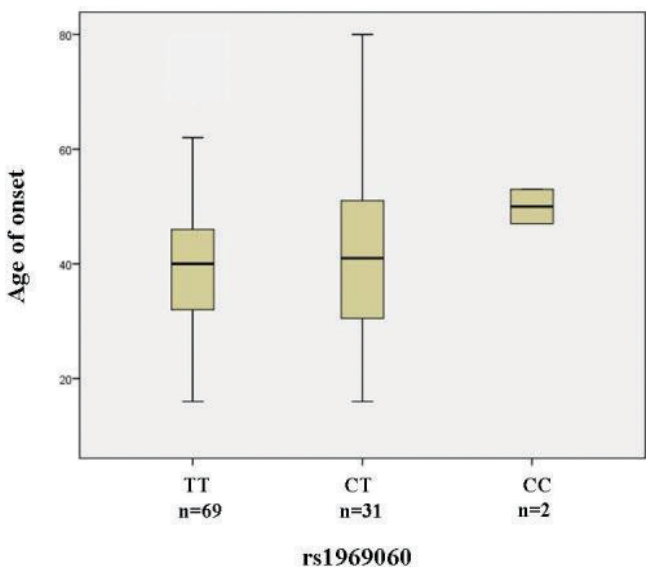

Figure 1. The AO distribution among rs1969060 genotypes is represented as box plots. For each genotype, the median AO is represented as a black bar, the quartile is shown as a solid box, and the range is indicated by margins.

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In addition to that, although GRIN2B rs180620 polymorphism did not reveal an important regression model, HD patients with genotype TT had lower mean AO (35 years) than those having the remaining genotypes (41.18 years). However, upon independent samples *t*-test, this difference was not found to be significant ($p = 0.23$), either.

The result of simple associations test (**Table 5**) has shown that the distribution of GRIN1 rs6293 GG genotype was significantly different in patient and control groups ($p = 0.01478$). Upon this finding, risk analysis was performed and the OR value was calculated to be 2663, indicating that the GG genotype could be a risk factor in HD.

SNP	A1	F_A	F_U	A2	CHISQ	*P*	OR	L95	U95
rs6293	G	0.348	0.2321	A	5.942	0.01478	1.766	1.115	2.796
rs1806201	T	0.3137	0.3333	C	0.171	0.6792	0.9143	0.5979	1.398
rs890	T	0.5049	0.4804	G	0.2452	0.6205	1.103	0.7481	1.626
rs1969060	C	0.1716	0.2059	T	0.7844	0.3758	0.7988	0.4856	1.314

Table 5. Association analysis of SNPs using PLINK (A1: minor allele, A2: major allele, F_A: Frequency of minor allele in patients (F_U: frequency of minor allele in controls, CHISQ: chi-squared test, L95 and U95: lower and upper bounds of 95% confidence interval).

4. Discussion

It has been well established that the AO is inversely correlated with the number of CAG repeats in the mutant HD allele. On the other hand, the negative correlation between AO and repeat size is stronger for higher repeat numbers (earlier AO) and weaker for lower repeat numbers (later AO). This indicates that although the CAG size is the major determinant of onset age particularly in juvenile patients, factors other than the repeat size should contribute to onset in later ages [50]. Previous studies have shown that the CAG repeat number alone explained 30–70% of the variance in AO [13–15, 17–20]. In various studies, many gene polymorphisms were defined as possible candidates for explaining the remaining variance. The genes encoding htt-interacting proteins or proteins that are found to be involved in disease pathology were regarded as the major candidate modifiers. In this study, we aimed to evaluate the hypothesis that polymorphisms in GRIN1, GRIN2A, and GRIN2B genes that code for NMDAR subunits may contribute to explain the variability in AO in Turkish HD patients.

According to our findings, expanded CAG repeat size explains 41.8% of the variance in AO, which is in accordance with the results from other populations. However, when the entire observed CAG repeat sizes are considered, the remaining variance could not be attributed to any of the studied SNPs. Classification of SNP genotypes into four CAG repeat length intervals did not reveal any important findings either, except GRIN1 rs6293 in the >50 CAG repeats group ($p = 0.016$). As a result, rs6293 SNP can be considered as an AO modifier for Turkish HD patients with 50 or higher CAG repeats in their IT15 gene. Also, the results of the association test indicated a significant association of this SNP to HD ($p = 0.015$) and further risk analysis revealed that the GG genotype constitutes a risk factor (OR = 2.663).

Prior to this study, the effects of the SNPs in NMDAR-coding genes in determining AO in HD were investigated in the German population [14, 23] and a replication study was performed with Venezuelan kindreds [24]. In the Arning's work, German HD patients having 41–45 CAG repeats in their mutant HD alleles were intentionally selected. In this cohort, the contribution of the expanded CAG repeat size to the AO was calculated to be 30.8%, while variations in the GRIN2A (rs1969060) and GRIN2B (rs1806201) genes accounted for 4.5 and 12.3% of the remaining variance, respectively. In addition to that, GRIN2B rs1806201 (C2664T) variation resulted in a difference of 2.8 years between the genotypes and patients having TT genotype that had earlier AO than those having other genotypes [17]. In our sample population, although GRIN2B rs180620 polymorphism did not reveal an important regression model, TT genotype did show lower mean AO (35 years) than the remaining genotypes (41.18 years). Arning and coworkers expanded their findings with 8 additional SNPs and 83 additional HD patients with repeat sizes varying between 39 and 61 [14]. In that study, when GRIN2A and GRIN2B genotype variations were considered together in the multiple regression analysis, 7.2% of the additional variance could be explained. Furthermore, the classification of patients according to sex revealed a significant difference and 5.6% additional variance was attributed to C2664T genotype variations in women. Female HD patients with the CC genotype tended to have delayed onset compared to the other two genotypes. In our study, we could not deduce any significant results out of gender stratification. In the Venezuelan kindreds, GRIN2B 1806201 association could not be replicated; however, GRIN2A rs1969060 SNP was found to be associated with AO, with a very slight increase in R^2 ($\Delta R^2 = 0.003$). They found 3.9 year difference in the mean age of onset between the CT and TT genotype classes. They found that TT genotype was protective. On the contrary, in our population HD patients with CC genotype have about 10 years higher mean AO than that of the remaining patients, which may imply toward a protection effect. However, due to very few numbers of patients with the CC genotype, this result does not reach statistical significance. As mentioned by Andresen et al. [24], heterozygosity at this polymorphic locus varies greatly by geographical variation and the results should be evaluated carefully. The C allele frequency was established to be 17% in European, 52–56% in Asian and African, and 24% in Venezuelan populations. The data from our population are in accordance with that of the European population (17.2% in HD patients and 20.6% in control subjects).

Apart from our study, GRIN1 rs6293 was investigated only with the German HD patients with repeat numbers 41–45 [17], which demonstrated a trend for explaining the variance in AO ($p = 0.055$). On the contrary, we established GRIN1 rs6293 as an AO modifier for Turkish HD patients with 50 or higher CAG repeats in their IT15 gene.

In summary, the effects of the genetic variations in the GRIN1, GRIN2A, and GRIN2B genes have been investigated in a very few numbers of populations until now and the results could not be replicated. The major reason for this discrepancy could be that the effect of these polymorphisms on AO may exist only in groups of similar genetic backgrounds or of specific ethnic origin. Therefore, further investigations involving HD patients of various ethnic populations with a wide range of CAG repeat sizes are required to clarify the issue. However, these negative findings are far from ruling out NMDA receptors as an important biological effector in the pathophysiology of HD. Ample evidence suggests an important role for NMDARs in

selective vulnerability of MSNs and neuronal cell death in HD. While there is evidence that NMDARs play an important role in the pathogenesis of HD, their exact roles and the modifying effects of the polymorphism remain to be further investigated. It is clear that numerous supplemental studies are needed to establish a confirmed association of GRIN genes as genetic modifiers of AO in HD.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 3

Pathogenesis of Huntington's Disease: How to Fight Excitotoxicity and Transcriptional Dysregulation

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66734

Abstract

Huntington's disease (HD) is a neurodegenerative disorder caused by an expanded CAG repeat in the exon-1 of the *huntingtin* (htt) gene. The presence of mutant htt (mhtt) results in multiple physiopathological changes, including protein aggregation, transcriptional deregulation, decreased trophic support, alteration in signaling pathways and excitotoxicity. Indeed, the presence of mhtt induces changes in the activities/levels of different kinases, phosphatases and transcription factors that can impact on cell survival. Many studies have provided evidence that transcription may be a major target of mhtt, as gene dysregulation occurs before the onset of symptoms. The greatest number of downregulated genes in HD has led to test the ability of a large number of compounds to restore gene transcription in mouse models of HD. On the other hand, mhtt engenders multiple cellular dysfunctions including an increase of pathological glutamate-mediated excitotoxicity. For that reason, targeting the excess of glutamate has been the goal for many promising drugs leading to clinical trials. Although advances in developing effective therapies are evident, currently, there is no known cure for HD and existing symptomatic treatments are limited.

Keywords: CREB, glutamate, HDAC inhibitors, excitotoxicity, transcriptional dysregulation

1. Introduction

Huntington's disease (HD) is a progressive, fatal, dominantly inherited neurodegenerative disorder [1] characterized by motor and cognitive dysfunction. Neuropathologically, HD is primarily characterized by neuronal loss in the striatum and cortex [2] together with hip-

pocampal dysfunction [3]. The disease is caused by an unstable expansion of CAG repeats in the huntingtin (htt) protein [4]. Htt is ubiquitously expressed [5, 6] and interacts with proteins that cover diverse cellular roles including apoptosis, vesicle transport, cell signaling and transcriptional regulation [7].

Although it is well established that the disease occurs as a consequence of an expanded polyglutamine repeats above 35 [4], the pathological mechanisms are not fully understood yet. Increasing evidence suggests that in addition to the gain of toxic properties, reduced htt physiological activity may render, in part, striatal neurons particularly vulnerable [8, 9]. The presence of mutant htt (mhtt) results in multiple pathophysiological changes, including protein aggregation, transcriptional dysregulation and chromatin remodeling, decreased trophic support, alteration in signaling pathways and disruption of calcium homeostasis and excitotoxicity.

Htt functions in transcription are well established. Htt has been shown to interact with a large number of transcription factors [10, 11], indicating a role of the protein in the control of gene transcription [12]. Htt is also believed to have a prosurvival role. Several *in vitro* and *in vivo* studies have demonstrated that expression of the full-length protein protected from a variety of apoptotic stimuli [13–17]. Currently, there is no known cure for HD and existing symptomatic treatments are limited. However, recent advances have identified multiple pathological mechanisms involved in the disease, some of which have now become the focus of therapeutic intervention; progressing toward developing safe and effective therapies which eventually may be successfully translated into clinical trials. These new prospects offer hope for delaying and possibly halting this disease. The aim of this chapter is to describe molecular pathways involved in HD, which offer new targets for the development of therapeutics focusing on the control of excitotoxicity and transcriptional alterations. Indeed, the presence of mhtt induces changes in the activity/levels of different kinases and transcription factors that can impact on cell survival and the selective vulnerability of medium spiny neurons in the striatum.

2. Transcriptional dysregulation in HD and potential therapies

Many studies have provided evidence that transcription may be a major target of mhtt [11, 18–20], as gene dysregulation occurs before the onset of symptoms [21]. Subsequently, a large number of studies showed transcriptional abnormalities in HD [21–23].

Initially, it was shown that mhtt establishes abnormal protein-protein interactions with several nuclear proteins and transcription factors, recruiting them into aggregates and inhibiting their activity [11, 24] (**Figure 1**), as occurs with CREB (cyclic-adenosine monophosphate (cAMP) response element (CRE) binding protein)-binding protein (CBP) [11, 24]. On the other hand, mhtt can also fail to interact with other transcription factors (**Figure 1**), altering their activity which could induce the repression of a large cohort of neuronal-specific genes [25, 26]. Mhtt fails to interact with repressor element-1 transcription/NRSE, so then the complex can translocate from the cytoplasm to the nucleus and bind NRSE repressing a large cohort of neuronal-specific genes, including the brain-derived neurotrophic factor (*bdnf)* [26].

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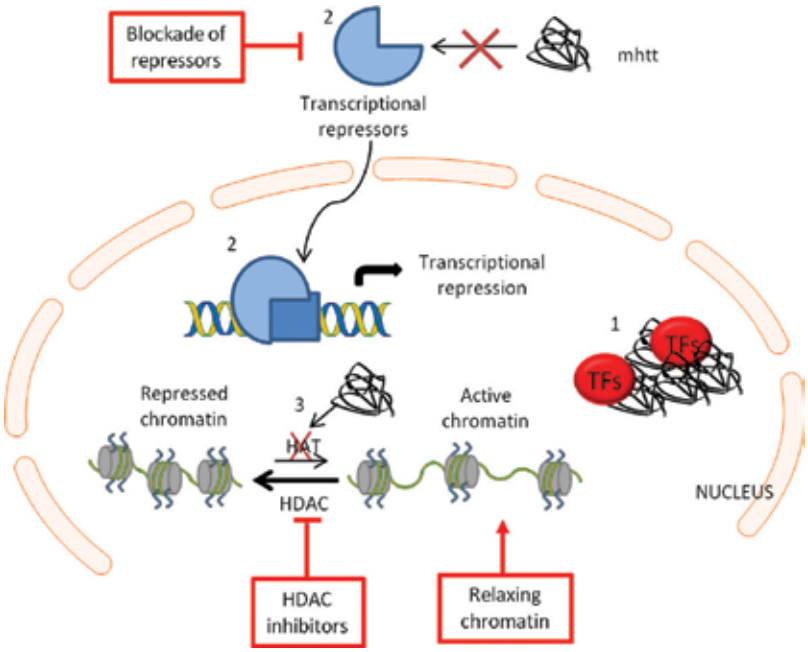

Figure 1. Mechanisms of transcriptional dysregulation in Huntington's disease. Different mechanisms by which mhtt disrupts normal transcriptional activity and possible therapeutic interventions. (1) Mhtt can bind transcription factors (TFs) and sequesters them into mhtt inclusions. (2) Mhtt loses the capacity to bind to transcriptional repressors allowing them to get into the nucleus and represses transcription. (3) Transcription depends on the acetylation status of histones, regulated by activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Mhtt interaction with HATs inhibits proper histone acetylation and causes repression of the transcription. Inhibition of HDAC, compounds promoting the detachment of histones from DNA and molecules targeting transcriptional repressors could represent promising therapeutic targets in HD.

Moreover, htt can also interfere in chromatin structure. Histone acetyltransferases (HATs) favors gene transcription through the opening of chromatin, whereas histone deacetyltransferases (HDACs) repress gene transcription through chromatin condensation. Mhtt binds to the acetyltransferase domain of some factors, such as CBP and p300/CBP-associated factor, blocking their activity [27, 28] (**Figure 1**).

The greatest number of downregulated genes in HD [21] has led to the initiation of new lines of research aimed at testing the ability of a number of compounds to restore gene transcription in mouse models of HD. However, the development of therapies targeting altered transcription faces serious challenges, as no single transcriptional regulator has emerged as a main factor of the disease. Nevertheless, potential therapeutic advances have emerged recently. Some of them include inhibition of HDAC [29, 30], compounds that directly interact with DNA [31], as well as drug-targeting proteins involved in the modulation of transcription [32, 33] (**Figure 1**).

Increasing evidence indicates that CREB is essential for activity-induced gene expression and memory formation [34]. CBP is a CREB-transcriptional coactivator that enhances

CREB-mediated transcription of specific genes [35] and can also act as a HAT allowing gene transcription [36]. Decreased levels of CBP due to sequestration into mhtt aggregates or increased degradation have been associated with striatal neurodegeneration in HD [20, 37]. Moreover, hippocampal-dependent cognitive deficits have been related to a reduced expression of CBP and reduced levels of histone acetylation [38]. Consistent with deficits in striatal and hippocampal CBP function, either CBP overexpression or HDAC inhibition could represent therapeutic strategies to improve transcriptional dysregulation. HDAC inhibitors have been under study for several years (**Figure 1**). Indeed, McCampbell et al. [20, 39] demonstrated that overexpression of CBP reduced polyglutamine-mediated toxicity in neuronal cell culture. CBP overexpression reversed the hypoacetylation phenomenon observed in polyglutamine-expressing cell which reduced cell loss. A similar effect was observed when cells were treated with HDAC inhibitors demonstrating that altered protein acetylation in neurons could play an important role in polyglutamine diseases [39]. Pharmacological treatments using the HDAC inhibitors, sodium butyrate and suberoylanilide hydroxamic acid (SAHA), significantly improve survival, motor performance, modulate transcription and delay neuropathology in the R6/2 transgenic mouse model of HD [29, 40]. In this line, benzamide-type HDAC inhibitor 4b, ameliorated motor and behavioral symptoms and corrected transcriptional abnormalities in R6/2 and N171-82Q transgenic mice [30, 41]. Moreover, 4b treatment induced DNA methylation changes that were inherited to the next generation. First filial generation offspring from drug-treated male HD transgenic mice shows significantly improved HD disease phenotypes compared with the offspring from vehicle-treated male HD transgenic mice [42]. Likewise, administration of the HDAC inhibitor trichostatin A (TSA) rescues hippocampal-dependent recognition memory deficits and increases the transcription of selective CREB/CBP target genes in HdhQ7/Q111 mice [38]. Moreover, more physiological approximations to increase CBP levels and reduce HDAC activity have been recently suggested. Moreno et al. observed that dietary restriction not only induces the expression of *Cbp* in WT and YAC128 mouse model of HD, but also reduces the expression of HDAC. These changes were accompanied by changes in the expression of different neuroprotective genes [43]. **Table 1** lists the different HDAC inhibitors, their specificity and the reported beneficial effects in HD models.

Inhibition of HDAC by 4b was shown not only to affect transcription but also posttranslational modification processes which can influence aggregate formation [41]. On the other hand, inhibition of HDAC4 resulted in a delay in cytoplasmic aggregate formation, together with restored Bdnf transcript levels, rescued neuronal function and improved phenotype in HD mouse models, pointing HDAC4 as a novel strategy for targeting htt aggregation [44]. This potential role of acetylation in mhtt degradation adds importance to HDAC inhibitors as a therapeutic target in HD pathology. These promising results have led to the enrollment of HD patients in clinical trials as HDAC inhibitors are safe and well tolerated [45]. However, these compounds can cause some side effects [46]. It is therefore important to improve our knowledge, to be able to generate effective and specific HDAC inhibitors. Sirtuins belong to the class III of HDAC enzymes and have been a recent focus of therapeutic development for neurodegenerative disease [47]. Interestingly, activation, instead of inhibition of sirtuins, with their ligand resveratrol, was found to be neuroprotective in HD worms [48, 49]. Resveratrol and other potent activators of sirtuins have been used in preclinical trials, but further experiments need to be performed to assess the therapeutic potential of these enzyme targets in HD [50].

HDAC	Compound	Model	Effect	Reference
1,2,3,4,5,7,8 and 9	Valproic acid	N171-82Q mouse and YAC128	↑ Survival	[189]
			Improve motor performance	
			↑ BDNF and Hsp70 levels	
1,2,3,4,5,7,8 and 9	Sodium butyrate	R6/2 mice	↑ Survival	[40]
			Improve motor performance	
			↑ Body weight	
1,2,3,4,5,7,8 and 9	Phenyl butyrate	N171-82Q mouse	↑ Survival	[190]
			↓ Brain atrophy	
			↑ Proteasome pathway	
			↓ Caspase activation	
All HDAC	TSA	HdhQ7/Q111	↑ CREB target genes	[38]
			Rescue memory deficits	
All HDAC	SAHA or vorinostat	R6/2 mice	Improve motor performance	[29, 191]
			↑ BDNF levels	
			↓ mhtt cortical aggregates	
3	RGFP966 (benzamide)	N171-82Q mouse	Improve motor performance	[192]
			↓ Striatal degeneration	
			↓ GFAP	
1 and 3	HDACi 4b	N171-82Q mouse	Improve phenotype	[41, 42, 30]
		and R6/2 mice	↓ mhtt aggregates	
Sirtuin	Nicotinamide	R6/1 mice	↑ BDNF and PGC-1α levels	[193]
			Improve motor performance	
Sirtuin (activation)	Resveratrol	*C. elegans*	Rescue mhtt toxicity	[49]

Table 1. HDAC inhibitors and effects of HDAC inhibition in different models of HD.

Apart from HDAC, other drugs like anthracyclines could produce a beneficial effect in promoting transcription in HD. Anthracyclines are DNA topoisomerase II inhibitors and are broadly used in cancer chemotherapeutics [51]. A novel function of these molecules has recently been identified. Anthracyclines can induce histone eviction from the DNA [31] making it more accessible to the transcriptional machinery and maybe being able to counteract the transcriptional inhibition that occurs in HD. Nevertheless, side effects promoted by these treatments should be taken in high consideration.

When thinking about potential genes downregulated in HD, Bdnf is considered to be one of the principal focuses of attention. BDNF has emerged as the major regulator of neuronal development, synaptic plasticity and neuronal survival and also a key molecular target for drug development in HD [9, 52]. When targeting BDNF deficits in HD, different approximations

have been developed. Several evidence suggest that HDAC inhibitors induce the expression of multiple downstream targets that might work collectively to elicit neuroprotective effects, like neurotrophins. For instance, it was observed that BDNF was induced by treatment with valproic acid, sodium butyrate, or TSA [53, 54]; thus, it is conceivable that restoring BDNF to their normal levels is part of the molecular mechanism underlying the beneficial effects elicited by HDAC inhibition in various HD models. Moreover, inhibition of HDAC6 increases vesicular transport of BDNF in a similar way to the cystamines, compensating for the transport deficit in HD [48, 55]. Focusing on BDNF deficits, identification of compounds or small molecules capable of antagonizing the repressive action of REST/NRSF in gene transcription has begun and represents a rational and promising target to break down with transcriptional repression present in HD [33, 56]. To this aim, Cattaneo's laboratory has developed a cell-based reporter assay to monitor re1 activity in brain cells and identify compounds that specifically upregulate BDNF expression in HD [57]. It has also been identified a benzoimidazole-5-carboxamide derivative that inhibited REST silencing in an RE1-dependent manner, the X5050 compound. X5050 targets REST degradation and produces an upregulation of neuronal genes targeted by REST. This activity was confirmed in human-induced pluripotent stem cells derived from an HD patient and in mice with quinolinate-induced striatal lesions [32].

3. Breaking signaling pathways

Protein kinases/phosphatases regulate most aspects of normal cellular function. Inhibitory or stimulatory actions at these signaling pathways strongly affect neuronal function by altering the phosphorylation state of target molecules and by modulating gene expression [58]. In fact, several kinases and phosphatases have been reported to be altered in HD patients and animal models. Some of these kinases altered in HD are closely related to synaptic plasticity, cell survival and transcriptional regulation such as cAMP-dependent protein kinase (PKA) [59], the kinase Akt [60, 61], the mitogen-activated protein kinases (MAPKs) [62–64] and kinases downstream MAPK pathway [65–67]. Furthermore, also several phosphatases are altered in HD mouse models. Some examples are the phosphatase calcineurin [68, 69], the PH domain and leucine-rich repeat protein phosphatases (PHLPP) [61] and the striatal-enriched protein tyrosine phosphatase (STEP) [61]. Therefore, therapies with potential to modulate cell signaling pathways could provide protection against neurodegeneration [70, 71].

3.1. Kinases and downstream targets

Numerous kinase signaling pathways are thought to contribute to HD pathophysiology. They are known to counter toxic metabolic changes induced by mhtt and help to maintain neuronal survival [72, 73].

3.1.1. Extracellular signal-regulated kinase (ERK)

Transcription of target genes is controlled by a series of transcription factors, which are, in turn, regulated by a number of kinases. Among the kinases implicated in HD, those involving ERK signaling cascades are of particular interest [74]). ERK 1/2 is a strong antiapoptotic and

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prosurvival mediator. Moreover, ERK 1/2 downregulation is linked to neurodegenerative conditions [75, 76]. Recent studies using HD mouse and cellular models provide strong evidence that activation of ERK has the neuroprotective effect, while the specific inhibition of ERK activation enhances cell death [62, 64, 71]. Supporting the neuroprotective role of ERK activation, we have previously reported that enhanced activity of the ERK pathway may participate in the reduced neuronal loss observed after quinolinic acid (QUIN) injection in R6/1 mice (**Figure 2**) [64]. When injected with QUIN, both WT and R6/1 mice display an increase in the phosphorylation of ERK levels, but activation of ERK was more prolonged in resistant R6/1 mice than in susceptible controls [64]. Moreover, inhibition of ERK has been found to block the induction of BDNF-regulated genes [77], thus implicating this pathway as an important regulator of BDNF-induced transcription. For that reason, the ERK pathway has been investigated as a potential neuroprotective modulator of HD pathology [62, 64]. In this context, it has been suggested that reduced levels of ERK in the cortex of HD models can lead to increased cell dead and reduction in the expression of BDNF. Then, less BDNF is available to striatal neurons, which activates, in response, compensatory mechanisms increasing the expression of ERK (**Figure 2**) [62].

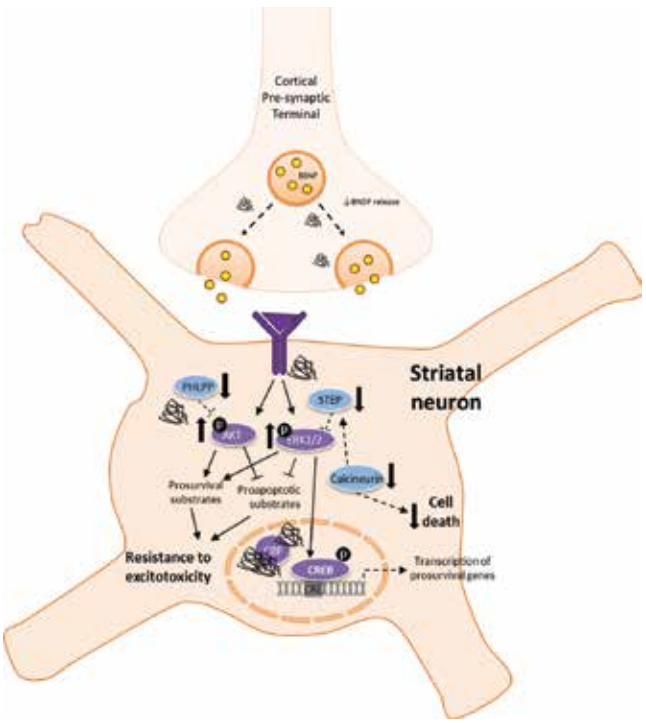

Figure 2. Proposed compensatory mechanism activated in the presence of mhtt in response to reduced cell death and increased resistance to excitotoxicity in HD mouse models. Decreased BDNF delivery from cortical neurons activates compensatory mechanism in striatal neurons by increasing ERK phosphorylation. Reduced STEP and calcineurin activity contribute to the maintenance of ERK activity. PHLPP levels are downregulated which contribute to the increased activation of Akt. Both ERK and Akt are proposed as a possible mechanisms related to the increase resistance to excitotoxicity observed in mouse models of HD, by activating prosurvival pathways (like CREB) and by the inactivation of proapoptotic factors.

Drugs, targeting the ERK pathway, may provide a basis for developing disease modifying therapeutic interventions for HD. Neuroprotective compounds identified using a neuronal cell culture model of HD in combination with a library of 1040 biologically active compounds were shown to prevent cell death by activation of ERK and Akt signaling, with the ERK pathway playing the major role [78]. More recently, results from another screening showed that pizotifen caused transient ERK activation in an immortalized striatal cell line expressing mhtt (STHdhQ111/Q111) and inhibition of ERK activation increases cell death in this *in vitro* model. In addition, R6/2 mouse treated with pizotifen showed increased activation of ERK in the striatum, reduced neurodegeneration and significantly enhanced motor performance [79]. To further test the hypothesis that pharmacological activation of ERK might be protective in HD, a polyphenol (fisetin), which was previously shown to activate the Ras-ERK cascade [80], was tested in three different models of HD: PC12 cells, *Drosophila* expressing mhtt and the R6/2 mouse model of HD [71]. Fisetin was able to reduce the impact of mhtt expression in each model. Likewise, the previously discussed resveratrol, a related polyphenol, could also activate ERK and was also protective in HD models [71]. Also activation of cannabinoid receptor type 1 protects PC12 and STHdhQ111/111 cells from mhtt-induced cell death in an ERK-dependent manner [81, 82]. Additionally, different antipsychotic drugs, such as clozapine and olanzapine, also promote and cause an increase in ERK phosphorylation [83].

3.1.2. p90 ribosomal s6 kinase (Rsk)

These aforementioned studies suggest that pharmacological intervention at the level of ERK activation or downstream ERK may be an appropriate approach in HD therapy. Most common kinases phosphorylated by ERK1/2 include Rsk and the mitogen- and stress-activated protein kinases (MSK) [84, 85]. In this context, we have reported changes in the expression of Rsk related to the presence of motor symptoms in HD. Meanwhile, an increase in Rsk protein levels was observed in the striatum of HdhQ111/Q111 and R6/1 mice at presymptomatic stages of the disease [67], they were downregulated in the same models when motor symptoms were present [65], indicating that Rsk downregulation is associated with the presence of motor impairment, the main clinical feature in HD [2]. Similarly, Rsk levels were increased in STHdhQ111/Q111 cells [67], but strongly decreased in postmortem caudal and putamen samples from HD patients [65]. Knockdown experiments indicated that Rsk activity exerted a protective effect against mhtt-induced cell death in STHdhQ7/Q7 cells transfected with mhtt and overexpression of Rsk in R6/1 mice at the onset of motor sympt oms rescues motor impairment, enhanced expression of synaptic markers and increased expression of genes related to synaptic plasticity, such as cfos and egr1 [65, 67]. We also observed that downregulation of Rsk was due, at least in part, to the depletion of BDNF in HD striatum suggesting that Rsk could be a downstream effector of BDNF function. These results place Rsk as a new element regulating striatal alteration that leads to motor phenotype in HD, making it a good target for neuroprotective therapies in HD.

Different drugs could be used to increase Rsk activation. As a downstream target of ERK [84], activation of ERK pathway could result in an activation of Rsk as an effector. In this line,

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previously proposed drugs could be also useful in promoting Rsk activation. As for ERK activation, clozapine treatment also increases levels Rsk phosphorylation in the cortex and striatum in an ERK-dependent manner, meanwhile Rsk activation by olanzapine and haloperidol is not concomitant with ERK signaling [83]. Although the Rsk pathway can be activated by increased ERK activity, more research focusing on specific drugs targeting Rsk should be carried out.

3.1.3. Activation of transcription factors: CREB and Elk-1

ERK 1/2 cannot only phosphorylate different kinases, but also some transcription factors such as CREB (**Figure 2**) [86, 87]. But CREB can also be phosphorylated by other kinases as Rsk [88–90] and PKA [91]. Once activated, CREB interacts with CBP and CREB-mediated gene expression is induced [92]. CREB is a widely expressed transcription factor known to mediate stimulus-dependent expression of genes critical for plasticity, growth and survival of neurons [93]. Activation of CREB is necessary for synaptic transmission [94] and CREB-mediated gene expression is sufficient for the survival of multiple neuronal subtypes [95, 96]. CREB may exert this prosurvival effect by regulating the transcription of prosurvival factors, such as *Bcl-2* and *Bdnf* [97].

Different studies observed that CREB signaling is compromised in different mouse and cellular models of HD and in human HD samples, where the expression of mhtt induces aggregation of its coactivator CBP (**Figure 2**) [11, 28, 98], reduces the levels of cAMP [72] and downregulates CRE-mediated transcription of numerous genes [19]. This decrease in CREB-induced transcriptional activity is believed to contribute to HD pathogenesis [97]. One of the genes regulated by CREB is *Bdnf* [97]. Reduced CREB-dependent transcription of *Bdnf* is a robust feature of HD pathology. In human samples, BDNF protein and mRNA levels are decreased in the frontoparietal cortex [99]. Reduced levels of cortical and striatal BDNF have also been reported in multiple mouse models of HD, including R6, N171-82Q, Hdh and YAC-72 lines [17, 19].

The beneficial effect of restoring CREB phosphorylation has been observed by us and others in both excitotoxic and genetic mouse models of HD [100, 101]; thus pathways targeting CREB activation can also lead to an increase in BDNF together with cognitive improvements in HD models [102]. Furthermore, regulation of possible downstream effectors of BDNF function also shows clearly motor improvements together with a restoration of CREB-mediated gene transcription and expression of synaptic markers in R6/1 mouse model of HD [102, 103].

ERK1/2 can also phosphorylate the transcription factor Elk-1, which, together with CREB, is considered to be one of the most important transcription factors in neurons [104, 105]. In the cortex, Elk-1 is activated after QUIN-induced lesion and has the capacity to prevent excitotoxic cell death [106]. Increased phosphorylation of ERK-activated transcription factors, such as Elk1, has been correlated with increased ERK phosphorylation in R6 striatum [107, 108]. However, the expression of *c-fos* and *egr-2*, two genes regulated by Elk-1 [109], was downregulated in these mice and in STHdhQ111/111 [108]. This downregulation was

correlated to a strong decrease in the expression and the phosphorylation of MSK-1 in R6/2 mice [107], a kinase that phosphorylates the histone H3 and promotes the expression of c-Fos [110]. Both MSK-1 and Elk-1 inhibition induced mhtt-specific cell death, with no effect on wild-type cells. Moreover, overexpression of MSK-1 restores c-fos expression and protects striatal cells against neurodegeneration induced by mhtt expression, showing a neuroprotective role of this protein in HD [107]. Reinforcing this hypothesis, the inhibition of Elk-1 in STHdhQ111/Q111, but not in STHdhQ7/Q7 cells, resulted in a decrease of *c-Fos* and *Egr-2* mRNA levels [108].

3.2. Regulating cAMP

To increase activation of CREB, it is also important to take into account the levels of cAMP. The major kinase that is in charge of CREB activation is PKA, which in turn needs cAMP to be activated [91]. The cAMP signaling pathway has a key role in the neurobiology of learning and memory and therefore could serve as a target for cognitive enhancers and to reduce memory deficits in HD. In support to this idea: (1) reduced levels of cAMP were reported in the cerebral spinal fluid of symptomatic HD patients [111] and (2) forskolin, which stimulates adenylyl cyclases to produce cAMP from ATP, was able to ameliorate mhtt-induced phenotypes in PC12 cells [112]. Reduced levels of cAMP were also observed in STHdhQ111/Q111 striatal cells together with a decreased nuclear localization of CBP [72]. Activation of cAMP/PKA signaling by forskolin restored a nuclear CBP expression in the mutant striatal cells [72] and could partially rescue the loss of neurite outgrowth and cell death due to reduced CRE-mediated transcriptional activity [112].

3.2.1. Role of phosphodiesterases

Different studies [113] suggest that phosphodiesterase (PDE) inhibitors might be good candidates for enhancing CREB activation. PDE inhibitors prevent the breakdown of cAMP to 5′-AMP, prolonging the activation of protein kinases that promote phosphorylation of CREB [114]. It has been shown that the expression of different PDEs is altered in the striatum [115, 116] and hippocampus [38] of HD mouse models. The use of drugs that maintains CREB phosphorylated, like the specific PDE4 and 10 inhibitors rolipram and T10, decreases striatal cell loss after the injection of QUIN in an excitotoxic model of HD [100, 117]. Following this research, the same group reported that administration of rolipram in R6/2 mice enhanced the expression of both phosphorylated CREB and BDNF in striatal neurons and ameliorated neurodegeneration, decreased mhtt inclusions preventing the sequestration of CBP, reduced microglia activation and rescue motor function [118, 119]. Likewise, beneficial effects of PDE inhibition on cognitive function were also observed in the hippocampus of HD mouse model [101]. We recently observed that papaverine, which is considerably selective for PDE10A, could improve spatial and object recognition memories in R6/1 mice and significantly increase phosphorylation of CREB and cAMP levels in the hippocampus [101].

Although PDE10A has been proposed as a therapeutic target for HD based on the observation that pharmacologic inhibition of PDE10A in transgenic HD mice significantly

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improved behavioral and neuropathologic abnormalities [101, 119], some conflicts appear when focusing on HD patients. Earlier work had shown that striatal PDE10A levels in HD mice already decline to minimal levels before onset of motor symptoms [115, 116]. In humans, decreased PDE10A levels were found in postmortem striatal tissue [115] and in PET studies from Huntington's disease patients with significant striatal atrophy [120] and premanifest Huntington's disease gene carriers [121, 122]. It is unclear how the alteration of PDE10A expression is related to the neuropathological out-standing networks. Depletion of PDE10A in HD striatum would at first sight seem hard to reconcile with a beneficial effect of PDE10A inhibitors in HD. However, a recent study reported a dramatic increase in PDE10A levels in the perikarya of striatal medium spiny neurons [123] and moreover, we did not observe changes in the expression of this protein in the hippocampus of R6/1 mice compared to controls [101]. Taking together all these results, it is important to determine whether PDE10A levels are affected in HD patients and in *in vivo* models of HD in the different brain areas and if these alterations are functionally significant in order to choose PDE10A inhibitors for use in clinical trials in HD.

3.2.2. Role of G protein couple receptors

G protein-coupled receptors (GPCRs) constituted a large family of receptors coupled to G proteins that activated two main signaling pathways: cAMP and phosphatidylinositol pathways [124]. GPCRs are involved in many diseases and are also the target of approximately 40% of all modern medicinal drugs [125].

In order to increase the levels of cAMP, molecules targeting GPCRs could be useful. Depending on the subunit of G protein that the receptors are coupled, they can activate ($G\alpha_s$) or inactivate ($G\alpha_{i/o}$) adenylate cyclases [125]. Therefore, drugs targeting the activation of $G\alpha_s$-coupled receptors or the inhibition of $G\alpha_{i/o}$-coupled receptors would result in an increase in the levels of cAMP and probably in turn an increase in the activation of CREB. In line with this idea, we have recently demonstrated that fingolimod (FTY720) treatment improves synaptic plasticity and memory in the R6/1 mouse model of HD, through regulation of BDNF signaling [103]. FTY720 targets GPCRs $G\alpha_{i/o}$ SP1 receptor and inhibits it [126]. Between the different effects of SP1 receptor activation there is a reduction on cAMP as $G\alpha_{i/o}$ inhibits adenylate cyclases [127]. Therefore, inhibition of SP1 receptor could result in increased levels of cAMP. Indeed, FTY720 treatment increased cAMP levels and promoted phosphorylation of CREB in the hippocampus of R6/1 mice [103].

Another approximation to increase cAMP levels is inducing the activation of $G\alpha_s$-coupled receptor. Prostaglandin (PG) receptors are well-known GPCRs [128]. EP2 prostaglandin receptor is known to stimulate cAMP and activation of the transcription factor CREB [129]. EP2 receptor activation is associated with neuroprotection and hippocampal-dependent synaptic plasticity [130] and can lead to the induction of BDNF [102, 131]. In terms of HD, we have recently shown that chronic treatment of R6/1 mice with misoprostol, an EP2 receptor agonist, ameliorated hippocampal-dependent long-term memory deficits in these animals [102]. Importantly, misoprostol treatment promoted the expression of hippocampal BDNF and increased cAMP levels, together with a recovery in the expression of different synaptic

markers. All these data suggest that mhtt leads to alterations of CRE-mediated gene transcription and reinforce the idea of a beneficial effect of increasing gene expression mediated by CREB could be a good therapeutic approach in HD.

4. Cycle of neurotoxicity

Ultimately, excitotoxicity contributes to neuronal degeneration in many acute as well as chronic central nervous system diseases [132]. Polyglutamine expansion produces a hyperactivation of N-methyl-D-aspartate receptor (NMDAR and kainite receptors) [133]; stabilizes NMDA receptors in the postsynaptic membrane [134]; inhibits the uptake and release of glutamate at the synapses [135]; and can also sensitize the inositol (1,4,5)-triphosphate receptor type 1 located in the membrane of the endoplasmatic reticulum [136]. In addition, mhtt can contribute to excitotoxicity by decreasing the expression of the major astroglial glutamate transporter (GLT-1) [137], which reduces the glutamate uptake (**Figure 3**) [138]. All these alterations promote glutamate-mediated excitotoxicity by a massive increase of intracellular Ca^{2+}, which affect the calcium homeostatic mechanism [139] and lead to deleterious consequences. Imbalance in the calcium homeostasis has been previously reported in different HD mice [140–142] that it is in agreement with consistent changes in the expression levels of many Ca^{2+} signaling proteins [143]. Moreover, different proteins involved in neuronal Ca^{2+} signaling have been proposed as attractive targets for developing therapies for HD [144]. Excitotoxicity and mhtt expression also promote the activation/inhibition of several pathways regulated by different kinases and phosphatases [74, 145]. In the following lines, we will review some of the mechanism implicated in this excitotoxic process that occurs in HD, together with the prosurvival mechanism activated in HD brains to fight against this process. Moreover, we will discuss about potential and new state-of-the-art therapies to fight neurodegeneration and reduce excitotoxicity.

4.1. Fighting glutamate

4.1.1. NMDA receptors

Alterations in proteins involved in glutamatergic signaling have been reported in mouse models of HD [146, 147]. Since the main hypothesis underlying striatal neurodegeneration in HD has been excitotoxicity, due in part to increase in glutamate release, NMDA receptors were the first glutamate receptors studied. At early stages of the disease, when cognitive and plasticity alterations are detected, no changes in the protein levels of any NMDAR subunit are observed in the striatum and hippocampus of HD mouse models [148–150]. Conversely, HD mouse models do not respond to intrastriatal NMDAR agonists (**Figure 2**) [141, 149, 151]; which support the idea that signaling downstream the receptor is affected in HD [152] and contributes to synaptic plasticity impairment. Not only the expression of these receptors is important, but also their location. Stimulation of synaptic NMDAR conveys the synaptic activity-driven activation of the survival-signaling protein ERK and triggers an increase in nuclear calcium, leading to the activation of the transcription factor CREB and the production of the survival-promoting protein BDNF [153]. In contrast, global or extrasynaptic NMDAR

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stimulation decreases ERK and CREB activation and BDNF production, promoting cell death (**Figure 3**) [153].

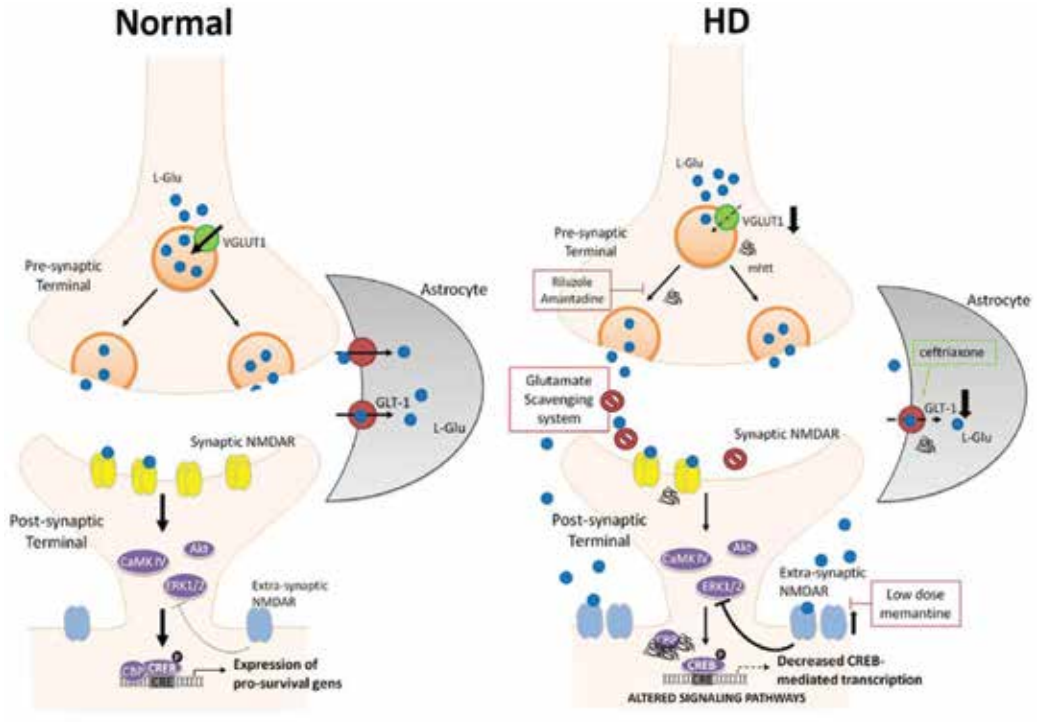

Figure 3. Changes in glutamate regulatory system in the presence of mutant huntingtin. In the presence of mhtt, there is an increase in the levels of glutamate together with an imbalance in the levels of synaptic and extrasynaptic NMDAR. Increased activation of extrasynaptic NMDAR leads to neuronal death by inhibition of ERK and the activation of the transcription factor CREB. Moreover, there is a downregulation/dysfunction of the glial glutamate transporter (GLT-1), which leads to an increase in glutamate at the synaptic cleft. Reduced VgluT1 transporter also affects glutamate recruitment into the synaptic vesicles contributing to deficits in synaptic transmission. Different drugs to modulate these mechanisms are shown.

4.1.2. Glutamate transporters

On the other hand, not only glutamate receptors but also glutamate transporters are altered in HD, such as the vesicular glutamate transporter 1 (VGluT1) [154] that contributes to the imbalance of glutamate in neurons could play a role in cell dysfunction in HD. Presynaptic expression of VGluT1 contributes to the proper expression of other synaptic proteins and reduced levels of this glutamate transporter, as occurs in the striatum of R6 mice [154, 155], can disrupt cortico-striatal synaptic transmission [154, 156]. The expression of glutamate transporters is also altered in glial cells. GLT-1 is the major molecule responsible for the clearing of glutamate from synaptic cleft [157], making it an attractive therapeutic target. Reduced mRNA levels of GLT-1 and decreased glutamate uptake have been described in HD postmortem brains [22] as well as in R6/2 mice [137], suggesting decreased glutamate removal at synapses in HD.

Moreover, alterations in the palmitoylation of this transporter were detected, which can alter its function [158]. In addition, strategies aiming at the upregulation of GLT-1, like ceftriaxone treatment [159], attenuate some behavioral alterations in the R6/2 mice model (**Figure 3**) [160].

4.1.3. Strategies to decrease glutamate excitotoxicity

Drugs inhibiting glutamate neurotransmission [161, 162], glutamate antagonists [163] and blockade of NMDAR [164, 165] have been used for the first time to attempt for blocking the excess of glutamate at the synapse. Riluzole and amantadine are two antiglutamatergic therapies that have been investigated in rigorous trials in HD [162]. Moreover, riluzole is already marketed for the treatment of amyotrophic lateral sclerosis. Riluzole is a drug that inhibits glutamate release and the current evoked by the stimulation of excitatory amino acid receptors [166]. Treatment of R6/2 mice with riluzole showed positive effects in reducing the progression of neurological abnormalities in this mice model of HD [161]. Specific blockade of NMDAR has been also extensively studied, but accuracy has to be taken into account. Drugs like memantine are shown to inhibit NMDAR [164, 165, 167], but their beneficial effects depend on the right dose. At high concentrations, memantine blocks synaptic and extrasynaptic NMDAR, inducing neuronal death, as NMDAR once at the synapse can activate prosurvival pathways [167]. When used in a lower dose, memantine can specifically block extrasynaptic NMDAR producing a potential therapeutic effect in mouse models of HD [164, 165]. A new technique to combat the glutamate exposure developed recently is the blood glutamate scavenging system (Braintact) [168, 169]. Braintact is developing a platform solution that overcomes the excess glutamate level in blood by using a new approach developing drugs that remain in the blood circulation and boost a natural mechanism that reduces glutamate levels in the bloodstream and leads to lowering of glutamate concentrations in the brain (**Figure 3**).

Although common strategy is to treat with NMDA glutamate antagonist for reducing excitotoxicity, their clinical viability has not been proven [162]. Some agents showed efficacy in terms of motor dysfunction, but no treatment has been identified as appropriated. Moreover, many present treatments considerable side effects or effects in cognitive improvement were not even considered. Therefore, there is a need to continue the research on antiglutamatergic drugs in HD for the treatment of excitotoxicity. Also cellular pathways and drugs trying to enhance or inhibit these cellular pathways related to survival will be discussed further in this section.

4.2. Role of kinases

Increasing our understanding on the pathways behind the excitotoxic events and neuronal dead occurring in HD is necessary in order to identify targets downstream glutamate receptors cascade that may represent useful therapeutic strategies do reduce or halt neuronal dysfunction. Alterations in numerous signal transduction pathways and aberrant activity of specific kinases have been identified in multiple cell and mouse models of HD, as well as in human HD brain. Unbalanced activities within these pathways provide a potential mechanism for many of the pathological events associated with HD. Aberrant kinase signaling

regulation in HD has a wide range of effects on multiple pro and antiapoptotic kinases, resulting in the activation of compensatory mechanisms to fight excitotoxicity or prodeath mechanisms triggered by excitotoxicity [74].

4.2.1. ERK

The ERK pathway is a strong mediator of antiapoptotic and prosurvival signaling. Although both protective and deleterious roles have been proposed for ERK activation in neuronal cells [170], recent studies using mhtt-expressing cells provide strong evidence that activation of ERK is neuroprotective, while specific inhibition of ERK enhances cell death [62]. The phosphorylation of ERK activates neuroprotective factors [62, 107] and inactivates proapoptotic mediators by phosphorylation [171]. Data derived from cell culture experiments showed that ERK is activated in response to mhtt and increases cell survival [62]. The ERK pathway is also upregulated in several transgenic animal models of HD. Significant ERK activation was observed in the striatum of R6/1 and R6/2 mouse (**Figure 2**) [64, 107]. The timing of ERK activation in HD mice supports the hypothesis that the ERK pathway might not be involved in a primary pathological process, but rather that it is a compensatory mechanism activated in response to mhtt and could participate in delaying striatal cell death because R6 mice show no significant cell loss [172]. Accordingly and as previously mentioned, ERK pathway activation in response to mhtt may participate in the reduced neuronal loss observed after QUIN injection in R6/1 mice (**Figure 2**) [64]. Moreover, changes in ERK levels and activation can modulate transcription in HD what triggers, in part, the neuroprotective role of ERK mediated by its downstream effectors.

Checking on the ERK mechanism along the different sections, we can conclude that ERK has a prosurvival role in the presence of mhtt, which can be achieved by the activation/inactivation of different proteins promoting survival and transcriptional regulation of protective genes. Therefore, ERK activation might provide a novel therapeutic approach to prevent neuronal dysfunction in HD.

4.2.2. AKT

The AKT signaling pathway has been extensively characterize in models of HD and its activation is considered to be antiapoptotic and neuroprotective in different models of acute and chronic neurodegeneration [72, 173]. A primary mechanism of AKT-mediated neuroprotection is by its phosphorylation and inactivation of proapoptotic machinery [61, 72, 174].

In HD, the AKT pathway has been proposed as a crucial neuroprotective pathway, because it is one of the serine/threonine kinases that phosphorylate Ser421 of mhtt, attenuating its toxicity [174]. Activation of the AKT pathway has been determined in several cells and mouse models of HD. Increased levels of phosphorylated AKT were observed in the striatum of full-length and exon-1 mouse models and also in striatal cells expression mhtt [61, 72]. We observed that enhanced AKT signaling correlates with decreased expression of PH domain leucine-rich repeat protein phosphatase (PHLPP), a phosphatase that dephosphorylates AKT (**Figure 2**) [61]. PHLPP1 protein levels were reduced in the striatum of HdhQ111/Q111,

R6 and Tet/HD94 mouse models of HD as well as in the putamen of HD patients. In addition, we showed that intrastriatal QUIN injection in R6/1, but not in control, mice upregulates the phosphorylated AKT protein levels, which can contribute to the absence of striatal cell death observed in these animals after an excitotoxic injury [61, 151]. This increase in the phosphorylated AKT is still detected at later stages of the neurodegenerative process, offering together with phospho-ERK, a mechanistic explanation to the small amount of neuronal death observed in these HD models (**Figure 2**). In accordance with our results, AKT prevents neuronal death induced by mhtt [174] and increasing AKT expression has beneficial effects on Drosophila models of HD [175]. Thus, on the basis of these results, it is not too daring to suggest that use of therapeutic approaches focusing on AKT prosurvival pathway could delay neuronal death in HD.

4.3. Role of phosphatases

Concomitantly to kinases, several Ser/Thr protein phosphatases activate to counteract the effect of kinases. They are of particular interest in this respect as several phosphatases are altered in HD mouse models [145] and, most importantly, in the caudate/putamen of HD patients [176]. Many of these altered phosphatases in HD play a role in memory and plasticity phenomena and then this imbalance likely contributes to synaptic alterations and cognitive impairment in HD.

4.3.1. Striatal-enriched protein tyrosine phosphatase (STEP)

Striatal-enriched protein tyrosine phosphatase (STEP) is a brain-specific phosphatase involved in neuronal signal transduction. STEP is enriched in the striatum and plays an important role in synaptic plasticity through the opposition to synaptic strengthening [177]. We and others recently reported reduced STEP protein levels in the striatum and increased inactivity in different HD mouse models [64]. Reduced STEP activity in HD can lead to an increase in the activity of the NMDAR [178]. Additionally, STEP has been implicated in susceptibility to cell death through the modulation of ERK1/2 signaling pathway, as we have previously reviewed [64]. The STEP pathway is severely downregulated in the presence of mhtt and participates in compensatory mechanisms activated by striatal neurons that lead to resistance to excitotoxicity (**Figure 2**) [64]. When injected with QA, R6/2 mice displayed a greater increase in STEP inactivation compared to WT together with decreased neuronal death, but overexpression of STEP in R6/2 animals increased QUIN-induced cell death [64]. Moreover, it has been suggested that an increase in STEP activation at the synapse in YAC128 mice together with calpain activation contributes to altered NMDAR localization (increased extrasynaptic localization of GluN2B receptors) and increases excitotoxicity [179].

In order to select STEP as a potential therapeutic target in HD different aspects have to be taken in consideration. In HD, STEP downregulation is initially neuroprotective to mhtt-induced glutamate excitotoxicity [64], but a decrease in synaptic plasticity and cognitive impairment still occurs. On the other hand, increased STEP activation produces alterations

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in the trafficking of NMDA and AMPA receptors, dephosphorylating them and producing an excessive internalization of these receptors which decreases synaptic plasticity [177]. On the basis of this evidence, a suitable expression of STEP might be a good therapeutic strategy in different neurodegenerative diseases. Pharmacological inhibition of STEP by a recently discovered inhibitor, TC-2153, reversed cognitive deficits in a mouse model of Alzheimer's disease, where STEP levels are increased [180]. But the effect of STEP activation is still not clear in a model like R6/1 mice, where STEP levels are reduced.

4.3.2. Calcineurin

The role of protein phosphatases in the cascade of events triggered during excitotoxic cell death has not been extensively studied, but some protein phosphatases, such as Ca^{2+}-dependent calcineurin, were found to contribute to excitotoxicity (because its inhibition is neuroprotective [181]). Calcineurin is a ser/thr protein phosphatase activated physiologically by calcium/calmodulin and it is highly expressed in the brain [182]. Calcineurin plays an important role in synaptic plasticity and learning and memory [183]. Interestingly, it is enriched in MSNs [182] and thus variations in its expression levels/activity can seriously alter their function. Some studies have shown that activation of calcineurin promotes apoptosis and pharmacological inhibition of calcineurin reduces the activation of excitotoxic molecules and decreases cell death after different toxic insults [184, 185].

Calcineurin levels are reduced in R6 and Tet-HD94 mice striatum [19, 69] and lower calcineurin activity has been shown in the striatum of YAC128 mice at 12 months of age (**Figure 2**) [186]. Inhibition of calcineurin with FK-506 drastically reduced cell death in an excitotoxic model of HD [69]. Moreover, calcineurin levels were downregulated during the progression of the disease in R6/1 mice and the induction of calcineurin after QUIN injection in these excitotoxicity-resistant mice [151] was lower than that in control animals [69]. These finding suggested that altered calcineurin activity contributes to the excitotoxic resistance observed in R6/1 mouse models (**Figure 2**). On the contrary, in HdhQ111/Q111 mice calcineurin activity was shown to be increased in the cortex [187] and higher expression and activity of calcineurin was also observed in STHdhQ111/111 cells [68]. These cells presented increased vulnerability to NMDAR stimulation, which was associated with higher calcineurin protein levels and activity [68] (**Table 2**).

However, controversial data have been reported about the role of calcineurin in HD. Although decreased calcineurin activity increases resistance to excitotoxicity [69] and high levels of calcineurin increase mhtt toxicity [68, 186, 187], it has been shown that inhibitors of calcineurin accelerates the neurological phenotype in R6/2 mice [188], which are resistant to excitotoxicity [151]. Moreover, decreased calcineurin activity appears when pathological symptoms are present in these animals and not in presymptomatic stages [69], suggesting a dual role of calcineurin during the progression of the disease and a possible involvement of this protein in the striatal neuronal dysfunction. Therefore, like it is occurring with STEP, it is reasonable to suggest that a therapy targeted to maintain normal levels of calcineurin could represent a good approach to delay neuronal dysfunction in HD.

Model		Calcineurin change	Age	Susceptibility to excitotoxicity	Age	Reference
Cellular models	STHdhQ7/Q111	Increased		Increased		[68]
	YAC128 primary cortical neurons	Not reported		Increased		[186]
	YAC72 primary striatal neurons	Not reported		Increased		[194]
Exon-1 mouse models	R6/1	Decreased	16 weeks	Decreased	8 weeks	[69, 141]
	R6/1: BDNF$^{-/+}$	Decreased	12 weeks	Decreased	12 weeks	[69, 150]
	R6/2	Decreased	10 weeks and earlier	Decreased	3 weeks	[141, 195]
	Tet/HD94	Decreased	22 months	Not reported		[69]
	N171-82Q	Not reported		Decreased	15 weeks	[149]
Full-length models	YAC72	Not reported		Increased	6 and 10 months	[194]
	YAC128	No change	3 months	Increased	1.3–6 months	[186, 196]
		Reduced	12 months	Decreased	10–18 months	[186, 21]
Knock-in models	HdhQ111/Q111	Increased	12 months	Not reported		[187]
	HdhQ7/Q111	Increased	12 months	Not reported		
	FVB/CAG140$^{-/+}$	Not reported		Decreased	12 months	[197]
	FVB/CAG140$^{+/+}$	Not reported		Decreased	4 months	
	C57Bl/6/ CAG140$^{-/+}$	Not reported		Decreased	4 months	
	C57Bl/6/ CAG140$^{+/+}$	Not reported		Decreased	4 months	
Human samples		Decreased				[69]

Table 2. Changes in calcineurin levels and resistance to excitotoxicity in different HD mouse and cellular models.

5. Discussion

As we have seen in this chapter, many pathways are interconnected and related between them, even making a "cycle." This "cycle" could be used for developing therapies that maybe targeting one or several proteins which can modify different pathogenic events. As an example, when increasing activation of some kinases, excitotoxicity can be counteracted and at the same time promote the activation of transcription factors that can burst transcription. Then, different expressed genes can contribute to further fight against excitotoxicity completing the "cycle." But, the development of therapies targeting altered transcription or modulation of cell signaling pathways face difficult challenges as, nowadays, no single transcriptional regulator has been identified as a main player of the disease. Nevertheless, potential therapeutic advances have recently emerged. Some of them include the inhibition of HDAC, compounds

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that directly interact with DNA and drugs targeting proteins involved in the modulation of transcription, representing promising therapies to protect against neurodegeneration. Also drugs inhibiting glutamate/NMDAR neurotransmission or glutamate scavenging systems have been used as a first attempt to block the excess of glutamate at the synapse. Altogether, these findings show us that although HD is a disease cause by a single gene mutation, multifactorial drug treatments could be applied in order to reduce or delay the symptoms and open a wide spectrum of research fields to reach the final cure to this de

Acknowledgements

Financial support was obtained from the University of Girona (MPCUdG2016/036). The authors declare that they have no competing interests.

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Chapter 4

Porcine Model of Huntington's Disease

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66353

Abstract

At present, we are probably the only research facility to be breeding transgenic Huntington's disease minipigs (TgHD). These minipigs express N-terminal part of human mutated huntingtin including 124Q under the control of human huntingtin promoter. The founder animal, born in 2009, gave birth to four subsequent generations with an equal contribution of wild-type (WT) and transgenic (TgHD) piglets in all litters. We take different approaches, some of which are unique for large animal models, to study the phenotype development comparing WT and TgHD siblings. In this chapter, we review these approaches and the phenotype progression in the minipigs. Additionally, we outline perspectives in generation of new models using novel methodology and the potential of pig models in preclinical HD studies.

Keywords: large animal models, pig, huntingtin, transgenic animal, Huntington's disease

1. Introduction

The cause of Huntington's disease (HD), an abnormal polyglutamine elongation of the gene encoding the huntingtin protein (Htt), is known for more than two decades, but the effective treatment is still lacking. Animal models of Huntington's disease represent an indispensable part of disease investigation. It offers better understanding of molecular mechanisms involved

in the disease pathology and above all testing novel potential therapeutic approaches in HD treatment. Many different HD animal models were created. Predominantly used rodent models represent an important tool for understanding the complex progression of HD phenotype and have been used for many preclinical studies. However, the rodent's small brain size, differences in neuroanatomy to humans and short lifespan limit their application for detailed modelling of the pathogenic features of human disease. Moreover, wider scale of models is desired especially for safety and tolerability tests of potential therapeutics and longitudinal studies of HD.

In consequence, large animal models of HD, including non-human primate [1], sheep [2] and pigs [3–5], have been generated. The advantages of pigs, in particular the minipigs, are the adult body weight of 70–90 kg, relatively large gyrencephalic brain with similar neuroanatomy to humans, longer lifespan of 12–15 years and other anatomical, physiological and metabolic similarities to humans [6]. Because of their sophisticated cognitive and motor abilities, pigs are suitable for longitudinal learning, memory and behavioural studies. Furthermore, pigs are excellent models for disease progression studies, because of their long lifespan. The pig brain size and neuroanatomy make them available to be used in neurosurgical procedures and non-invasive imaging methods similar to those used in human diagnostics [7, 8]. In addition, pigs are farm animals, and thus, the social tolerance for using them as experimental animals is higher than in other large animal models such as non-human primates or animals regularly kept as pets. Moreover, the minipig's litter size is usually six to eight pups, thus providing a good experimental group. Hence, the creation of HD minipig model offers many advantages for the HD studies.

Up to now, three attempts to generate HD transgenic pig models have been reported. In 2001, porcine mutant huntingtin (75Q) cDNA was microinjected in the pronucleus of fertilized eggs [3]. Several copies of the transgene were incorporated in the porcine genome, but there might have been problems with the gene intactness or transcriptional silencing. No HD phenotype was reported. In 2010, HD transgenic minipig expressing the N-terminal part of human mutated huntingtin with 105Q was created using the somatic cell nuclear transfer strategy (N208-105Q) [4], but these piglets died 53 h after birth, which could be due to the incomplete reprogramming during somatic cells nuclear transfer.

The transgenic HD minipig (TgHD) model created in Libechov expresses N-terminal part of human mutated huntingtin (N548-124Q) under the control of human huntingtin promoter. It was generated using strategy based on lentiviral infection of porcine embryos [5]. The mutant huntingtin was detected at the level of RNA and protein in central nervous system (CNS) as well as in peripheral tissues. The fluorescent *in situ* hybridization (FISH) and genomic analysis confirmed the incorporation of one copy of mutant huntingtin (mHTT) into non-coding sequence of the first porcine chromosome (1q24-q25) not interrupting any coding sequence in the pig genome. However, TgHD minipigs possess both endogenous alleles coding wild-type Htt (wtHtt). Because the reducing amount of wtHtt has substantial influence on disease development in HD patients [9], the physiological level of wtHtt in TgHD minipigs may postpone a phenotype progression. Most of the minipigs are now in the preclinical stage of HD. However, we observe the disease progression in the oldest animals based on behavioural, immunological, immunohistological and biochemical methods.

Our transgenic minipig model represents an advantageous model for studying wide range of aspects of HD like molecular mechanisms of HD in primary cells isolated from TgHD and WT animals, brain and other organ structure using high-resolution imaging techniques or post-mortem, preclinical symptoms of the disease and longitudinal non-invasive studies. Importantly, the minipig model is feasible for preclinical therapeutic studies (unpublished results) and thus can serve as the link between rodents and humans.

2. Impairment of male fertility: testes and sperm pathology

Although HD is characterized mainly by neurodegeneration, we at first discovered a reproductive failure in TgHD boars, starting at the age of 13 months and worsening with age.

In general, wild-type and mutant forms of Htt are expressed in many tissues, mostly in brain and testes [10]. Furthermore, the testes and brain display the most comparable gene expression pattern compared to other tissues [5]. Testicular degeneration was a long time unknown feature of HD. Interestingly, the first reports of testicular degeneration were described in mouse models. R6/2 mice showed dramatic atrophy of testes, which started at 4 weeks of age [11]. Also in YAC128 mouse models, mHtt causes testicular atrophy and male fertility problems occurring before neurodegeneration [12–14]. In human patients, only analysis of testes from post-mortem samples was performed [13]. This study showed a decreased number of spermatocytes and spermatids in HD patients; the seminal tubules of the patients were thicker than the seminal tubules of the healthy controls.

In our TgHD minipig model, we observed reduction in spermatozoa [5] and also their functional defect. *In vitro* penetration assay showed impairment of TgHD spermatozoa to penetrate oocytes with intact zona pellucida, which was in accordance with lower sperm motility and progressivity. Electron microscopy (EM) revealed sperm and testicular morphology defects. Deformity of nucleus associated with incomplete chromatin condensation, abnormal acrosome,and also the absence of residual bodies were seen in TgHD spermatozoa. Proximal cytoplasmic droplets were often associated with disorganized mitochondrial sheaths (**Figure 1**). The presence of the polyglutamine-containing proteins was observed in structures in the spermatozoa tail of TgHD boars using immunocytochemistry (ICC) and Western blot (WB) [14].

Moreover, detailed examination of TgHD testes showed degenerative changes in seminiferous tubules. Apoptotic spermatogonia and Sertoli cells were detected. EM of 24- and 36-month-old boars showed degenerative changes. The increased density of cytoplasm of Sertoli cells associated with its vacuolization, swollen mitochondria and dilated endoplasmic reticulum and clumps of heterochromatin in the nucleus were observed. Lamina basalis was often thick and undulated, made up of several layers due to the reduction in tubules diameter in the absence of spermatogenic elements (**Figure 2**). Seminiferous tubules of testes from the WT siblings were intact [14].

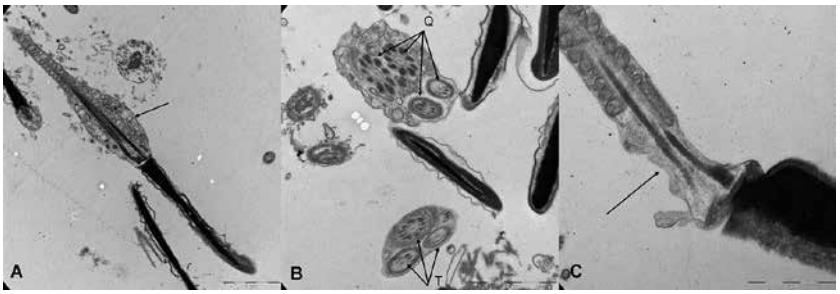

Figure 1. Morphologic defects of spermatozoa in TgHD boar's sperm. (A) Cytoplasmic droplet on midpiece of sperm. (B) Double, triple-T, quadruple-Q axoneme with fused mitochondrial sheaths. (C) Break on midpiece of sperm tail.

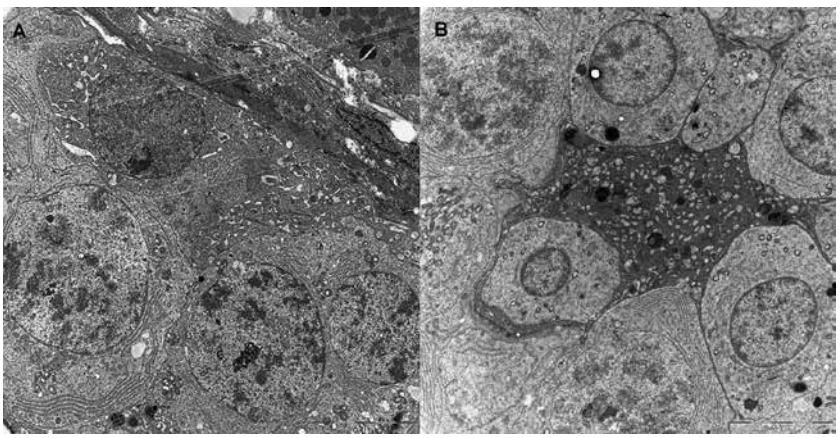

Figure 2. Seminiferous epithelium of TgHD boar at the age of 36 months. (A) Spermatogenic cells and (B) Sertoli cell undergoing apoptosis, increased chromatin condensation, increased density and vacuolization of cytoplasm.

In addition, the testes from 24-month-old TgHD boars were analysed using non-invasive methodology of 31P magnetic resonance spectroscopy (MRS) [15]. The results of this study showed significant reduction in relative phosphodiester (PDE) concentration in testicular parenchyma of TgHD boars compared to wild-type ones of the same ages. A decreased level of PDE/γ-ATP ratio in TgHD minipigs may be related to decreased concentration of seminal fluid or the changes in sperm motility. This hypothesis agrees with the observed sperm pathology in both our previous studies discussed above [5, 14].

3. Markers of neurodegeneration

3.1. Aggregates and fragmentation

It is known that the most affected organ in HD is the brain, especially the medium-sized spiny neurons in striatum, and the pyramidal cells in the cortex are disrupted [16]. The hallmark of

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the disease is an accumulation of misfolded proteins, resulting in the formation of aggregates [17]. Nevertheless, the precise role of the aggregates in pathogenesis of HD is still not clear. Recent studies suggest their protective role against the effects of mHtt [18]. Moreover, smaller soluble forms of mHtt and huntingtin oligomers were described to be toxic to the cells and to be the key factors of cellular dysfunction [19]. Furthermore, the inhibition of the mHtt proteolysis reduces neurotoxicity [20]. In affected areas of the brain in human patients, the expanded Htt is found rather in fragmented, oligomerized and polymerized forms [21].

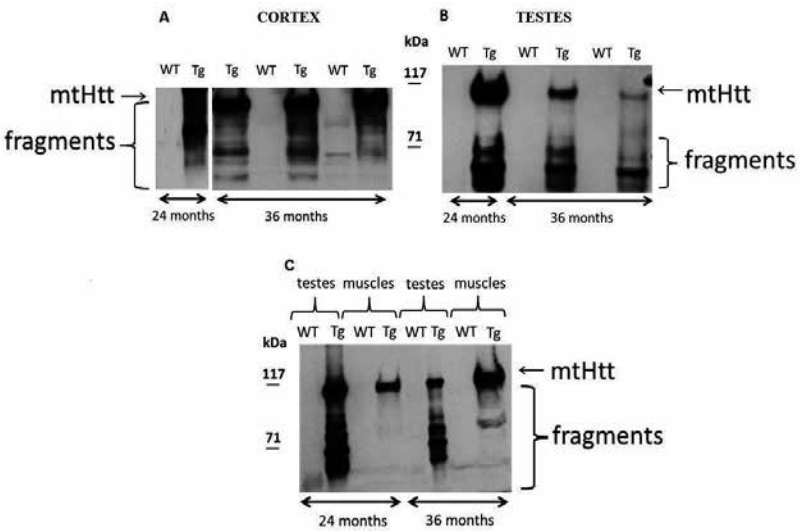

Figure 3. Fragmented mHtt detected in 24- and 36-month-old TgHD minipigs compared to their WT siblings in different tissues by polyQ 3B5H10 antibody. Western blot analysis shows fragmented forms of mHtt in (A) cortex and (B) testes of TgHD minipigs. (C) Comparing to muscles of TgHD minipigs, testes show increased amount of fragmented mHtt of 24- as well as 36-month-old minipigs. Reprinted with permission of CzMA JEP adopted from CeskSlovNeurol N2015; 78/111 (Suppl 2).

In our TgHD minipig model, we continuously test the aging animals for aggregates by several methods, which have been previously used in other animal models. The main focus is on the visualization of aggregates by immunohistochemistry (IHC) using anti-Htt antibodies, some of which are specific to higher molecular formation. For example, MW8 is a commercial antibody against N-terminal end, recognizing alpha-helical, random coil and extended conformations of huntingtin. In addition to the antibodies, certain dyes like Congo red stain protein aggregate by binding to fibrils with enriched β-sheet conformation [22, 23]. Another biochemical method suitable for detection of aggregates is the filter retardation assay based on the fact that very large polymers cannot pass through a 0.2-μm cellulose acetate filter and therefore can be identified [24]. Seprion assay uses beads binding amyloid structures, which can be then revealed on WB [25]. Velocity sedimentation method is based on fractionation of the sample by ultracentrifuge and detection of proteins in different fractions by SDS-PAGE and WB [26]. Htt oligomers and monomers can be detected by WB, taking advantage of oligomer retardation in stacking gel. Also, SDS agarose gel electrophoresis (AGERA) [27], 1%

agarose gel with a lower SDS concentration (0.1%) without reducing agent and heating of the samples, can be used. Another approach is an immunoprecipitation with oligomer-specific antibodies (OC, A11). In order to distinguish between soluble and insoluble forms stabilized by covalent bonds, formic acid that cannot dissolve covalent but can dissolve non-covalent bonds can be used.

Up to date, we have detected only a very few aggregates in 24- and 36-month-old TgHD brain sections by IHC (unpublished data) in comparison with the massive incidence of aggregates in the R6/2 mice brain sections. We suspect that the aggregate formation will progress in older animals. Nevertheless, we could detect N-terminal mHtt fragments in 24 months of age in brain and testes increasing with the age. Interestingly, most of the other tissues such as heart and muscles do not show fragmentation at 24 months. Only a small amount of fragments can be detected in muscles of 36-month-old animals, suggesting progression of the disease with age (**Figure 3**) [28]. Furthermore, we detected smears in the stacking gels of 3–8% polyacrylamide gels in TgHD cortex and testes samples, also starting at 24 months of age [28].

3.2. Magnetic resonance imaging (MRI) and spectroscopy (MRS)

Neurodegeneration in minipigs can be observed by several approaches. One of them is a non-invasive approach by MRI. MRI including objective motor measures (Q-motor) showed a relationship between a decrease in brain volume and progression of HD in patients [29–31]. It was shown that MRI applicable for the brain volume assessment can be performed also with Libechov TgHD and WT minipigs [32].

Magnetic resonance spectroscopy (MRS), also known as nuclear magnetic resonance (NMR) spectroscopy, is a non-invasive method used in research and clinical practice that allows an evaluation of *in vivo* metabolism at the molecular level [33]. Concentrations of metabolites, such as *N*-acetylaspartate, creatine, phosphocreatine, glutamate, glutamine, choline-containing compounds, inositol, γ-aminobutyric acid and others, can be determined by ^{1}H MRS [34]. ^{1}H MRS has previously been considered as a biomarker method in pre-manifest and early stages of HD [35–37]. In order to evaluate *in vivo* brain metabolite differences, single-voxel spectroscopy (SVS) has been primarily used [35, 38, 39]. However, several studies have also used two-dimensional chemical shift imaging (2D-CSI) [33, 40, 41]. Studies measuring changes by MRS revealed different values of metabolite concentrations in patients with HD [34, 35, 42, 43]. Recently, several longitudinal clinical studies have been performed. They showed a decrease in creatine and other metabolites (myo-inositol, *N*-acetylaspartate and choline) in striatum, white matter axial diffusivity and connectome changes in HD gene carriers during disease onset [44–46]. Various changes in brain metabolite concentration have also been found in different HD animal models (mice, non-human primates). Moreover, our scientific team also determined changes in the brain of TgHD minipigs before HD onset using ^{1}H magnetic resonance (MR) spectroscopy. Measurements were performed on a 3 T MR scanner using a single-voxel spectroscopy sequence for spectra acquisition in the white matter and chemical shift imaging sequence for measurement in the striatum, hippocampus and thalamus. Similarly to HD patients and HD animal models, we revealed significant decrease in total creatine (tCr) in the thalamus of 2-year-old TgHD boars accompanied with a non-significant

decrease in tCr in all examined brain areas. This aspect resulted into significant changes in metabolite ratios (increased metabolic ratios of total choline tCho/tCr in the striatum, thalamus, hippocampus as well as white matter). Creatine represents an important marker for brain energy metabolism, and we had supposed that the majority of the observed changes were predominantly related to changes in energy metabolism and mitochondria functions in TgHD caused by the presence of transgenic human mutated huntingtin [15].

3.3. Further markers of HD progression

The Htt protein is part of many cell processes and it interacts with various proteins in cells [16]. Different markers can be used for characterization of HD progression in large animal models. Typical markers in the brain include medium-sized spiny neuron marker (DARPP32), marker for activated microglia (IBA1), an astrocyte marker (GFAP), marker associated with cell apoptosis (Cas3), markers of Golgi complex (ACBD3 and RHES) and marker of neuronal secretion (BDNF). We use coronal brain section containing striatum for immunohistochemical staining and measure intensity of the labelling in different brain areas: (1) motor cortex, (2) somatosensory cortex, (3) insular cortex, (4) caudate nucleus and (5) putamen, according to 3D view model of pig brain (**Figure 4**).

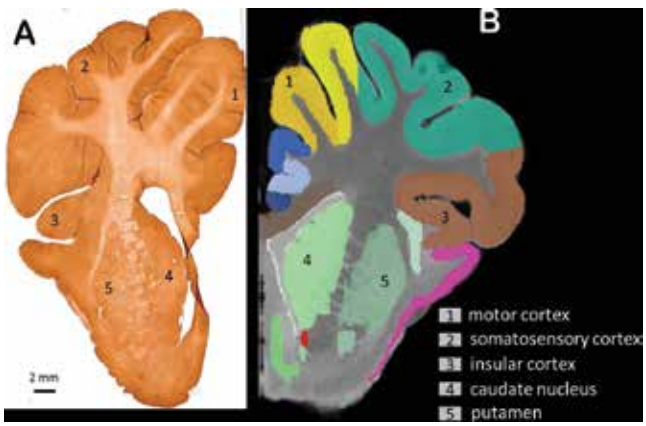

Figure 4. Identification of brain regions in porcine coronal brain section for evaluation. (A) Digitalized section staining with interest antibody. (B) Virtual section from 3D view model of pig brain (from program 3D slicer—slicer.org). 1. Motor cortex, 2. somatosensory cortex, 3. insular cortex, 4. caudate nucleus and 5. putamen.

Among the markers tested, medium-sized spiny neuron marker (DARPP32) was shown to be decreased in the striatum of our transgenic minipig model at 16 months [5]. DARPP32 mediates the response of medium-sized spiny neurons localized in the striatum to the activation of a dopamine receptor D1 [47]. The loss of DARPP32 was also shown in the brain of 7-month-old ovine transgenic HD model [2]. These results are in agreement with loss of D1 receptor detected in HD patients [48].

Next, apoptosis can be detected by higher expression of caspases. Caspase 3 was elevated in brain of HD minipig (N208-105Q), which died 53 h after birth [4]. Caspase 3 belongs to effectors

of apoptosis, and its activation mediates apoptotic cell death in HD [49]. Activated caspase 3 was also found in post-mortem human HD brain [50].

4. Immune response in central nervous system (CNS) and in the periphery

The principal aim of current research in Huntington's disease is focused on detection of the pre-manifest disease stages [51].

Not all aberrant changes in HD are secondary to the neuronal dysfunction, but they might be caused by the expression of mutant Htt in the peripheral tissues [52, 53]. It is known that the immune system is implicated in the pathogenesis of HD [54–57]. Therefore, inflammation is a growing area of research in HD.

The ubiquitously expressed mHtt may likely cause parallel inflammation in central nervous system (CNS) and in the periphery [58, 59] (**Figure 5**). Björkqvist et al. showed that immunomodulatory molecules IL-6 and IL-8 are over-expressed in the striatum and also in plasma of HD patients [54].

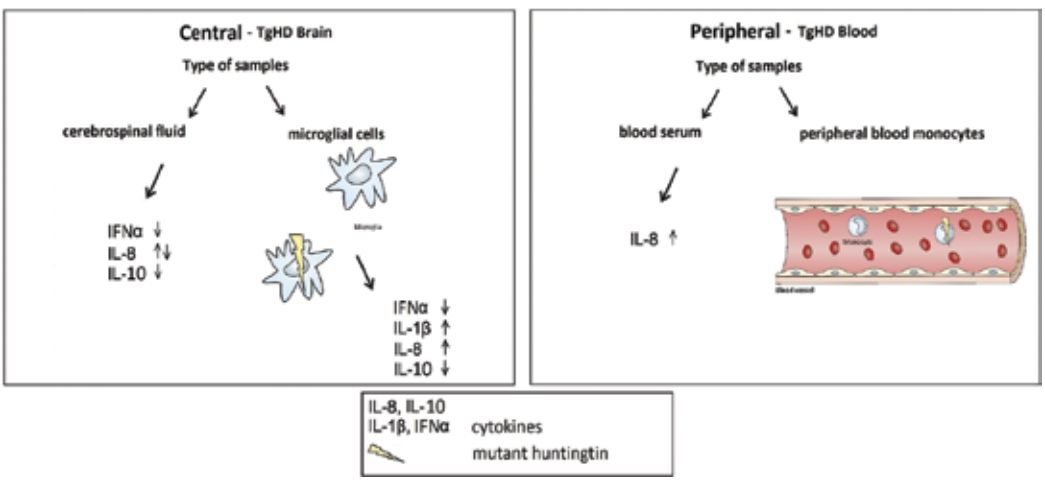

Figure 5. Immune activation in TgHD minipigs. The activation of immune system induced by mutant huntingtin, in CNS (central) and in the periphery (peripheral) as well.

In order to identify the mechanisms of immune system dysfunction in HD, our porcine model of HD was used [60]. The advantage of this large animal model is the ability to obtain samples from WT and transgenic TgHD animals with similar genetic background. The CNS immune response was measured by the levels of cytokines in CSF and in the secretome (in culture media) of cerebellar microglial cells. The inflammation in the periphery was simultaneously measured by the cytokine levels in serum and culture media of CD14+ blood monocytes. The samples were collected from WT and TgHD minipigs at the age of 9–36 months. TgHD minipigs at this age represent the pre-symptomatic stage of HD, thus offering the opportunity

to study early pathologic mechanisms before the disease onset. Multiplexing bead-based assay allowing the measurement of seven different porcine cytokines (IL-1β, IL-4, IL-8, IL-10, IFNγ, IFNα and TNFα) was used. Microglia secretomes and CSF of TgHD minipigs showed decreased levels of IFNα and IL-10, whereas microglia secretome as well indicated increased levels of IL-8 and IL-1β compared to WT animal controls. The difference in cytokine production in TgHD vs. WT samples is possibly caused by the presence of mutated Htt in TgHD microglia. Furthermore, increased levels of IL-8 were observed in TgHD serum samples.

This study suggested IFNα, IL-10, IL-8 and IL-1β as promising biomarkers reflecting immunopathological mechanisms of HD minipig model in the disease pre-symptomatic stage. Identifications of these candidate biomarkers in CSF and serum could be valuable for monitoring the HD progression and therapy. Better understanding of the earliest changes in brain tissue as well as in periphery system may lead to preventive or disease-modifying therapies [60].

5. Behavioural, motoric and cognitive studies

Behaviour is an important parameter in several neuroscience disciplines. People with HD have great difficulties with coordination, focusing and learning. The symptoms include involuntary chorea-like movements, poor balance, slurred speech, difficulty swallowing, cognitive difficulty and personality change. HD patients often have depression, anxiety, irritability and apathy. Interestingly, not all symptoms are experienced by all patients [61]. Mice models also exhibit difficulties in a number of tasks, namely swimming, beam traversing and maintaining balance on the rota rod at the fastest rotating speeds [62].

Several behavioural, cognitive and motoric tests using mainly F3 generation of Libechov TgHD and WT minipigs were established in George Huntington's Institute in Muenster [61]. The list includes GAITRite automated acquisition system, a carpet denoting walking, which can be used to detect imbalance and disturbance in walking. Hurdle test aims also at assessing motor coordination of gait and tongue, and coordination test detects tongue protrusion. A colour discrimination test, dominance test and a startbox back and forth test assess cognitive deficits in minipigs. The tests are easily done with Libechov TgHD and WT minipigs, and they are reproducible. Nevertheless, this group of tested minipigs, where the oldest were three and half years old, did not show any differences in performing above-mentioned tests in TgHD compared to WT. We have also performed some of these tests in Libechov using the oldest F0 and F1 generations of the minipigs. We established several additional tests involving a little stressful situation, for example, climbing on a balance beam or a pullback test. The oldest two animals, starting at 5 years, exhibit motoric defects and anxiety behaviour (data not published); however, this does not constitute a significant group of animals yet.

Additionally, we established, together with Technical University in Prague, vocalization—grunting test of the TgHD minipigs (**Figure 6**). Transgenic mHtt songbirds (145Q), created by lentiviral injection into the embryos, reflect severe vocal disorders associated with HD neuropathology [63]. It is also known that large majority of HD patients, more than 90%,

develop voice and speech dysfunction, abnormalities in speech timing, articulation deficits and irregular loudness [64]. Some of these symptoms start already before the onset of the disease [64–68]. Pigs have similar articular organs as humans, and thus, similar motor disturbances like rigidity, chorea and bradykinesia can be expected. A preliminary data and the experiment set-up were published [69].

Figure 6. A pig with a fixed microphone. Microphone records sounds with the MP3 player. These recordings are then transferred to a computer for audio analysis.

We observed three types of grunting:

1. Single grunts—associated with investigatory behaviour or contact calls in group.
2. Single squeals—higher level of arousal, but function is similar as single grunts.
3. Rapidly repeated grunts—appear to have either a greeting or threat function [69].

Several minutes long recordings of different grunting were taped, and the results will be analysed using acoustic software Praat used in evaluation of humans [70].

5.1. Telemetry

The long-term collection of neurobehavioral and other physiological data using telemetry devices represents a critical component of differently focused animal studies. Such devices have to be implanted in a location that is safe, well tolerated and functional. The major advantage of telemetry approach is the collection of biopotentials from freely moving experimental animals without the presence of disturbing factors—e.g. researchers—during different time periods (even in nonstop mode) that results into relatively objective data, which can be analysed by software.

Pigs, piglets and especially minipigs represent convenient large animal models for biomedical studies also in relation to the telemetry approach thanks to their relatively small size, characterized health status and ease of training and handling [71–74].

Important early features identified in HD patients include sleep deficits and disrupted circadian organization; these also correlate with symptom severity. Similar observations were also made in R6/2 mouse model [75] and ovine model of HD [76]. Mutated Htt action and loss of wild-type Htt function affect not only the brain structures, but also peripheral tissues or organ systems like testes, heart, pancreas, skeletal muscle, etc. [11, 52, 77, 78]. A major cause of death in HD patients includes heart disease. Moreover, in the R6/1 model, strong dysfunction of the autonomic cardiac nervous system was detected resulting in cardiac arrhythmias and sudden death [79].

The telemetry approach was applied to detect and analyse a pathological pattern in physical activity of TgHD boars at the age of 3 years [80]. In this study, we included five TgHD and five wild-type (WT) animals for comparison. The physical activity was measured by the telemetric system rodentPACK2 (emka TECHNOLOGIES, France), whereas transmitters were placed into the collar. For reducing collar influence on minipig activity, the boars wore collars without transmitters a few days before beginning of the study. The analysis showed significant decrease in total acceleration representing physical activity in TgHD boars between 4:40 and 5:30 a.m. (after night sleep and before morning feeding) in comparison with WT boars (**Figure 7**). This could be explained with disturbed energy metabolism.

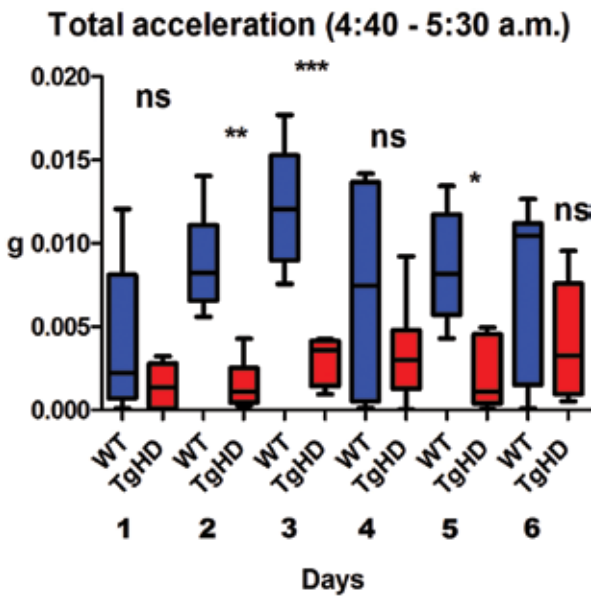

Figure 7. Total acceleration of TgHD and WT animals between 4:40 and 5:30 a.m. during six following days. Each column (blue—WT, red—TgHD) represents averaged total acceleration of five animals (TgHD or WT). Reprinted with permission of CzMA JEP adopted from CeskSlovNeurol N2015; 78/111 (Suppl 2).

6. Perspective and engineering of new models

A variety of HD animal models has been already engineered; nevertheless, it is necessary to generate improved models. While each model has some of the typical markers of HD, no model recapitulates the full phenotype of HD patients. The advantage of HD for model engineering is the conditionality of a single gene by the mutation in the polyQ stretch of HTT gene. However, the mHtt cytotoxicity may include more factors influencing the disease development, and it is not yet clear what mechanism causes the HD pathology. So the question is what should the ideal HD model carry out in the genome? New approaches of gene editing allow the sequence-specific targeting of genome and the design of modifications in the endogenous HTT locus. Recently, the CRISPR/Cas9 system has become the most widespread system due to the universality of the available engineered nucleases, and there are a lot of recent improvements in its application.

The CRISPR/Cas9 system enables the site-specific modification at a desired region in the genome. This system, just as programmable nucleases, namely the transcription activator-like effector nuclease (TALEN) and the zinc-finger nuclease (ZFN), is feasible to combine with the several genetic engineering applications. The somatic cell nuclear transfer (SCNT) together with the microinjection into the zygote belongs between the widely employed methods of generating genetically modified porcine models. There are advantages and disadvantages in both approaches. The SCNT gives us an opportunity to select the genetically modified cells and transfer only embryos with modified genome into a recipient sow. On the other side, genetic diversity of offspring is conserved using microinjection into the zygotes [81]. First genome edited pig was generated in 2011 followed by many other porcine knockout (KO) models [82]. However, the generation of porcine knock-in (KI) model remains a huge challenge for the researchers due to certain limitations such as incompetence of porcine embryonic stem cells and induced pluripotent stem cells for SCNT [83], founder mosaicism [84], abnormal epigenetic programming resulting in prenatal and early postnatal death [85] and other technical difficulties. Nevertheless, Peng et al. [86] successfully generated CRISPR/Cas9-mediated knock-in pig. They inserted human albumin (ALB) cDNA into porcine ALB locus. This approach is giving a hope to replace one wild-type allele and to create the DNA configuration of HD patients.

7. Conclusion

The Libechov TgHD minipig constitutes an animal model with slowly progressing phenotype similar to HD patients. The piglets are born with no evident defects, and the first detectable changes start at the age of 13 months. The TgHD boars have testicular degeneration and show a reproductive failure with low number of spermatozoa incompetent to penetrate the oocyte. MRS analysis of testes detected a significant reduction in relative PDE concentration in testicular parenchyma of 24-month-old TgHD boars, which could be related to changes in sperm motility. At 16 months, we detect lower expression of DARPP32 in TgHD striatum.

Fragmentation of mHtt, especially in the brain and testes, starts at 2 years and increases with age, similar to the appearance of oligomeric smears containing mHtt. The study of immune response in CSF and periphery suggested IFNα, IL-10, IL-8 and IL-1β as promising biomarkers reflecting immunopathological mechanisms of the pre-symptomatic stage of HD in the minipig model. Several non-invasive methods have been established in Libechov as well as in the George Huntington Institute, testing motor coordination, behaviour and cognition of TgHD minipigs. These methods can be applied to porcine models generated for various neurological diseases. MRI and MRS are the methods of choice to track any changes in clinical studies. Thus, relevant values obtained in porcine preclinical MRI studies can be easily translated to clinics. Moreover, we have already described some changes in the preclinical stage, namely significant decrease in total creatine (tCr) in the brains of 2-year-old TgHD animals. Also, telemetric studies showed differences in physical activity patterns of 3-year-old TgHD compared to WT minipigs between 4:40 and 5:30 a.m. The oldest two animals, starting at the age of 5 years, show motoric defects and accentuated anxiety behaviour. Therefore, we expect the clinical onset of HD in TgHD animals with the N-terminal part of human mHtt at the age of approximately 6 years. This fact needs to be confirmed using a higher number of animals reaching this age. Meanwhile, due to the availability of novel transgenic technologies, we are attempting to generate a knock-in humanized minipig in order to produce even better large animal model for HD.

The large animal HD models are the missing link between the mouse models and human patients; they may identify early dysfunctions of HD pathophysiology that could be used for future HD treatment approaches. There is an urgent need to identify specific biomarkers and to generate disease-modifying treatments that could be able to delay the HD onset or even reverse the disease progression [87, 88]. Our porcine HD model described here has already been used in preclinical testing of therapeutic strategies to reduce the amount of mHtt and thus proved to be important in this field.

Acknowledgements

This study was supported by National Sustainability Programme, project number LO1609 (Czech Ministry of Education, Youth and Sports).

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Chapter 5

Pluripotent Stem Cells to Model and Treat Huntington's Disease

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66589

Abstract

Stem cell therapies hold considerable promise for the treatment of neurodegenerative diseases. Pluripotent stem cells (PSCs) have been of particular clinical interest because of their ability to generate neuronal cells and to be used in animal models of neurodegenerative disease as well as for testing new drugs. Several PSCs isolated from humans and animals that carry the genotype of Huntington's disease (HD) have been used in aforementioned studies. HD-PSCs obtained can produce *in vitro* neural progenitor cells (NPCs). These NPCs applied in HD models show several advantages: they engraft into the brain in animal models and differentiate into neuronal cells, thus promoting behavioral recovery and motor impairment. Although progress has been made using PSCs, additional tests should be done to overcome several limitations as, for example, tumorigenicity, before their clinical application. We focus this chapter on current knowledge regarding HD-PSC lines and their helpfulness as an *in vitro* model for basic research. Next, we discuss the advances of disease-free PSCs in preclinical HD models aiming to their potential application in patients. Additionally, we discuss their potential use as a test system for anti-HD drug screening by the pharmaceutical industry, especially considering HD patients' welfare.

Keywords: Huntington's disease, neural progenitor cells, pluripotent stem cells, stem cell transplantation

1. Introduction

HD is an autosomal dominant neurodegenerative genetic disease caused by an expansion of polyglutamine (CAG) repeats in the huntingtin (HTT) protein. Clinically, HD patients present cognitive decline, motor dysfunction and psychological problems. The age of onset for these

symptoms is directly associated with the number of repeats. Pathological threshold is reached when patients present more than 36 repeats [1, 2]. Conventional therapies have no effect on HD [3–6]. Stem cells, which have amazing potential to develop into many different cell types in the body during early life, may offer new therapeutic approaches for treating HD disease [7–9]. Fetal neural grafts, neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) have already been used in several preclinical and even in preliminary clinical trials [10–14]. Other options of stem cells to be used in HD studies are embryonic stem cells (ESCs) and, especially, induced pluripotent stem cells (iPSCs), which have recently been developed in the field of human stem cells [15–19].

In devastating HD, the loss of neurons and the incapacity to mobilize inherent regenerative mechanisms to recover from progressive damage underlies the pathology and prognosis [1, 2]. Stem-cell-based therapies hold promise for the future treatment of these symptoms and to study the progress of disease. The establishment of *in vitro* cellular HD models for testing new drugs is under development and is of great importance. Furthermore, *in vitro* HD cell models help to better understand HD at the molecular and cellular levels and to identify new HD biomarkers [20–22]. Recently, NPCs have been derived from HD-iPSC [23]. The present chapter discusses PSCs use as a model study HD, and to carry out drug screening and study stem cell-based therapy in animal models of HD.

2. HD clinical aspects

HD has been reported in almost all countries and occurs in all races, equally affecting both genders. The diagnosis of HD depends on a detailed clinical evaluation and positive family history, which may be confirmed through the use of molecular genetic techniques. The average age of onset varies between 35 and 45 years, although it may manifest at any age. In about 10% of cases, the onset of symptoms occurs before 20 years of age, when patients are said to have "juvenile HD" and, in 25% of cases, the onset arises after 50 years of age—the so-called "late HD" [24]. The median survival time in HD ranges from 14 to 17 years, while it may be as long as 40 years [25]. The most frequent complaint in HD patients is a lack of "coordination" and occasional involuntary tremors in several body segments, which can usually be attributed to the presence of chorea [26]. Other early motor abnormalities include interrupted saccadic eye movements or hypometric balconies, motor impersistence of tongue protrusion and difficulty performing rapid alternating movements. Patients are described as being excessively irritable, impulsive, unstable or aggressive. The most common early symptom is mental depression. Symptoms of emotional nature or personality changes, preceding or concurrent with the onset of tic movements, are reported in half of the patients with HD [27]. A striking feature in a large number of HD patients is the intense weight loss. Cause of this weight loss is unclear. Premature aging is another obvious feature of HD [26].

3. HD genetic and pathophysiological aspects

HD is a hereditary autosomal dominant condition. The Htt gene is located on chromosome 4p16.32 and the genetic alteration is an increase in the number of repetitions of three nucleotides

http://dx.doi.org/10.5772/66589

(C, A and G) in the coding region of the first exon of the HD gene [1]. The CAG "triplet" is normally repeated about 20 times in humans, but an estimated doubling in the number of repeats (40 CAG repeats or more) results in the development of HD. Intermediate numbers of repeats, between 27 and 35, are not associated with the HD phenotype. Expansions above 36 are most frequent in the paternal lineage, due to the instability of the number of CAG repeats during spermatogenesis. The presence of 36–39 repeats is related with reduced penetrance, whereby HD may develop or not, and is considered uncertain [1]. The majority of adult onset cases have 40–50 CAG repeats, whereas expansions of 50 or more repeats generally causes the juvenile form of the disease [28, 29]. Additionally, the greater the number of CAG repeats in the Htt gene, the earlier the disease will manifest [30]. In HD, the number of CAG repeats explains many of the genetic features of this disorder, including its progression and severity.

CAG is a codon that codes for glutamine, and the mutation leads to an abnormally expanded polyglutamine tract in huntingtin [1]. In HD, the expanded polyglutamine is cleaved, resulting in an N-terminal fragment containing the polyglutamine expansion [31]. Huntingtin (HTT) is a protein of approximately 300 kDa, which is located in the cytoplasm of all somatic cells, except for neurons, where it is found in both the cytoplasm and nucleus. Normal gene function and how this mutation produces HD is still unknown, however, huntingtin is essential for life. These aggregated proteins accumulate in excess in neuron axons or dendrites and may block neurotransmitter action, impairing the normal neuronal function and leading to onset of behavioral deficits [32]. During disease progression, HTT protein aggregates accumulated in neuron cells cause cell death. Striatum and cerebral cortex are the structures that have most prominent neuronal loss. In fact, the most striking anatomopathological feature of HD is the degeneration of the basal ganglia, especially the caudate nucleus and putamen, with progressive and intense atrophy and gliosis [30].

PSCs are an important tool for HD *in vitro* studies, since these cells can model the disease, informing, for instance, the outcome of different CAG repeat numbers in HD neurons, including the possible interactions of the mutant HTT with different proteins. Conversely, PSCs can also be used *in vitro* to study the effect of different number of CAG repeats in PSCs development and ability to differentiate.

4. Mouse pluripotent embryonic stem cells: variability and heterogeneity

Pluripotency is a transient property of stem cells during early embryogenesis. It refers to an unrestricted developmental potential of the cells to give rise to all three embryonic germ layers: endoderm, ectoderm and mesoderm, and to contribute to the formation of all tissues of the developing organism. After isolation from early embryos at morulae or at blastocyst stage, PSCs are able to retain pluripotency during long-term *in vitro* cultivation [33, 34]. In mice, there are two different PSC types isolated from early embryo, which are naive and primed ESCs. Naive cells are extracted from the inner cell mass (ICM) of preimplanted embryos at day 4.5 [33, 35], while primed cells are obtained from the epiblast of postimplantation embryos around day 7 [36, 37]. Naive ESCs and primed epiblast stem cells (EpiSCs) differ in the expression levels of pluripotent key markers, such as the POU-family transcription factor Oct-4, the homeodomain DNA-binding protein Nanog and the Sox-family transcription factor Sox2 [38]. Naive female

ESCs have both X chromosomes active; in contrast, female-derived EpiSCs have only one of the X chromosomes activated. When injected into immunocompromised or syngeneic mice, both naive and primed EpiSCs are able to produce teratomas which contain the derivatives of all three germ layers [36]. Reintroduction of naive and primed EpiSCs into the mouse blastocyst leads to the formation of chimeras (animals composed by donor and recipient cells) with a high percentage of donor cell contribution, thus demonstrating their ability to participate efficiently in normal development. Only naive ESCs are able to generate germline-competent chimeras, which are able to pass on their donor cells genotype to the next generations [39–42].

5. Human pluripotent embryonic stem cells: variability and heterogeneity

In humans, so far only primed ESCs are known. They are isolated from the ICM of preimplantation human blastocysts [33]. They express several key markers of pluripotency, such as OCT4, NANOG and SOX2 [33, 43–45], and are able to generate teratomas *in vivo* [33, 46–48]. Due to ethical considerations, live chimeras cannot be obtained from hESCs. Nevertheless, efficient hESC integration into the postimplantation mouse epiblast has been shown, although, at a later stage, these cells were rapidly eliminated during embryo development probably because of the difference in cell cycle timing between the two species [49].

The production of human ESCs involves destruction of human embryos, which is of ethical concern. An alternative, the generation of iPSCs by adult somatic cell reprogramming, has been proposed. These cells are initially obtained *in vitro* using a defined cocktail of transcription factors (Oct3/4, Sox2, c-Myc and Klf), called reprogramming factors, which are able to restore pluripotency when introduced into terminally differentiated cells or into adult cells [17, 19, 20, 50]. These human iPSCs are able to produce teratomas that contain the derivatives of all three germ layers. Recent studies showed that X chromosome reactivation, an important event in cell reprogramming, occurs in hiPSCs [51, 52]. Additionally, hiPSCs are able to integrate into different anatomic sites in mouse embryos at E10.5 [52]. Both these studies suggest that, hypothetically, hiPSCs might form chimeras, thus showing the characteristic of primed PSCs.

Given the pluripotent ability of hESCs and hiPSCs, both cell types are of great interest to generate HD *in vitro* models that can be used in basic research characterizing juvenile and adult HD molecular and cellular mechanisms as well as in the pharmaceutical industry, to screen new drugs. The capacity of hiPSCs to differentiate into neural cells and produce functional neurons [53–55] has potentially great impact given the possibility of the use of these cells in cell therapy and tissue regeneration. However, due to the potential risk that these cells can derive teratomas, hiPSC application in patients is still under the investigation.

6. Isolation of pluripotent hESCs from HD embryos

Primary cell cultures from adult tissues can be obtained from HD patients. However, this is not always possible, can pose a risk for patients and there is a limited variety of tissues that can be used for cell isolation. Therefore, frequently HD cells are isolated post mortem from

tissue samples. Thus, isolation of hESCs from HD embryos was a cutting-edge discovery in HD cellular models. These hESCs with genetic disease inheritance that have unlimited proliferating and self-renewing potential are unique sources to reproduce heredity of diseases *in vitro* [56, 57]. However, only a few studies reported isolation of these cells so far [57–61].

The first derivation of hESCs from HD embryos occurred in 2005 [57]. Since then, other HD lines have been obtained from donated embryos that mainly contain 37–51 CAG repeats. These cells express the Htt gene, and mutated Htt mRNA and protein levels, and thus have the potential to model HD pathology at the cellular level. The HD-hESCs isolated so far (**Table 1**) can be considered primed hESCs according to the existing classification [43, 62]. They express core pluripotency markers and present a normal karyotype [58–61]. Only one study demonstrated that HD-hESCs are able to form teratomas [59]. *In vitro*, HD-hESCs are able to differentiate into neurons and astrocytes [58–61] through neurosphere formation by the cells positive for the neuroectodermal marker Pax6 [60]. Another study showed that HD-hESCs differentiate preferentially into astroglial cells [58]. Glial cells comprise 90% of the brains cells and provide support neuroprotective for neurons. In healthy brain, astroglial cells protect against excitotoxicity by removing excess of glutamate from the extracellular space [63]. However, in the HD brain, mutant HTT accumulates in glial nuclei and decreases the expression of glutamate transporters in neurons and atroglial cells (**Table 1**). This is an important outcome for further HD studies that investigate the effect of mutant HTT on astroglial cells and the potential therapeutic potential of these cells in HD.

HD is considered as a disease of the striatum, characterized by vulnerability to degeneration and death of the medium spiny neuron (MSN) [64]. Thus, the ability of HD-hESCs to differentiate into gamma-aminobutyric acid GABAergic MSNs, which are susceptible to neurodegeneration in HD, has also been tested. MSNs receive a massive combination of dopa-

Ref	Source	CAG repeats	Number of lineages	Pluripotent markers expressed	Teratoma formation	Formation of Htt aggregates	Neuronal differentiation
[59]	HD embryo	40–48	4	OCT4, SSEA3, SSEA4, TRA-1-60, and TRA-1-81	Positive	N/A	Neurons
[60]	HD embryo	37 and 51	2	SSEA-3, SSEA-4, Oct-4, TRA-2–39, TRA-1–60 and TRA-1–80	N/A	absent	Neurons and astrocytes
[61]	HD embryo	37 and 51	3	TRA-1-60,D9	N/A	N/A	Neurons and GABAergic neurons
[58]	HD embryo	47	1	POU5F1, SSEA3 and 4, TRA1-61 and 1-80CD9	N/A	N/A	Neurons and astrocytes

N/A, non available.
Chromosomal abnormalities were absent in all derived cell lines.

Table 1. Human ESC lines derived from HD patients.

minergic and glutamatergic inputs, which result in preferential vulnerability of these cells to the toxicity of polyQ-HTT [65]. However, only one report has shown that HD-hESCs are able to differentiate into GABAergic MSNs [61].

As discussed before, repeat size of CAG is a major determinant of the severity and pathology in HD. The longer the repeats, the more severe the symptoms [66]. After differentiation, neuronal precursor populations derived from HD-hESCs do not present any alteration in the incidence of CAG repeats [58, 60]. These findings indicate that the presence of Htt mutation does not prevent HD-hESCs from differentiating into neural cells *in vitro* [57, 59, 60], implying that HD-hESCs can be used as an *in vitro* model of HD. This model has the potential to increase the understanding of the mechanisms of neurodegeneration and can be used for efficient screening for new anti-HD drugs, selecting only the most efficient for further testing in human clinical trials.

7. Isolation of induced pluripotent stem cells from HD patients

HD-iPSCs that carry different number of huntingtin gene repeats (from 39 CAGs to 180 CAGs) have been isolated [23, 67–72] (**Table 2**). To produce HD-iPSCs, the most common original cell type isolated from HD patients is fibroblasts. Fibroblasts from HD patients show HD-related phenotypes, such as alterations in proteasome activity and altered Htt gene expression [23, 67–73]. The majority of HD-iPSCs have been generated by retroviral infection that promotes the expression of four transcription factors: Oct-4, Sox2, c-Myc and Klf4 [23, 67–69, 71, 72] (**Table 2**). After retroviral infection, HD-reprogrammed fibroblasts gain hESCs-like morphology, start to express markers of pluripotent cells, such as OCT4, NANOG, SSEA4 and alkaline phosphatase (AP) [23, 67, 68, 71, 72]; TRA-1-60 [67, 71, 72, 74], SSEA3 [67, 72]; and TRA-1-81, REX1, GDF3 and hTERT [67]. At present, the pluripotency of human HD-iPSCs is less studied when compared with that of human iPSCs derived from healthy donors. Only few studies perform the teratoma formation assay, which is essential for the characterization of the pluripotency of any reprogrammed cell. This assay is a reliable method to verify the *in vivo* differentiation potential of HD-hiPSC [71, 74, 75]. An important aspect of HD-iPSC technology is a unique possibility to study the mechanism of HD patient-specific neuronal differentiation, since HD-iPSCs are able to form neurospheres that express neuronal progenitor markers [67, 71, 72, 74]. These neurospheres are able to produce neurons, including GABAergic MSN, and glial cells [23, 68, 71, 72, 74]. Overall, these studies show that the Htt mutation and the number of CAG repeats seem not to affect neural cells fate *in vitro*, although HD *in vivo* is associated with changes in neural function and survival.

In order to use autologous HD-iPSCs therapeutically, it is critical to develop reprogramming methods that can provide a correction of the expanded Htt allele in iPSCs in these cells upon their expansion *in vitro*. A gene targeting technique has been used to achieve a correction of the expanded Htt allele in HD-iPSCs, replacing the expanded CAG repeat with 21 repeats (within the normal, non-pathological range, which varies from 6 to 34) using homologous combination [72]. The resulting cells maintain the pluripotent characteristics and can differentiate into MSNs *in vitro* and *in vivo*. This study demonstrated that non-pathological iPSCs potentially can be produced from diseased patients for stem cell replacement therapy [72].

Ref	Source	CAG repeats	Inducing method	Correct allele CAG repeat	Number of lineages	Pluripotent markers expressed	Teratoma formation	Formation of mHtt aggregates	Neuronal differentiation	Chromosomal abnormalities
[72]	HF	72	Retroviral iPSC (*OCT3/4, SOX2, KLF4, C-MYC*)	21 CAG	8	NANOG, SOX2, OCT4, SSEA4, and TRA-1-60	N/A	Absent	GABAergic neurons	Absent
[67]	HF	72	Retroviral iPSC (*OCT3/4, SOX2, KLF4, C-MYC*)	N/A	N/A	TRA-1–81, TRA-1–60, OCT4,NANOG, SSEA3, and SSEA4 REX1, GDF3, and hTERT	N/A	N/A	N/A	Absent
[74]	HF	60 and 180	Retroviral iPSC (*OCT4, SOX2, C-MYC, KLF-4*) and *LIN 28*	N/A	14	OCT4, SSEA4, or TRA-1-60, PAX6	N/A	Absent	GABAergic, neurons and astrocytes	Absent
[23]	HF	72	Not informed	N/A	N/A	OCT4, NANOG, SOX2 and SSEA4	N/A	N/A	GABAergic neurons	N/A
[68]	HF	39 to 44	Retroviral and lentiviral iPSC (*OCT4, KLF-4, SOX2, C-MYC*)	N/A	5	OCT4, SOX2	Positive	N/A	GABAergic neurons	Positive
[71]	HF	50 and 109	Retroviral iPSC (*OCT3/4, SOX2, KLF4, C-MYC*)	N/A	N/A	NANOG, OCT3/4, SSEA4 and TRA 1-60	Positive	N/A	Astrocytes neurons	N/A
[75]	MF	72	Retroviral iPSC (*OCT4, SOX2, KLF-4*)	N/A	N/A	OCT4, SSEA4, TRA1 -60, RIPS3 alkaline phosphatase	Positive	Positive	Astrocytes neurons	N/A

N/A, non available; HP, human fibroblasts; MF, monkey fibroblasts.

Table 2. Human iPSC lines derived from HD patients.

http://dx.doi.org/10.5772/66589

8. HD animal models

Models of HD recapitulate disease pathogenesis and predict response to experimental treatments. In general, there are two ways of generating animal models of HD: use of toxic/chemical pharmacological agents or of genetically modified animals [76, 77]. The majority of studies *in vivo* with NPCs from PSCs used quinolinic acid (QA)-HD models [69, 78–81].

QA can be found endogenously, where it binds and activates the *N*-methyl-D-aspartate receptor, a glutamate receptor and ion channel protein found in nerve cells. At high concentrations, QA is neurotoxic by over-exciting the same receptors, eventually leading to neuronal cell death [76]. This toxin mimics several aspects of human HD, such as extensive degeneration in striatum, death of dopamine-expressing GABAergic neurons [82, 83], weight loss [84] and motor and cognitive deficit [85]. However, motor deficit is discrete, the main motor alterations including tremor, seizures, eventual paralysis and recumbence [84]. Another aspect of the QA-HD lesions are symptoms that mimic deficits seen in early stages of HD (but not later). The lesions produce hyperactivity in animal models, but the hypoactivity that occurs later in the disease is not modeled by any dose of the toxin [86].

With the discovery of the Htt mutation in 1993, it became possible to create animal models with a similar genetic background as that found in humans with HD [1]. Hayden and colleagues used a yeast artificial chromosome these YAC vector system to express the entire human Htt gene under control of the human Htt promoter [87] YAC mouse strains contain either 72 or 128 CAG repeats. The resulting YAC mice present more hallmarks of human HD than toxic models, with a decrease in the number of GABAergic neurons in the striatum, decrease in body weight and pronounced motor deficit (ataxia, gait abnormalities, hind limb clasping) and increased nuclear *Htt* staining. Interestingly, only the YAC 128 CAG shows positive staining for inclusion bodies—a feature found in human HD—at 18 months [88].

Rodent and non-rodent studies *in vitro* and *in vivo* show the potential of these HD models, but there are limitations as to how these models may benefit patients. It is important to choose appropriate animal models according to the question under investigation. Chemical toxicity models, such as QA, are reliable to reproduce neuronal regeneration when associated with massive cells loss; however, they are not appropriate for assessment of later stages of the disease (similar to chronic). Whereas genetic animal models have similar HD symptoms as patients in later stages of the disease, thus allowing investigators to study HD progression.

9. Isolation of pluripotent stem cells from HD transgenic animals

Several PSCs have been established as *in vitro* models of HD. Somatic cells, such as fibroblasts and NPCs isolated from HD-transgenic animals (monkeys and rodents), have been reprogrammed using the Oct4, Sox2 and Klf4 transcription factors, producing HD-iPSCs. These cells preserve both the HD-related genotype and phenotype: they express mutant HTT protein and show formation of intracellular HTT protein aggregates [75, 89, 90]. In addition, PSCs have been generated using cell fusion as a tool for reprogramming: transgenic HD monkey

skin fibroblasts and wild-type non-transgenic monkey oocytes were fused and the pluripotent hybrid cells selected after fusion were found to express mutant *Htt* and to have HTT protein intracellular inclusions after the induction of *in vitro* neural differentiation [91]. These studies teach us that HD pluripotent cells can recapitulate the genotype and cellular phenotype of HD-patient cells, which is crucial for the production of cell systems that closely resemble HD. These models can then be used for the screening of anti-HD drugs in pluripotent cells and neurons derived from these cells.

10. Transplantation of hESCs and rodent ESC-derived NPCs in HD animal models

Studies have shown the therapeutic potential of hESC-derived NPCs in HD chemical rodent models (**Table 3**). NPCs have been transplanted directly into the striatum at between 10^4 and 10^6 cells per animal [79–81]. These cells were able to survive and graft into the striatum in a QA-induced HD animal model [79]. After transplantation, the cells were shown to differentiate into GABAergic MSN [80] and astrocytes [79–81]. However, the stage of NPC maturation reflects on their specification. Thus, rosette-forming NPCs are not able to differentiate *in vivo* into MSN, while striatal progenitor cells effectively generate striatal neurons [80]. The main problem in using PSCs in the clinic is the need to control neural cell proliferation, avoiding xenograft overgrowth, which may compromise postgrafting safety. Although published data suggest the beneficial action of NPCs in striatal injury regeneration, the role of hESC-derived NPCs in this process needs to be better elucidated.

Only one study so far has demonstrated an efficient recovery of motor deficit after hESC-derived NPCs transplantation in the QA rat model [81]. The animals treated with NPCs exhibited a significant behavioral improvement in the apomorphine-induced rotation test as compared to sham 3 weeks' posttransplant. None of the studies investigated long-term motor functional recovery following NPC transplantation or the possible mechanisms of therapeutic action of these cells besides differentiation [79–81]. There is no doubt that more ample and rigorous studies using chemical and transgenic animals must be performed to demonstrate the efficiency and stability of hESC-derived NPCs to promote neural tissue restoration and functional recovery of motor deficit in HD animal models.

NPCs derived from rodent ESCs have similar beneficial effects as human NPCs when transplanted into the chemical rodent model. They are able to differentiate into neurons and the animals that receive rodent NPC transplantation show rotation behavior improvement as compared with untreated animals [13, 77, 92].

11. Transplantation of hiPSCs-derived NPCs in HD animal models

The beneficial effect of hiPSC, as well as hiPSC-derived NPCs has also been tested in HD animal models aiming to the future clinical application of these cells [69, 70, 78]

Immunosuppression	Immunorejection symptom	Decreased striatal atrophy	Number of cells	Graft survival	Neuronal differentiation *in vivo*	Behavior improvements	Aggregate formation *in vivo*	Aberrant cell differentiation *in vivo*	Time course (weeks)
Cyclosporine A	N/A	N/A	1×10^5	Yes	Nestin ,MAP2, DARPP-32, Gaba	Rotation activity	Present	N/A	12 and 33
Cyclosporine A	N/A	N/A	1×10^5	Yes	Nestin ,MAP2, DARPP-32	Learning and motor activity	Absent	N/A	12
Absent	Microglia activation	reduced lesion	1×10^6	Yes	NeuN ,Darpp32,GFAP,Iba-1	Memory learning	N/A	Absent	4–6
Cyclosporine A	N/A	reduced lesion	N/A	Yes	Pax-6, NeuN, MAP2,GFAP	N/A	N/A	Present	4–8
Absent	Microglia activation	N/A	1×10^4	Yes	MAP2, NeuN, DARPP32, GFAP, MAP2, Pax6, NCAM	N/A	N/A	Present	4–6 and 13–21
Absent	N/A	N/A	1×10^4	Yes	Nestin,TuJ1,GAD6	Learning and motor activity	N/A	Absent	3

N/A, non available.
Neurotrophic action was not evaluated in any of the studies.

Table 3. HD treatment by stem cell transplantation in animal models.

(**Table 3**). Using the ipsilateral ventricular route, these cells were transplanted into both chemical [69, 78] and transgenic HD rodent models [70]. Similar to ESCs, the hiPSCs differentiate *in vivo* into neurons, including GABAergic specification neurons [69, 70, 78], and astrocytes [78]. Such transplantation caused a modest reduction in striatal neuronal atrophy, a hallmark of HD disease that starts long before the onset of motor symptoms [19, 78]. NPCs derived from iPSCs are of particular interest to be used in HD, since patients are dominated by chorea (involuntary movements) and cognitive disability that should improve by the presence of healthy neurons [20]. The ability of these transplanted cells to reverse HD symptoms in animal models was assessed using several motor and memory tests, such as the using rotarod performance test, the staircase test, the stepping test and the Morris water maze spatial memory task. They showed that experimental animals receiving iPSC-derived NPCs showed short- and medium-term functional motor improvements in different Skills, exhibiting a significantly better performance than sham group animals [69, 70, 78]. However, the long-term (<12 months) stability of such behavioral improvements still needs to be demonstrated.

12. Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) protein expression is found in the brain and the spinal cord [93, 94]. This protein promotes the survival of nerve cells (neurons) by playing a role in the growth, maturation (differentiation) and maintenance of these cells. In the brain, BDNF is active at the connections between nerve cells (synapses) where cell-to-cell communication occurs [93, 95]. The BDNF protein helps regulate synaptic plasticity, which is important in learning and memory, and is found to be expressed in regions of the brain that control eating, drinking and body weight [96–100].

The deficits in BDNF signaling contribute to the pathogenesis of several major diseases and disorders such as HD and depression [30, 101, 102]. The decrease in BDNF expression that is observed in HD impairs dopaminergic neuronal function [77], which may be associated with HD motor disturbances. In transgenic HD models, the level of BDNF in cortical tissues can be reduced to 45% of that of controls [103]. Such reduction of BDNF levels is attributed to a mutation in *Htt* which prevents BDNF transcription [104]. Additionally, BDNF transport from the cortex to striatum is decreased in HD transgenic models [105, 106].

The significant role of BDNF in neuronal HD cells is also evident *in vitro*. After removing BDNF from the cell culture medium, neurons derived from HD-iPSCs (109 and 180 CAG repeats) have a robust increase in 3/7 caspase activity and die [107]. This and many other studies indicate that BDNF is a critical factor in the pathology of HD and is a putative candidate for HD treatment [108–110]. However, it is difficult to find an ideal dose for each patient because of the variability in neurodegenerative disease manifestation between individuals. Overdoses of BDNF may induce tumor formation in the brain; on the other hand, low BDNF doses may not provide an efficient treatment.

Mouse ESCs have been genetically manipulated by use of knock-in technology and clones overexpressing BDNF-GFP have been generated. These cells differentiated into neural cells

in vitro and gave rise to an increased number of neurons as compared to control unmodified ESCs. BDNF-GFP-expressing ESC-derived neurons have a more complex dendritic morphology and differentiate into GABAergic cells more efficiently than control cells. These BDNF-GFP-expressing ESC-derived neurons show similar electrophysiological properties as cortical neurons and release BDNF in an activity-dependent manner [111].

BDNF-secreting iPSCs that were produced using a virus-free reprogramming method can differentiate into neural cells that overexpress BDNF. In this study, mice which were exposed to a stressor regimen and received BDNF-secreting iPSC-derived neural progenitors via intracerebroventricular transplantation reversed the impact of stressor challenge by subventricular zone adult neurogenesis [112].

Both of these studies demonstrate that PSCs may be used to investigate the effects of BDNF in cell transplantation in various neuropathological conditions. Indeed, neurons derived from HD-iPSCs may provide a model to study the role of BDNF secretion in HD, as well as may help to understand whether the number of repeats and the level of mutant Htt protein expression affect the production of BDNF. Furthermore, these cells can be used as a model to develop different pharmacological, genetic and cellular strategies of BDNF delivery into patients, providing potential new treatments for this orphan disease.

13. Limitations on neuronal cells derived from pluripotent stem cells in the treatment of HD

A major concern regarding cell treatment in HD is the propensity of grafted PSCs or their derived cells to form tumors [33]. Two studies showed that after transplantation of neuron progenitors derived from hESCs and iPSCs into HD animal brains, there occurred the formation of teratoma-like cell masses [79, 80]. These studies teach us that PSCs-derived NPCs can be contaminated with residual PSCs, which maintain their pluripotency and may contribute to tumor formation *in vivo*. Coincidentally, in both studies, neural progenitor stem cells expressed paired box 6 (Pax6). Pax6 is a marker of immature NSCs, which play a role in the development of human neuroectodermal tissues; this transcription factor also has an important regulatory function in cancer cell proliferation and tumor progression [113, 114].

Another disadvantage of PSC transplantation is the stimulation of the host immune system, which could lead to rejection of the cell graft [115]. The majority of *in vivo* studies which transplant human PSCs into HD animal models used immunosuppressive drugs [69, 70, 79], making these studies hard to interpret, since these drugs may relieve HD symptoms [116].

The studies conducted with the absence of immunosuppressive protocols show microglia activation in host tissues after transplantation with hESC-derived striatal and NPCs [78, 80]. Neuroinflammation, characterized by activation of microglia and astrocytes, occurs acutely after traumatic injury, and is a main factor contributing to secondary injury in the central nervous system (CNS). Thus, microglia activation can be considered an important parameter to measure the anti-inflammatory process in stem cell therapies [117]. Alternatively, a study claims that microglia activation may be indicative for an immune response, which suggests donor cell rejection [118].

http://dx.doi.org/10.5772/66589

The use of HD-iPSCs as therapeutic tools has significantly increased over the last years. However, autologous HD-iPSCs are not a good choice for stem cell-based therapy since they carry the HD mutation, which compromises such therapy [69, 119]. Thus, transplantation of HD72-iPSC-derived neural precursors, where HD72 is the number of CAG repeats, into a QA rat model showed that a long time after transplantation (33 weeks), grafted cells showed the formation of huntingtin aggregates. Furthermore, in spite of initial improvement in HD, the disease returned after 33 weeks [69]. Later, the formation of aggregates was evaluated using the same cells (HD72-iPSC-derived neural precursors) a short time after transplantation, and no evidence of aggregates was found in the mouse transgenic model. Recently, Jeon et al. [119] performed more studies and confirmed that the mutant HTT protein derived from NPCs generated from iPSC-HD is able to proliferate *in vivo* in fetal host tissue. They associated this effect to the activity of exosomes, since it has been demonstrated *in vivo* and *in vitro* that exosomes can transport mutant Htt.

Previously, it had been considered that the mutant HTT protein causes cellular dysfunction in a cell-autonomous manner that results in aggregation, inclusion body formation and cell death [120]. However, more recent publications suggested that the pathology does not occur purely at the cellular level. Observation of aggregates of mutant HTT within fetal striatal allografts in patients with HD provides strong evidence for the existence of a non-cell-autonomous mechanism of action, which accounts for the HTT protein to spread via pathological cell-cell communication [119, 121].

All studies demonstrate that HD-iPSC transplantation is a very powerful model which should be more intensively explored. More research is still needed to assess the ability of HD-iPSCs with varying number of CAG repeats to form huntingtin protein aggregates as well as to evaluate the disease pathology after short- and long-term cell transplantation.

14. Final considerations

A small number of studies have focused on isolation of HD-PSCs and their use in preclinical studies and have already shown that these cells are an appropriate *in vitro* model for studying molecular and cellular expects of HD. Interestingly, most HD-ESCs derived so far have 40–50 CAG repeats in *Htt*, a number of repeats usually associated with adult onset of HD (**Table 2**). In contrast, the majority of HD-iPSCs established to date present a variable number of CAG repeats, all ≤50 (**Table 3**), which is associated with juvenile-onset HD (prior to age 20). Furthermore, although, a subgroup of 5% of juvenile-onset HD patients have a CAG repeat number greater than 60, none of these have derived HD-iPSCs [28].

NPCs derived from ESCs and iPSCs at different stages of maturation (rosette-forming NPCs and striatal progenitor cells) have mainly been used in transplantation studies in chemical and transgenic HD animal models. However, these studies must be interpreted with care due to the limited number of animals used.

Another consideration worth mentioning regards injection route in transplantation assays. Several studies transplant NPCs via parenchymal brain injection. Although these NPCs demonstrate the ability to engraft into brain, to reduce striatum lesion and to differentiate into GABAergic neurons, such intracerebral injection route is strongly invasive, and it is

not advisable to be used in humans. Thus, other routes, for example, the intravenous route, should be explored in NPC transplantation [11, 122–124].

Additionally, though behavior improvements have been achieved after transplantation of NPCs derived from hESC and hiPSC, these improvements were observed during short- and middle-term periods (until 12 weeks), whereas long-term studies are lacking and would be more useful in reproducing sort after effects for human treatment [69, 70, 72, 81].

Some safety aspects regarding future transplantation of PSC-derived NPCs into humans need to be reevaluated. Recent studies suggest that huntingtin aggregates formed in one cell can be transmitted to neighboring cells [125]. Since PSC-derived NPCs show robust engraftment into the injury site and differentiation to neurons, the ability of huntingtin aggregates formed in the neuronal cells of HD animals to pass into donor-derived neurons should be investigated thoroughly before clinical trials are started.

The majority of studies attribute clinical benefits of PSC-derived NPCs in HD animal models mainly to robust cell graft and tissue regeneration [69, 70, 72, 79, 81]. However, previous studies that used fetal NSCs and MSCs derived from adult tissues attribute the clinical improvements observed after cell transplantation to the paracrine action and neurotrophic support provided by these cells (reviewed in [12]. In these contexts, strategies that provide neuroprotective effect for HD neurons are essential for future clinical intervention in HD. Also, recent studies carried out with NPCs show that these cells are sensitive to BDNF withdrawal *in vitro*, thus NPCs could be an appropriate model to carry out NPC-BDNF dose-response assays.

Preclinical studies which used PSC-derived NPCs in HD animal models do not present enough information to support safety and efficiency of these cells for use in humans. It should be also considered that they presented many limitations in their use in rodent models, thus justifying the delay in clinical studies with PSC-derived NPCs until better data are collected.

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Chapter 6

Transplantation in HD: Are We Transplanting the Right Cells?

Sophie V. Precious and Claire M. Kelly

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66490

Abstract

Cell replacement therapy is a viable option for the treatment of Huntington's disease (HD), where the aim is to replace the lost medium spiny projection neurons of the striatum. The intra-striatal engraftment of developing striatal precursors harvested from the foetal brain has provided proof of concept in both rodent models and human patients that these primary foetal tissue grafts can bring about a degree of functional recovery in a HD-degenerated brain. With the advent of pluripotent stem cell technologies, novel, potential alternative donor cell sources have become available. Ongoing studies are assessing the capacity of these cells to differentiate towards striatal precursors for transplantation in HD. Here, we review the characteristics of potential donor cells for HD with respect to available cell markers, functional properties and maturity of cells upon transplantation. We consider the optimal composition of the donor cell population, that is, whether a heterogeneous population containing all cell types from the developing striatum (the whole ganglionic eminence) is preferable to a more homogeneous population of striatal projection neurons, as directed by differentiation protocols applied to pluripotent stem cells. Furthermore, we consider what might be required to improve transplant efficacy and success, with respect to striatal differentiation of transplanted cells and functional improvement.

Keywords: neural transplantation, primary foetal tissue, pluripotent stem cells, striatal medium spiny neurons, DARPP-32

1. Introduction

The application of cell transplantation as a therapeutic for the neurodegenerative disease Huntington's disease (HD) offers to replace those striatal cells lost to the disease. Medium-sized spiny projection neurons (MSNs) of the striatum are the predominant cell type lost, and

it is these cells which we endeavour to replace in order to initiate reconstruction of the damaged circuitry and alleviate some of the symptoms associated with the disease. This specific and focal loss of MSNs in HD makes it an ideal candidate for cell replacement therapy.

Cells from the developing striatum (named the whole ganglionic eminence (WGE); the striatal primordia), harvested during the window of striatal neurogenesis and implanted into the HD brain, have shown beneficial effects with a degree of functional recovery in preclinical rodent studies and in 'proof-of-principle' clinical trials, see [1–3]. This indicates that intrastriatal transplantation of developing MSNs has the potential to alleviate some aspects of this disease. All clinical investigative studies of transplantation in HD have, to date, utilised human primary foetal tissue as the donor tissue [2, 3], where developing striata are harvested from multiple embryos obtained after elective termination of pregnancy. This donor tissue source has many limitations associated with it, leading to the ongoing quest to find an alternative cell source that can fulfil the requirements for successful transplantation, integration and functional improvements.

In this chapter, we will discuss the use of human primary foetal tissue, and what we know to date with respect to intra-striatal transplantation of this donor tissue source in the HD paradigm. The unanswered questions related to this donor source will be assessed, including what the optimal parameters might be for transplantation. We will consider the need for alternative donor cell sources and will look at the characteristics of potential alternative donor cell sources, and in particular, their ability to generate striatal MSNs *in vitro* and post-transplantation and the factors potentially influencing their ability to improve function following transplantation.

2. Primary foetal tissue

It is well documented that the gold standard donor cell source for neural transplantation in HD is primary foetal tissue [4], where cells are taken from the developing brain from the region of origin of the desired mature cells and within an appropriate gestational window. Striatal MSNs originate in the WGE, which is situated within the developing telencephalon, and can be harvested easily using microdissection techniques [5, 6]. This can be straightforward depending on the method of tissue collection (i.e. medical versus surgical termination of pregnancy: MTOP and STOP, respectively), CNS tissue being more accurately dissected from MTOP-derived tissue than STOP-derived tissue due to less fragmentation of MTOP tissue, thereby enabling easier identification of different regions [7]. Thus, as a source of donor cells for transplantation, there has been a progressive move to the use of MTOP rather than the much more limited supply of STOP tissue. This in part reduces, albeit to a small extent, some of the logistical burden associated with the use of foetal tissue for cell replacement therapy. However, there are unknowns and limitations associated with the use of primary foetal tissue, which will be discussed in detail later.

Initial studies of cell transplantation in HD have provided accumulative evidence of the conditions for safety and preliminary evidence for clinical efficacy. There have been seven small clinical transplantation studies reported to date, all of which have used primary foetal striatal

tissue as the donor cell source [8–14]. Safety and feasibility of bilateral intra-striatal transplantation in HD patients have been shown [8–11].

Utilising magnetic resonance imaging (MRI), the trial based in California, USA [8], indicated graft survival in all three of their transplanted patients at 1-year post-transplantation. A separate trial, based in Florida, USA, reported a decrease in the Unified Huntington's disease rating scale (UHDRS) score at 12-months post-transplantation, suggesting an improvement in motor function [11]. The *INSERM* trial, conducted in Créteil in France, provided more solid efficacy data [15]. Out of five patients with HD who received bilateral striatal implants of foetal WGE tissue, three had surviving grafts as evidenced by changes in MRI signal and increased metabolic activity in the graft regions on ^{18}F-FDG positron emission tomography (PET). The three patients with surviving grafts were reported to show substantial motor and cognitive improvements at 1-year post-transplantation, as assessed using the Core Assessment Program for Intracerebral Transplantation in Huntington's Disease (CAPIT-HD) [15]. At 6-years post-transplantation, again using the CAPIT battery, the over-riding message from this trial was the stability of the disease progression; in particular, the choreatic movements experienced by the patients remained stable, at an improved level for 4–6 years [16]. Further reports employing imaging techniques following striatal transplantation in HD have shown metabolically active tissues [14] and increased striatal D2 receptor binding with PET [12]. In addition, data from the Florence cohort of eight patients showed a degree of stabilisation or improvements in some neurological indices over 18- to 34-months post-transplantation [14], whilst the London cohort revealed some clinical improvement over 5-years post-transplantation [12].

The longest clinical follow-up assessment post-transplantation reported comes from the Cardiff-Cambridge, UK trial [17]. Data are presented for clinical outcome measures up to 10-year post-transplantation. They report a 'trend towards a slowing of progression', and although there were improvements found on certain measures for individual patients, there were no overall statistically significant improvements found in CAPIT scores between grafted patients and a non-grafted reference group. However, data obtained from PET imaging showed no obvious surviving graft tissue, and the authors postulate that the grafts were insufficiently large to produce a clinical benefit. Overall, the aforementioned trials have suggested that intra-striatal grafting is feasible and largely safe; disease progression has not been reported to accelerate in transplanted patients [10], and for patients showing no indication of graft benefits, progression of the disease appears similar to that seen in non-grafted patients [16]. These studies have also shown that human foetal striatal transplants can survive long term and can bring about functional benefits to symptomatic HD patients in at least some cases. What is less clear currently is what factors are important for producing graft-related benefit in a more reliable fashion. Potential contributing factors that need to be considered for successful primary foetal striatal transplants include gestational age of donor tissue, tissue dissection, tissue preparation, number of cells transplanted and selection of graft recipient, among others, reviewed in [18].

In addition to the clinical data discussed above, there are a number of published reports of post-mortem analyses from these transplant trials [13, 19–23]. The earliest post-mortem time was 6-month post-transplantation [13], and in this study, the authors reported graft-derived

DARPP-32 (dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32 kDa), NeuN, calretinin, somatostatin and GFAP, as well as graft innervation of host-derived tyrosine hydroxylase fibres. Markers of more immature precursors were also present, including doublecortin, Sox2 and Ki67 [13]. One patient from the Florida cohort died 18-month post-transplantation due to cardiovascular disease, and post-mortem analysis showed surviving graft tissue, which was positive for striatal markers such as acetylcholinesterase (AChE), calbindin and calretinin, as well as innervating tyrosine hydroxylase-positive processes [21]. Moreover, there was no evidence of immune rejection in the graft region or evidence that the graft was affected by the underlying disease progression [21]. Analysis of a graft from the California cohort also showed no signs of rejection or evidence of HD-related pathology in the graft [20]. However, this latter study, reporting on one patient at 10-year post-transplantation, revealed the presence of multiple mass lesions and cysts, suggestive of graft overgrowth. Additionally, although calretinin, calbindin, parvalbumin and neurofilament markers were reported, only rare neuronal projections traversing the graft-host boundary were noted [20]. One post-mortem study from the Florida trial, 10-year post-transplantation, demonstrated graft survival with expression of markers of striatal projection neurons and interneurons and evidence of synaptic connections between transplanted neurons and host-derived dopaminergic and glutamatergic neurons, but also suggested some degeneration of grafted neurons [22]. A further post-mortem analysis from the Florida cohort, up to 12-year post-transplantation, observed that there were both fewer blood vessels and fewer astrocytes in the graft compared with the surrounding host tissue, which together may result in reduced trophic support to the graft and impact on graft survival [23]. However, these grafts also showed some typical striatal graft morphology in which there were regions of the grafts that were positive for AChE, termed p-zones, as well as areas with no expression of AChE, termed non-p-zones [23].

Overall, it can be said that the data obtained from these limited numbers of transplant trials are somewhat mixed, in terms of both clinical outcomes and post-mortem analyses. It is difficult to draw any direct comparisons between data from the various studies because of the differences between studies in the protocols for tissue dissection, preparation, transplantation, immunisation and patient assessments, thus highlighting the need to undertake better controlled studies with common protocols to allow comparison of results between centres, reviewed in [3, 18].

3. What do we still need to know about human foetal WGE in order to improve graft reliability?

Although both animal research and clinical research into foetal striatal transplantation for HD span over two decades, several important issues relating to the optimal conditions for use of this tissue as a donor source of cells remain. The success of neural transplantation depends on harvesting the foetal tissue from the appropriate part of the developing CNS, at the appropriate gestational age, and for the preparation to be optimised to maximise cell viability.

The first unknown is how to optimise the dissection of the developing foetal striatum. During development, the striatum forms as two ridges in the floor of the embryonic lateral ventricles:

http://dx.doi.org/10.5772/66490

the lateral and medial ganglionic eminences (LGE and MGE, respectively). DARPP-32-positive MSNs derive predominantly from the LGE [24], whilst striatal interneurons are predominantly derived from the MGE [25]. Based on this, it has previously been proposed that deriving donor cells from the LGE, rather than WGE, would generate a purer population of MSNs and that this would produce an improved graft [26–29]. However, studies of rodent-to-rodent grafts show similar behavioural improvement in both LGE- and WGE-derived grafts, although the overall striatal graft volumes and mean numbers of striatal-like neurons were greater in the WGE-derived grafts. Thus, contrary to expectation, it is suggested that the presence of interneurons from the MGE may facilitate graft survival and integration, thus favouring a WGE-derived cell population for transplantation [29–31]. Studies of human foetal brain samples show DARPP-32-positive MSNs beginning to appear in the LGE from 7-weeks post-conception with the number increasing over the following 2 weeks [32, 33], but to date there have been no systematic studies using human foetal donor tissue in animal models to address the issue of 'optimal dissection', largely due to the scarcity of tissue.

It is known that the foetal gestational age is important in deriving donor cells that will go on to produce a functional graft, but a second unknown is the optimal foetal donor age for this purpose. In rodent studies, it has been shown that grafts derived from embryonic day (E) 14–E16 rat donors generate a higher proportion of striatal-like tissue compared with grafts derived from older embryonic tissue [30]. However, functional recovery was only seen in those recipients who received transplants from the younger E14 donors [34]. This has not been systematically investigated to date in any one, single study for human foetal samples. Thus, it is necessary to draw what we can from the published literature in which a range of ages from 6- to 14-week gestation has been used [35–38]. It has been shown that human foetal WGE cells harvested at 7- to 9-week post-conception [37] and also at 14-week post-conception [36] are able to ameliorate the apomorphine-induced deficits seen in animals having received unilateral excitotoxic striatal lesions, which mimic the pattern of cell loss seen in HD. In agreement with these earlier studies, we too see an improvement in apomorphine-induced rotations using human foetal WGE at 8-week post-conception [38]. Furthermore, improvement was also seen in the vibrissae-evoked forepaw placing test, as well as stabilisation over time in the adjusting steps test [38]. Together these studies build on the histological assessments of cell survival and integration. As described above, clinical trials have utilised tissue in the range of 6- to 12-week gestation, making this a potentially significant source of variation. Thus, despite the logistical difficulties (largely due to the uncertainties of foetal tissue availability) of undertaking comparisons of different gestational ages of human foetal WGE human to rat grafts, this is clearly a critical factor that needs to be extensively and systematically addressed.

A third factor to be considered is the way in which the foetal donor tissue is prepared prior to transplantation. Two broad approaches have been used to date: the crude chopping of the tissue into smaller pieces [8, 9, 11] and the mechanical dissociation of the tissue with the aid of enzymes [10, 12, 14]. As with the previous issues, there is again limited systematic evidence supporting either method. One study that examined this issue directly (using rodent tissue) reported a greater proportion of striatal-like tissue in conjunction with more DARPP-32 immunopositive neurons within grafts derived from dissociated cell suspensions compared

to grafts derived from tissue fragments [39]. Conversely, a modest improvement in functional recovery on the paw-reaching test was seen in animals receiving tissue fragment grafts compared to suspension grafts [39]. With current legislation pertaining to good manufacturing practice (GMP), there is a need to replicate this study using non–animal-derived products and so replacing the classical trypsin approach with GMP-compatible products.

In light of what has been discussed above, it is clear that there are many unknowns when it comes to the transplantation of human foetal striatal tissue in HD. It is critical that the questions raised above are not dismissed as new cell sources are investigated, and one key step in preclinical validation of these alternatives will be to compare them to primary foetal tissue transplants. One important consideration relates to the functional readout from such studies, especially given the limited data thus far generated from transplants of human foetal tissue [35–38]; some rodent studies have highlighted the plasticity of striatal grafts, which can have implications on functional effects post-transplantation [40–44]. It has been shown that animals post-transplantation need to 're-learn' a task that had been well established prior to induction of the lesion, using the *9-hole box* operant chamber [40]. Here, it was reported that simply reforming the circuitry in the brain was not enough to achieve functional benefit, but instead the animals needed a period of time to re-train in order to make use of the reconstructed circuitry. A similar strategy using a different task, the paw-reaching/staircase test, also showed the benefits of additional training post-transplantation [41].

Another important consideration which might enhance functional recovery in human foetal striatal transplant studies is the role for environmental enrichment which has been shown to favourably affect the behavioural readout in rodent allograft experiments [45]. Housing animals in an enriched environment post-transplantation resulted in larger projection neurons with increased spine density and better graft re-innervation [45, 46]. In addition, levels of BDNF in the intact side of the brain were increased in both transplanted and non-transplanted animals that were exposed to environmental enrichment compared with those in standard housing [43, 45, 46]. Furthermore, the impact of the enriched environment on the plasticity of striatal grafts has also been shown electrophysiologically, by measurement of long-term potentiation (LTP), which indicates persistence of synaptic strength. LTP was more readily induced in the grafts where hosts had received enrichment compared with those where hosts were in standard conditions [44, 47, 48].

In the studies of human foetal tissue transplants in animal models described above, the behavioural effects reported have been limited to drug-induced rotations, vibrissae-evoked touch test and adjusting steps test, and so far no effect has been reported on the paw-reaching test [35–38]. However, to date, neither the approach of additional training to allow transplanted animals to re-learn a task post-transplantation, and learn to use the graft, nor the environmental enrichment strategy has been applied to human foetal striatal transplant studies. One limitation of behavioural analysis in xenotransplantation studies is the restricted time window post-transplantation due to the need for daily immunosuppression. Despite the presence of DARPP-32-positive cells in the brains of these animals upon post-mortem analysis, transplanted human foetal striatal cells might require a longer time *in vivo* in order to achieve a functional readout as seen in the equivalent rodent studies. One way of overcom-

http://dx.doi.org/10.5772/66490

ing this would be to use the 'neonatal desensitisation' approach, which negates the need for daily immunosuppression [49]. However, this too has its own limitations not least the lack of understanding behind its mechanism of action.

4. Are foetal donor cells a long-term prospect?

There are a number of advantages associated with the use of human foetal striatal donor cells. The prime advantage is, as discussed above, the generation of MSNs that have been exposed to patterning signals during natural development and are thus likely to be 'authentic' MSNs with the greatest ability to bring about functional improvement in HD models and HD patients. However, there are additional advantages associated with the lineage-restricted nature of these cells, in particular, that there is a reduced risk of non-neural cells arising from the graft, and thus a much reduced risk of graft overgrowth and/or teratoma formation. These factors are why, currently, human foetal WGE cells are the 'gold standard' with which newer donor sources need to be compared. However, the continued use of human WGE cells in both animal and human studies extends beyond the simple comparison of efficacy. Given the uncertainties of the current clinical studies outlined above, there is a need for further proof-of-concept studies and to gain further insight into factors important for graft optimisation, including not only considerations of the donor cells, but also factors such as optimum host age and stage of disease. Moreover, understanding in more detail how foetal cells survive and integrate will be crucial in learning how to generate effective cells from other starting sources such as human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells.

Nevertheless, although it is important to continue to study human foetal WGE for the reasons above, it is unlikely that they will be sufficient to achieve widespread clinical application due to several ethical and logistical issues. First, the scarcity of this tissue supply is limiting. This is complicated by the requirement to use multiple donors per patient; some studies have used up to eight foetal donors per patient for a bilateral transplant, albeit that many studies have used 1–3, see [3]. It is also further limited by the need to harvest cells at the point of peak MSN neurogenesis, believed to be in the range of 8- to 12-week post-conception, thus further reducing the number of suitable, potential donor tissue retrievals. Moreover, the shift in working practices at gynaecological units means that the STOP tissue source is becoming even more rare and the MTOP tissue, due to the very nature of the procedure, can in some cases be completed in the comfort of the person's own home, thus limiting the supply being procured through hospital facilities. A second issue is that following dissection, the tissue cannot be stored for long periods of time (maximum of 8 days) [50, 51]. Therefore, coordinating the tissue collection and transplantation can be logistically challenging. The organisational network that needs to be in place in order for clinical transplants to take place is exceptionally complex, in particular, the coordination of timing of foetal tissue collection (which it is not possible to manoeuvre), with the neurosurgical procedure. Another point to be mentioned (related to the inability of this tissue to be hibernated) is that the cells cannot be subject to full screening, tissue typing, etc, as they can't be stored for long enough to complete such assessments, prior to transplantation. These considerable limitations associated with the use of primary foetal

tissue have led to the search for possible alternative donor cell sources to permit more widespread and better controlled transplant processes for the future.

5. Alternative donor cell sources

Desirable characteristics of donor cells to replace foetal WGE cells include: (i) the potential to proliferate *in vitro* whilst ensuring stability of the quality; (ii) the capacity to be expanded *in vitro* so as to generate large numbers of cells to overcome the issue of tissue supply; (iii) the ability to be stored, ideally cryopreserved, so that batches of cells with the same quality may be generated and frozen for subsequent use; (iv) having the capability to be responsive to inductive developmental cues (i.e. exogenous factors) in order to generate the target cellular phenotypes for neural transplantation (i.e. for HD, striatal MSNs); and (v) following transplantation, being able to repair the circuitry damaged in the disease process and bring about functional recovery.

Together, these desirable traits would sidestep the issue of tissue supply and the quality control caveats that come with the use of primary foetal tissue, as well as standardisation of cells for implantation. They would also circumvent the logistical hurdles with respect to retrieval of tissue for dissection and preparation, and coordinating with neurosurgical teams for implantation procedures. However, it is paramount that any alternative donor cell source be able to achieve the goal of generating the specific, authentic mature phenotype following transplantation and then differentiation and maturation *in vivo* in the adult striatum.

The catalogue of alternative donor cell sources for potential use in cell replacement strategies for neurodegenerative diseases is predominantly comprised of expandable cells that may be derived from embryonic, foetal or adult tissues and may be pluripotent, multipotent or theoretically, even unipotent.

Foetal neural precursors (FNPs) are multipotent cells, which are already restricted to a neural lineage, see [52]. Specifically, striatal FNPs are derived from foetal WGE, can be expanded *in vitro* to increase cell numbers and because of their origin of derivation may have the potential to differentiate more readily to a striatal phenotype. In terms of therapeutic application, the rationale is to expand the WGE-derived cells *in vitro* to increase the total cell population and be able to perform one, or more, complete, bilateral transplants per foetal donor.

However, assessment of human FNPs expanded *in vitro* has revealed differential gene expression between relatively early and late time points, 4–8 weeks and 20 weeks, respectively; in particular, revealing differences in genes is a key for providing information on positional identity, whilst expression of the neural markers, Nestin and Sox2, remains stable [53, 54]. Furthermore, there is a negative correlation between length of time in expansion culture conditions and yield of neurons upon subsequent differentiation; that is, with increased time *in vitro*, FNPs have a propensity to yield fewer neurons [55]. In addition to this, post-transplantation, human striatal FNPs that have been expanded in culture and undergone passaging produce fewer surviving grafts, with reduced neuronal differentiation and a lower yield of striatal neurons [53, 54, 56, 57]. Thus, it appears that *in vitro* expansion of striatal FNPs, at

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least long term, limits the differentiation potential of these cells, which could be due to a loss in positional identity over time in culture. This, in turn, would mean that when long-term expanded cells are placed in an environment such as the adult brain and are not exposed to the developmental signals that they would see in the developing brain, they are unable to differentiate into phenotypes appropriate to the site from which they were derived (e.g. MSNs from striatally derived FNPs).

On the other hand, short-term expanded striatal FNPs maintained in culture for 10 days, without passaging, yielded 41% neurons, 70% of which were immunopositive for the striatal MSN marker DARPP-32 (unpublished observations). Further to this, we have previously compared survival and axonal outgrowth of transplants of human primary foetal striatal tissue with short-term expanded (10 days) striatal FNPs, where we found richer cellular outgrowth from the FNP-derived grafts [58]. Recently, we have reported that striatal-derived FNPs expanded for short periods in culture prior to transplantation yield the same number of DARPP-32-positive neurons in grafts as those derived from primary foetal WGE [59]. Furthermore, we provided evidence to suggest that short-term expanded (2 and 9 days) striatal FNPs can bring about a degree of functional recovery, specifically on the corridor task (testing bias towards the ipsilateral side and neglect of the side contralateral to the lesion and transplant), following transplantation into an HD rat model [59]. Collectively, this indicates that FNPs, as a potential donor cell source for application in clinical transplantation, should not be overlooked, but should be further investigated to establish their true potential.

Pluripotent stem cells (PSCs) include ES cells and iPS cells, which have the capacity to generate any cell of the three germ layers: mesoderm, endoderm and ectoderm. Mouse ES cells were first identified in 1981 [60, 61], and more recently, human ES cells were also derived [62] from the inner cell mass of the blastocyst. iPS cells, derived from adult somatic tissues, were first generated in 2006, when mouse fibroblasts were re-programmed using retrovirus-mediated transfection and the transcription factors Oct3/4, Sox2, c-Myc and Klf4 [63]. Later, human iPS cells were generated from human adult fibroblasts using the same four factors [64]. This seminal paper on derivation of human iPS cells showed that these cells are similar to human ES cells with respect to proliferation capacity, pluripotency, gene expression, morphology and telomerase activity [64]. The last decade has been fruitful in the publication of research looking at pursuing PSCs (both ES and iPS cells) as potential donor cell sources for clinical application. It is imperative to remember that whatever the donor source, the cells need to be directed to a striatal MSN phenotype.

6. Directed differentiation of PSCs towards striatal MSNs

With a focus on human-directed differentiation studies of PSCs, we will discuss the development of protocols utilised in attempts to achieve striatal MSN phenotypes i*n vitro* and *in vivo*. The key identifier used in these studies for confirmation of MSN production is DARPP-32, which is expressed by more than 95% of striatal MSNs. However, it is important to note that DARPP-32 is only expressed in mature MSNs and not precursor cells. Thus, when developing protocols for the generation of such cells, particularly from PSC sources, it is vital to have

other markers available in order to determine that the differentiation progression is appropriate to the desired lineage and phenotype. Two such candidate markers, FoxP1 and CTIP2, have been identified as important markers, both of which label MSN precursor cells and co-label with mature DARPP-32 immunopositive MSNs [65, 66].

Initial studies that aimed to generate striatal MSNs applied factors to influence the neural induction of ES cells and downregulate the pluripotent and proliferative traits of these cells. Neural lineage induction of human ES cells was achieved using a variety of methods, including culture on feeder cells prior to adherence on substrate for further differentiation, feeder cell-conditioned medium and feeder-free suspension culture [67–70]. Human ES cells cultured using defined neural induction medium in free-floating suspension generated cells expressing markers of immature neural precursors such as Sox1 and Pax6 [70, 71]. Terminal differentiation of these precursor cells yielded β-III-tubulin immunopositive neurons that expressed GABA (gamma amino butyric acid; the principal neurotransmitter of striatal MSNs) after 70 days in culture [71].

More recently, a highly robust method of enhancing neural conversion of human ES cells has been developed utilising SMAD signalling inhibitors [72]. Specifically, addition of both noggin and SB431542 (a BMP inhibitor and Activin/Nodal inhibitor, respectively) was shown to increase the yield of cells expressing the neural markers Pax6, Foxg1 and Sox1, whilst expression of the pluripotent marker Oct4 decreased [72]. This method is now widely used as the first stage in the generation of neural cells from PSCs and has successfully been applied to the initial stages of striatal differentiation protocols [73–75] (discussed below, and see **Table 1**).

The process of striatal neuron generation from PSCs requires exposing the cells to various inductive stages and 'patterning' them so they may obtain the desired identities, by introducing signalling molecules indicative of regionalisation and specification, appropriate to the striatum. Following neural lineage induction, the cells need to be directed towards a striatal precursor lineage and then differentiated to generate the specific cell fate, that is, mature striatal MSNs.

Table 1 highlights studies that have reported protocols for differentiation of PSCs towards striatal neuron phenotypes, with analysis of both cultured cells *in vitro* and transplanted cells *in vivo*. The earliest report describing successful yield of DARPP-32-positive neurons from human ES cells used the mouse stromal feeder-cell method to generate neural rosettes, which were then directed towards striatal precursors and then terminally differentiated to neurons, a protocol that required more than 62 days [68]. The growth factors used in this study during the striatal patterning phase included SHH (sonic hedgehog), DKK-1 (Dickkopf) and BDNF (brain-derived neurotrophic factor), followed by dbcAMP (dibutyryl cyclic AMP), VPA (valproic acid) and BDNF for the terminal differentiation. Efficient generation of striatal MSNs *in vitro* was described, with cells expressing the striatal neuronal markers DARPP-32, GABA, calbindin and calretinin. Of the 22% of total cells that were MAP2+ neurons, 53% expressed DARPP-32. Although analysis of these cells following intra-striatal implantation into a rat excitotoxic model of HD revealed DARPP-32-positive neurons within the graft region (21% of total neurons), transplants contained cells expressing markers of persistent proliferation and showed teratoma-like overgrowth [68], thus raising issues about the efficacy of this protocol

Study	Cell lines	Protocol	DARPP-32+ neurons *in vitro*	DARPP-32+ neurons *in vivo*
			Day of analysis/ observations	**Day of transplantation/ observations**
Aubry et al. [68]	Human ES cells: SA-01 & H9	Neural induction in serum-free and N2-supplemented medium for 21–23 days; striatal patterning with addition of BDNF, SHH & DKK-1 (days 46–59); neuronal differentiation with dbcAMP, VPA & BDNF (continued > day 62)	Between days 62–72: 22% total cells were MAP2+ neurons, 53% neurons were DARPP-32+	Day 59: DARPP-32+ cells at 4–6 weeks and 13–21 weeks post-transplantation; DARPP32+cells made up 21% neurons at 13 weeks, with no difference at later time points
Ma et al. [76]	Human ES cells: H9	Neural induction in serum-free and N2-supplemented medium for 10–12 days; striatal patterning with SHH or purmorphamine (to day 26), then VPA (to day 32); neuronal differentiation with BDNF, GDNF, IGF, AA &cAMP (to day 47)	Day 47: 93% total cells were β-III-tubulin+ neurons, 90% neurons were GABA+, ~90% GABA+ were DARPP-32+	Day 40: DARPP-32+ cells at 4 months post-transplantation; DARPP-32+/GABA+ neurons were 58% total graft-derived cells
Delli-Carri et al. [73]	Human ES cells: H9 & HS401 Human iPS cells: DF3F & WT iPS 3F-1	Neural induction in serum-free medium with increasing concentration of N2-supplementation and addition of dorsomorphin, noggin, SB431542; addition of SHH & DKK-1; neuronal differentiation with N2, B27 & BDNF	Day 45: 80% of cells were β-III-tubulin+ neurons, majority were GABA+ some of which were DARPP-32+. Day 80: 51% total cells were Map2ab+ neurons, 20% neurons were DARPP-32+	Day 38: DARPP-32+ cells at 9 weeks post-transplantation were 0.05% of total graft-derived cells
Nicoleau et al. [74]	Human ES cells: H9 & RC9 Human iPS cells: I90c17	Neural induction with LDN (or noggin) & SB431542 for 10 days; further 10 days in N2/B27 medium; neuronal differentiation with BDNF, dbcAMP & VPA; (also tested addition of SHH or cyclopamine, and Wnt3a, DKK-1 or XAV-939)	Day 20 differentiated for a further 25 days: DARPP-32+ neurons present (optimal with 1μm XAV) Longer term for >60 days: DARPP-32+ neurons, with 23 fold more expression at day 60 than day 10	Day 25: DARPP-32+ neurons extensive throughout grafts 5 months post-transplantation
Arber et al. [75]	Human ES cells: H1 & H7 Human iPS cells: 2F8 & 4FH	Neural induction in N2/B27 medium with SB431542 (up to day 5), LDN (or noggin) & dorsomorphin (up to day 9); addition of Activin A from day 9 (to day 20); terminal differentiation with BDNF & GDNF	Day 36–40: DARPP-32+ neurons, QPCR,5 fold increase with Activin treatment than without; ICC,20–50% DARPP-32+ (depending on cell line)	Day 20: DARPP-32+ cells 8 weeks post-transplantation (very few); 16 weeks post-transplantation, 49% HuNu+ cells were DARPP-32+

Abbreviations: BDNF, brain-derived neurotrophic factor; SHH, Sonic Hedgehog; DKK-1, Dickkopf; dbcAMP, dibutyryl cyclic AMP; VPA, valproic acid; GDNF, glial-derived neurotrophic factor; IGF, insulin growth factor; AA, ascorbic acid; FGF, fibroblast growth factor; XAV-939, chemical antagonist of Wnt/β-catenin pathway (substitute for DKK-1) (Wnt inhibitor molecule); QPCR, semi-quantitative real-time polymerase chain reaction analysis; ICC, immunocytochemistry; HuNu, human nuclei; DARPP-32, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32 kDa; GABA, gamma amino butyric acid.

Table 1. Studies reporting *in vitro* and *in vivo* analysis of striatal differentiation protocols with human pluripotent stem cells.

and highlighting the importance of *in vivo* verification post *in vitro* differentiation. A shortened neural induction period for generation of neuroepithelial cells followed by addition of SHH or purmorphamine (a SHH agonist), then VPA and finally BDNF, GDNF (glial-derived neurotrophic factor), IGF (insulin growth factor), ascorbic acid and cAMP yielded striatal neurons after 47 days [76]. Specifically, of the β-III-tubulin-positive neurons (93% of total cells), 90% expressed GABA, and 90% of these GABAergic neurons expressed DARPP-32 [76]. DARPP-32 immunopositive cells were observed *in vivo*, at 4 months after transplantation into an excitotoxic lesioned mouse model, with DARPP-32-positive/GABA-positive neurons making up 58% of total grafted cells. Behavioural analysis revealed improvements in transplanted mice on various tests including the rotarod test of motor control, open-field measures such as crossings and total distance moved, and increased stride length on the Treadscan test for gait analysis. Again, it is important to note that whilst the authors claim no graft overgrowth, the number of total cells in the grafts at the time of analysis was greater than 3 million, whilst only 100,000 cells were transplanted. Taken together, these data showed the successful generation of MSNs that had the capacity to alleviate some of the locomotor deficits seen in this model of HD. Reproduction of this effect has not been reported, despite numerous attempts by others, and so caution must be taken in the interpretation of these findings.

The first report of striatal differentiation from PSCs that utilised the dual SMAD protocol for neural induction, previously mentioned [72], used SB431542, noggin and dorsomorphin, for the initial neural induction phase, with subsequent addition of SHH and DKK-1, and later BDNF [73]. After 45 days *in vitro*, GABA-positive/DARPP-32-positive cells were observed, and by day 80, 20% of Map2ab+ neurons (51% of total cells) expressed DARPP-32. Following transplantation into a lesioned rat striatum, a modest functional improvement was reported (on an apomorphine-induced rotation test), as early as 3-week post-transplantation, which was maintained at 6 weeks, but fell short of significance at 9-week post-transplantation. Additionally, FoxP1-positive cells and a small number of graft-derived DARPP-32-positive neurons were seen [73]. A total of 4 million cells per graft were reported at 9-week post-transplantation (following transplantation of 500,000 cells), with approximately 2000 DARPP-32-positive cells per graft (0.05%). Again, caution must be conveyed in interpreting these data as the number of animals was low for confirmation of a significant effect, and with respect to the *in vivo* DARPP-32 staining presented, the numbers seen were very low.

Another study that employed the dual SMAD inhibition protocol using SB431542 and LDN or noggin also looked at the effects of addition of SHH or cyclopamine (a SHH antagonist), and Wnt3a, DKK-1 or XAV-939 (the latter two being Wnt pathway inhibitors) [74]. Striatal neurons expressing DARPP-32, calbindin and calretinin were yielded after 45 days in culture, with a combination of XAV-939 (1 μM) or DKK-1 (100 ng/ml), and SHH (50 ng/ml) resulting in optimal numbers of DARPP-32 immunopositive cells (~25% of MAP2-positive neurons). With longer time periods in culture (>60 days), increased expression of DARPP-32 was observed, as well as expression of other striatal neuron markers including CTIP2, dopamine receptors D1 and D2, calbindin and substance P [74]. Analysis of these cells at 5-month post-transplantation showed expression of DARPP-32-positive neurons throughout grafts that co-expressed FoxP1 and CTIP2. In addition, grafts were seen to take over most of the host striatum, although

http://dx.doi.org/10.5772/66490

assessment of proliferative markers, markers of cells from different lineages or total cell numbers were not reported [74].

A novel striatal conversion protocol, still utilising dual SMAD inhibition for the initial neural induction phase, but introducing Activin A in the patterning stage and reporting the redundance of SHH, resulted in DARPP-32-positive neurons after 36–40 days *in vitro* [75]. The numbers of DARPP-32-positive neurons yielded *in vitro* ranged from 20 to 50%, depending on the PSC line being assessed, and expression of striatal markers calbindin and CTIP2 was also reported. Although no functional effect was seen *in vivo* after transplantation of these cells, the striatal neuronal differentiation reported was considerably better than in previous studies [75]. Most graft-derived cells expressed GABA, and DARPP-32-positive neurons comprised 49% of total graft-derived cells. Other striatal phenotypes observed were calbindin, FoxP2, dopamine receptor D2 and substance P. The proliferative marker, Ki-67, was detected at 4-week post-transplantation, but was absent from grafts at 16-week post-transplantation. Furthermore, the largest graft comprised approximately 500,000 cells (determined by assessment of human nuclei immunostaining), at 16-week post-transplantation, following engraftment of 400,000 cells per graft [75].

The characteristics of human-derived MSNs at an electrophysiological level are not well described. We have previously used calcium imaging analysis to look at neuronal differentiation and functional cellular activity of primary human foetal-derived MSNs [7]. Exposing the *in vitro* differentiated neurons to various stimuli and neurotransmitter applications resulted in rises in intracellular calcium concentration. Stimuli used included GABA, NMDA, AMPA, kainate, L-glutamate, all of which are indicators of striatal function. A noteworthy finding is that observed following application of GABA, which showed an increase in intracellular calcium and therefore demonstrated a voltage-activated calcium influx in response to a degree of depolarisation. This is indicative of a foetal phenotype, rather than adult. Thus, this study showed that even after 24 days *in vitro*, the differentiated neurons still exhibited a foetal phenotype [7]. Apart from this preliminary analysis, little is known about the functional capacity of human-derived MSNs *in vitro*.

In comparison, some of the studies described above that generated MSNs from human PSCs have progressed further in understanding such characteristics. ES cell-derived neurons were reported to be mature and functional after 4 weeks in culture [71]. In this study, where there was no specific patterning towards striatal cell fates, neurons exhibited whole-cell currents including fast, voltage-activated and rapidly inactivating inward currents followed by slowly activated but sustained outward currents, and when stimulated generated action potentials. When differentiation of PSCs was directed towards a striatal MSN phenotype, generation of functional striatal neurons from PSCs has been confirmed [73, 75, 76]. Specifically, generation of GABAergic neurons was confirmed by stimulation with a high-potassium solution and subsequent measurement of the levels of GABA released, which showed that these cells produced a significantly greater amount of GABA than GABA interneurons [76]. In addition, these cells had the potential to generate action potentials following whole-cell patch clamping. Striatal neurons derived using protocols combining the dual SMAD inhibition method for neural induction followed by striatal patterning were shown to have the capacity

to function in a network, forming synapses and showing responsiveness to GABAergic and dopaminergic stimulation [73]. Furthermore, PSC-derived MSNs showed the ability to form GABAergic synapses and exhibited responses to a stimulus and delayed action potential firing typical of striatal MSNs [75]. These are crucial steps in validating the potential of these cells for use in transplantation.

One issue with the use of PSCs as donor cell sources is the exclusivity, initially with respect to neuralisation, and later 'striatalisation', of the 'induction-patterning-differentiation' protocols applied. Thus, this begs the questions: 'How heterogeneous is the resultant population with respect to cell types of other, perhaps, unwanted lineages?' and 'How much of a problem is this?' Certainly, the continued presence of undifferentiated PSCs and/or unrestrained proliferative cells in the culture system immediately prior to engraftment makes these cells less attractive as a prospect for transplantation due to the potential risk of uncontrolled overgrowth and even generation of teratomas.

It is clear that the directed differentiation of PSCs *in vitro* can yield functional striatal MSNs, albeit with varied times in culture and application of different signalling combinations at the different stages of the differentiation process. Transplantation of PSCs into animal models of HD has met with mixed fortunes with the majority of studies exhibiting overgrowth and teratoma formation in the host brain post-transplantation, thus emphasizing the importance of *in vivo* validation of *in vitro* cell generation. Identifying a more refined growth factor/signalling molecule cocktail may, perhaps, be a necessary prelude to using these cells for clinical transplantation application. This work is actively ongoing in many laboratories and forms a key part of the European funded 'Repair HD' consortium. What is lacking at this point is a detailed understanding of the potential of human foetal-derived MSNs to function, as the analysis to date is limited.

7. Concluding remarks

We have discussed here the current status of neural transplantation in HD and considered the promise shown by clinical trial data, which have provided proof of principle that the approach works in many cases. However, it is evident that there is still a long way to go, and the challenge for generation of successful, efficacious, reproducible transplants is still large. We have highlighted the importance of assessing functional readouts of grafts and not relying solely on histological assessments. Equally, with potential alternative donor cells, it is critical to undertake *in vivo* assessments of cells differentiated from these sources, understanding that the *in vitro* data are just a prelude to the necessary *in vivo* analysis.

Furthermore, we highlighted the limited preclinical data with respect to human-to-rodent investigations, which would advance our understanding of transplanted striatal MSNs derived from both human primary foetal tissue and PSCs. In addition, we see the requirement for future transplant experiments to seek to incorporate neurorehabilitation post-transplantation, in the form of training the graft and also environmental enrichment, as this may well impact on the findings pertaining to the donor cell source.

http://dx.doi.org/10.5772/66490

It appears very probable that an expandable donor cell source will be utilised in future clinical transplant trials, and we have discussed here reports of directed differentiation of such sources to MSNs, albeit with varying degrees of success. However, it is important to continue to gain understanding of human primary foetal striatal cells, including aspects of their development, physiological assessments both *in vitro* and *in vivo*, and their ability to generate effective transplants, restoring functional deficits seen in different HD models. This will be the foundation against which all possible alternatives should be compared as part of any preclinical validation.

Acknowledgements

The authors are very grateful to Anne Rosser for guidance and feedback during the preparation of this chapter.

Abbreviations

HD	Huntington's disease
MSN	Medium-sized spiny neurons
WGE	Whole ganglionic eminence
MTOP	Medical termination of pregnancy
STOP	Surgical termination of pregnancy
MRI	Magnetic resonance imaging
UHDRS	Unified HD rating scale
PET	Positron emission tomography
CAPIT-HD	Core Assessment Program for Intracerebral Transplantation in Huntington's Disease
AChE	Acetylcholinesterase
DARPP-32	Dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32 kDa
ES	Embryonic stem
iPS	Induced pluripotent stem
PSC	Pluripotent stem cell
LTP	Long-term potentiation
GABA	Gamma amino butyric acid
SHH	Sonic hedgehog
DKK-1	Dickkopf
BDNF	Brain-derived neurotrophic factor
GDNF	Glial-derived neurotrophic factor
VPA	Valproic acid
dbcAMP	Dibutyryl cyclic AMP
IGF	Insulin growth factor
NMDA	N-methyl-D-aspartic acid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

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Edited by Nagehan Ersoy Tunalı

Huntington's disease is a progressive neurodegenerative disorder of the brain. It is one of the quite devastating and currently incurable human conditions. Degeneration of specific types of neurons in the brain results in a triad of clinical features: serious behavioral disturbances, uncontrolled movements of body parts, and deterioration of intellectual capabilities. The underlying complex mechanisms and molecular players of the cellular cascades still need to be deciphered in detail despite considerable advances. Once solved, the related molecular mechanisms will not only enlighten the HD story but will also shed light on other polyglutamine diseases and similar brain disorders. This book, Huntington's Disease-Molecular Pathogenesis and Current Models, is planned to cover recent scientific achievements in understanding the cellular mechanisms of HD. The chapters provide comprehensive description of the key issues in HD research. In this regard, this book will serve as a source for clinicians and researchers in the field and also for life science readers in increasing their understanding and awareness of the clinical correlates, genetic aspects, neuropathological findings, and potential therapeutic interventions related to HD.

ISBN 978-953-51-4897-5

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