# DO COGNITIVE PROBLEMS START BEFORE MOTOR DYSFUNCTIONS IN ANIMAL MODELS FOR HUNTINGTON DISEASE

***Dissertation***

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## Declaration of original authorship

I hereby declare and confirm that the work presented in this thesis is entirely the result of my own original research, except where otherwise indicated. I have given references for all sources of information that are not my own, including words, ideas and pictures. I have appropriately acknowledged any assistance I have received in addition to that provided by my supervisors.

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# ABBREVIATIONS

**3 NP** = 3-nitropropionic acid
**ANOVA** = Analysis Of Variance
**ASO** = Anti Sense Oligonucleotides
**ATP** = Adenosine Triphosphate
**BAC** = Bacterial Artificial Chromosome
**B.O.S =** Base of support
**C-A-G, C-A-A** = Cytosine – Arginine – Guanosine (Glutamic acid codons)
**CRH** = Corticotrophin Releasing Hormone
**CS** = Conditionned Stimulus
**CVLT** = California Verbal Learning Test
**DRPLA** = Dentatorubropallidollusysian Atrophy
**EDS** = Extra Dimensional Shift
**EPM** = Elevated Plus Maze
**ESCs** = Embryonic Stem Cells
**IGF-1** = Insulin-like Growth Factor 1
**iPSCs** = induced Pluripotent Stem Cells
**EZM** = Elevated Zero Maze
**fl-mHTT** = full length mutated huntingtin
**fMRI** = functional Magnetic Resonance Imaging
**GABA** = Gama Amino Butyric Acid
**HAP-1**: Huntingtin Associated Protein 1
**HD** = Huntington Disease
**Hdh** = Huntington disease homologue
**HSG** = Huntington Study Group
**HTT** = huntingtin (gene)
**htt** = huntingtin (protein)
**IT** = interesting transcript
**kDa** = kilo Dalton
**mHTT** = mutant huntingtin

**MMSE** = Mini Mental State Examination

**MSN** = Medium Spiny Nerves

**MWM** = Morris Water Maze

**NMDA** = N-Methyl-D-Aspartate

**OCD** = Obsessive Compulsive Disorder

**OLT** = Object Location Test

**ORT** = Object Recognition test

**PolyQ** = polyglutamine

**PPI** = Prepulse Inhibition

**RAVLT** = Rey Auditory Verbal Learning Test

**SBMA** = Spinobulbar Muscular Atrophy

**SDMT** = Symbol Digit Modalities Test

**SCA** = Spinocerebellar Ataxia

**TBZ** = Tetrabenazine

**TG** = Transgenic

**UHDRS** = Unified Huntington’s Disease Rating Scale

**US** = Unconditioned Stimulus

**WT** = Wild Type

**WCST** = Wisconsin Card Sorting Test

**WMS** = Wechsler Memory Scale

**YAC** = Yeast Artificial Chromosome

# I. General introduction

# General Introduction

## A. Huntington disease

Huntington disease (HD) or Huntington chorea is an inheriting neurodegenerative disorder leading to multiple symptoms, like choreic movements and psychiatric disturbances. HD includes a triad of motor, cognitive and psychiatric symptoms. In general, clinical symptoms appear in patients between the age of 30 and 45 and death occurs 15-20 years later. The term "Chorea" is derived from Greek word χορεία (choreia) for dance and has been used in middle ages to describe the jerky, involuntary muscle movements of the face and extremities. In the 17$^{th}$ century it was used to describe disorders such as Sydenham's chorea that occurs in children, or chorea *Sancti Viti* (Wood, 1816; Stewart, 1953). However, HD is different from these choreas, although descriptions of uncontrolled motor movements in these disorders are similar to those observed in Huntington chorea. The full range of the inheriting chorea symptoms remained vague until the exact description by George Huntington in 1872.

### 1. *George Huntington and the hereditary chorea history*

George Huntington (1850-1916), was the son of Dr. George Lee Huntington and Mary Hoogland.[1] He saw the first cases of the hereditary disorder at the age of eight, when he accompanied his father on his rounds of visiting patients from Easthampton to Amagansett. The impact of the disease upon two women, mother and daughter, both bowing, twisting, grimacing, was such that his "interest in the disease has never wholly ceased" (DeJong, 1953). After his graduation in 1871 from the college of Physicians and Surgeons (Columbia University, New York), he took particular interest in the previously treated chorea cases by his father and grandfather. He studied the clinical notes and the medical history of several generations, and wrote a clinical treatise

[1] For detailed information about George Huntington life and factors that influenced him to pursue this career, refer to Durbach and Hayden, 1993.

entitled “On chorea”. He presented his assay before the Meigs and Mason Academy of Medicine at Middleport, Ohio, February 15, 1872. Three specific marks of the disease were described*: 1-Its hereditary nature; 2- A tendency to insanity and suicide; and 3- It’s manifesting itself as a grave disease only in adult life* (Huntington, 1872).

All the features of the disease with the fatal outcome and mental impairment could be recognized through the assay. Sir William Osler, called the ‘father of modern medicine’, acknowledged his work in these words: *“In the history of medicine, there are few instances in which a disease has been more accurately, more graphically, or more briefly described”* (Osler, 1908).

Although George Huntington did not pursue any further research, he has undoubtedly made a profound contribution to medical research in isolating and describing with accuracy this form of chorea that now bear his name. Since then, all the works over the next century were mainly focused on giving more details about the symptoms with a keen desire to understand the pathological and genetic aspects of the disease.

## **2.** *Cause, prevalence and diagnosis of HD*

In the description of the hereditary transmission of the disease, George Huntington indicated that a person who did not develop the illness during its entire life could not transmit it to subsequent generation. This introduces the basic Mendelian dominant inheritance pattern of the disease.

The HD gene is the first autosomal defect mapped and was localized on the short arm of chromosome 4 (4p16.3) (fig. 1; Gusella et al.1983). A decade later, the mutated protein and the gene Huntingtin (HTT) interesting transcript locus15 (IT15), containing the unstable C-A-G trinucleotide repeat, was isolated. This was possible through the Venezuela project, where most cases of HD were identified (HD collaborative research group 1993). HTT codes for 3144 amino acids and the protein, of approximately 340 kDa, is ubiquitously expressed. The huntingtin (*htt)* protein is cytoplasmic and has many functions in the cell. Although its normal function is not completely understood, many reports suggest it has a role in vesicle trafficking (exocytosis and endocytosis) and that it is essential during development (Caviston and Holzbaur, 2009; Cisbani and Cicchetti, 2012; Trushina et al. 2004; Zuccato et al. 2001).

The disease is caused by an expansion of C-A-G repeats located within exon 1 of the HTT gene. This mutation translates into a polyglutamine (polyQ) stretch and becomes pathological when the critical length of 35 ± 2 C-A-G residues is exceeded.

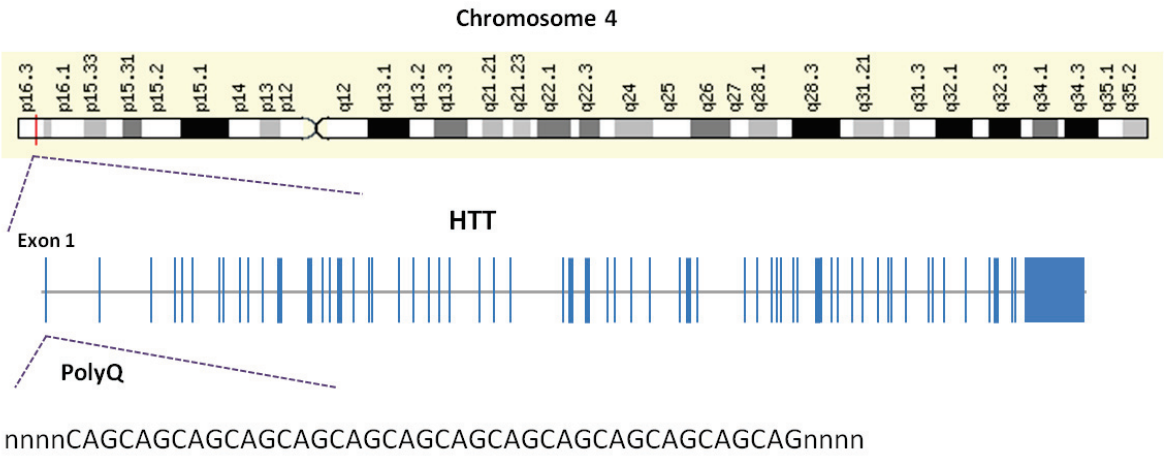


**Fig. 1** Chromosome 4 map and the huntingtin (HTT) gene. The HTT gene is located on the short arm of chromosome 4 at position 16.3. The HTT locus spans 180 kb and consists of 67 exons. The instable polyQ stretch is located in Exon 1 (Goldfrank et al. 2003; http://www.genecards.org/cgi-bin/carddisp.pl?gene=HTT).

HD is one of the nine neurodegenerative disorders (the others are: DRPLA, SBMA, SCA1, SCA2, SCA3, SCA6, SCA7 and SCA17) that have been identified and shown to possess the same type of mutation (i.e. expansions of the trinucleotide C-A-G repeats) in coding regions of their respective genes (Teive, 2009). However, the mutation in the HTT gene results in a selective atrophy and loss of pyramidal neurons in the cerebral cortex and medium spiny neurons (MSN) in the striatum (Vonsattel et al. 1985). The mechanisms by which the mutation in HTT causes neurodegeneration are not yet well understood. Postulated mechanisms are, amongst others, massive dysregulation of glutamate and dopamine signalling pathways promoting cytosolic and mitochondrial $Ca^{2+}$ overload and transcriptional dysregulation mediating a decrease in BDNF mRNA level (Bezprozvanny and Hayden, 2004; Ehrlich, 2012; Tang et al. 2007).

The disease affects males as well as females. Therefore, since the disease is transmitted in a dominant manner, each child of a person with the mutation has a 50% chance of inheriting the fatal gene, and will develop the disease unless he/she dies before it presents itself. The disease manifestation or age of onset in a person is inversely correlated with the glutamine tract expansion (Fig 2; Gusella and MacDonald, 2009).

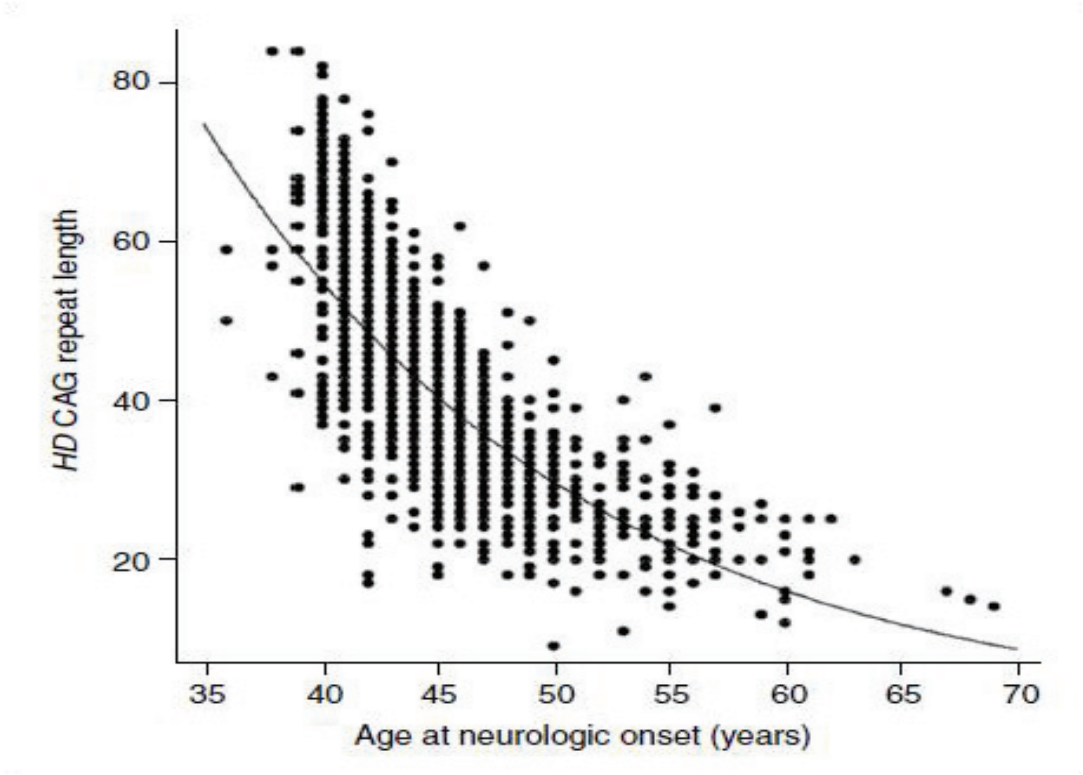


**Fig. 2** Relation between CAG repeats length and age of disease onset. The plotted data points are from 1200 HD subjects. The longer the CAG track is, the earlier the disease manifestations occurs (Gusella and MacDonald, 2009).

HD is relatively rare and appears to occur more frequently in the white race. Geographic differences in prevalence estimations have been reported (Fig.3; Warby et al. 2011). In general, the prevalence rate in Western Europe is between 3 and 7 persons per 100,000 and 3 to 4 persons per 100,000 in the Middle East (Scrimgeour, 2009). A recent meta-analysis conducted on 11 studies from Europe, North American and Austria, reported a prevalence of 5.70 HD cases per 100,000; whereas the prevalence

reported for Asia from 3 studies was much lower: 0.40 cases per 100,000 (Pringsheim et al, 2012). One explanation of the large geographic difference in HD prevalence between Europe and Asia resides in HD chromosome haplogroups; in fact, there are 3 haplogroups (A, B, C) and the high risk HTT haplotypes (A1 and A2) are absent from Asian HD population (Warby et al.2011).

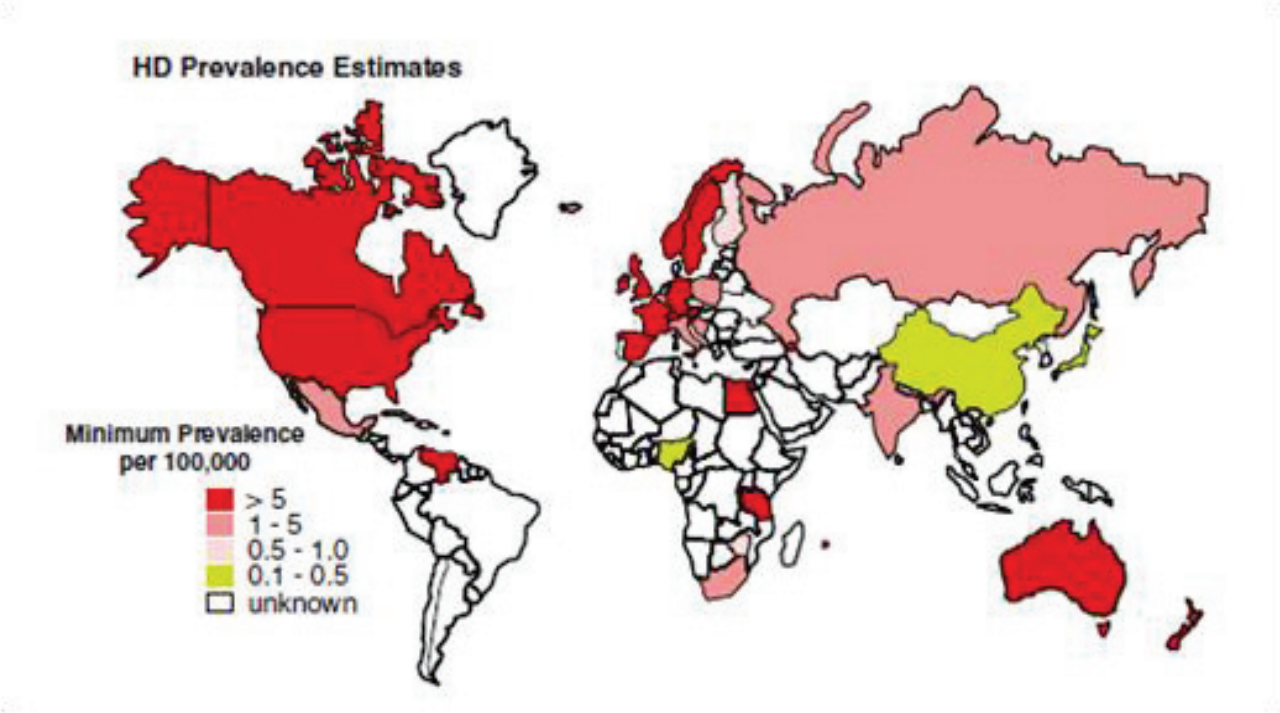


**Fig. 3** Worldwide prevalence estimation for HD (Warby et al. 2011). Note that in Africa studies were conducted in small sample sizes. An existing HD like-2 phenotype - similar to HD but not a polyglutamine disease - may lead to an overestimation of the prevalence in some regions (Margolis et al. 2005).

Sophisticated genetical approaches are used today to determine the number of polyglutamine repeats present in the HTT gene and predict whether a person will suffer from HD later in life. Analysis of DNA extracted from a blood sample is sufficient (Williams et al. 1999; Myers, 2004). The requirements for a full diagnosis also include a medical examination, familial history research, and brain imaging testing (fMRI). In general, physical examinations are combined with psychological tests in adults to determine whether the disease has begun.

### *3. Clinical assessment of HD symptoms*

Affected people can show the symptoms of HD at any age (juvenile versus adult HD onset). Most people develop symptoms between the age of 35 and 55 years; the disease manifests itself by progressive motor dysfunction, psychiatric disorders and cognitive symptoms, and this triad of symptoms has a severe effect on activities of daily living (Rosenblatt et al. 2011; *A.D.A.M. Medical Encyclopedia*, 2011).

Robust and sensitive clinical tools that can enable quantitative measurement and detect changes within individuals are essential in order to address effective treatments. A number of tests are in use worldwide. In 1996, an international Huntington Study Group (HSG) developed a comprehensive and reliable instrument to assess the clinical features of HD, named: the Unified Huntington's disease Rating Scale or UHDRS (HSG, 1996).*The UHDRS* is a collection of questionnaires allowing comprehensive clinical rating of HD severity. Four main domains are assessed: motor function, cognitive function, behavioral abnormalities, and functional capacity. A summary is given in Table1.

- *Motor symptoms*

Movement disabilities can be easily identified, but initially may be subtle, mild or limited to extremities. The UHDRS covers all the main motor signs in HD; higher scores often correlate with the later stages of the disease and indicate that motor disorders worsen over time. Choreic movements occur as rapid, irregular and arrhythmic complex involuntary movements. In general, HD patient's movements often begin normally, but then become jerky and irregular (Smith et al. 2000).

Other assays can also be useful to evaluate (sensori) motor abnormalities. Video motion analysis is often used to address motor abnormalities in HD. The advantage of using video recording is that spatial and temporal parameters can be assessed. Video recordings allow detection of specific gait abnormalities in patients such as delays in movement initiation and difficulties in executing movements. This technology has revealed akinesia, bradykinesia, reduction in stride length, in speed and cadence in

manifest HD. Finally, gait abnormalities with hyper and hypokinesia lead to excessive trunk sway and falls in HD (Delval et al. 2006; Georgiou et al. 1995; Grimbergen et al. 2008; Koller and Timble, 1985). For the measurement of sensorimotor gating, prepulse inhibition of acoustic and tactile startle reflex (PPI) can be used. In PPI, a weak prepulse inhibits a reflex response to a powerful sensory stimulus. A deficit in PPI has been reported in HD and other psychiatric disorders such as schizophrenia and OCD (obsessive compulsive disorder). A deficit in PPI can indicates that choreic movements may involve a loss of motor inhibition by striatal GABA projections and abnormalities in cortical-striatal pallidal-pontine circuitry (Ahmari et al. 2012; Kohl et al. 2013; Swerdlow et al. 1995).

| MOTOR | COGNITIVE | BEHAVIORAL | FUNCTIONAL CAPACITY | FUNCTIONAL ASSESSMENT | INDEPENDENCE SCALE |
|---|---|---|---|---|---|
| Ocular pursuit | Verbal fluency test | Sad/Mood | Occupation | Yes or No questions related to daily living obligations: | Level of subject's independence. Only 0 or 5 selections are acceptable from a list of 10 items: |
| Saccade initiation | | Low self-esteem/Guilt | Finances | | |
| Saccade velocity | Symbol digit modalities test | Anxiety | Domestic chores | | |
| Dysarthria | | Suicidal thoughts | ADL | employment, | 10→« no special care needed » |
| Tongue protrusion | Stroop Interference test (color, word) | Disruptive or Aggressive behavior | Care level | work, | |
| Maximal dystonia | | Irritable behavior | | finances, | to |
| Maximal chorea | | Obsessions | | transaction, | 1→« Tube fed, total bed care » |
| Retropulsion pull test | | Compulsions | | car driving, | |
| Finger taps | | Delusions | | shoping, | |
| Pronate/supinate Hands | | Hallucinations | | take care of children, | |
| Luria | | | | self care … | |
| Rigidity-Arms | | | | | |
| Bradykinesia-body | | | | | |
| Gait | | | | | |
| Tandem walking | | | | | |

**Table 1. UHDRS**. This clinical test consists of a collection of scales and questionnaires which allow comprehensive rating of HD severity (source: HSG, 1996).

- *Neuropsychiatric symptoms*

Emotional symptoms

The most frequently reported emotional symptoms are depressed mood, anxiety, perseverative preoccupations, irritability and apathy. Apathy is thought to be positively related to the disease progression and can be use as an end-point in clinical trials (Hamilton et al. 2003; Thompson et al. 2012).

Cognitive symptoms

Early and late stages of HD patients have a wide range of impairments in executive and mnemonic functions (Lawrence et al. 1996, 1998, 1999, 2000). Patients showing impairment in executive functions have difficulty with tasks that demand to plan, organize and shift cognitive set.

The cognitive tests listed in table 1 (UHDRS) have remarkable sensitivity to detect different aspects of brain damage or changes in cognitive functioning:

The Stroop color word test for example, consists of reading words, naming colors and then naming the color of ink of the words that describe colors. The score is the number of correctly identified items within a short period of time (less than 45s) for each condition. For the verbal fluency test, participants have to say as many words as possible of a given category in a short period of time (usually 60s). Finally, in the symbol digit modalities test (SDMT), the examinee has a short period of time (90s) to pair specific numbers with given geometric figures. The latter has the advantage to be culture free, and can be administered to people with speech disorder or linguistics.

In addition to those described above, other tests are used to evaluate executive functions. Among them are: the mini mental state examination (MMSE) scale and the Tower of London task which assess respectively dementia, planning and problem-solving; the Wisconsin card sorting test (WCST), the attentional set-shifting (EDS) task, the pattern and spatial recognition tasks are told to be dedicated for Visual retention, memory flexibility, perceptual interference, information processing speed, memory efficiency and recall for logical memory assessments (Lange et al. 1995; Lawrence et

al. 1996, 1998, 1999). We will describe the pattern and spatial recognition tasks procedure in more detail, since similar tests exist in animal research and are therefore relevant from a translational point of view:

The pattern recognition task consists of presenting subjects with a series of 12 abstract patterns that they have to remember. Following a delay of 5s, the 12 patterns are re-presented in reverse order, paired with a novel pattern. Subjects are then asked to touch the pattern they had seen previously.
In the spatial recognition task, five squares are presented sequentially in different locations around a screen. During the test, each location is re-presented, paired with a novel location, and subjects are asked to touch the location at which they had seen a square appear. Lawrence and colleagues (1996, 2000) have demonstrated impairments in HD patients in both tests.

A strong relationship between motor impairment and neuropsychological symptoms is present in HD (Thompson et al. 2002). Also, cognitive changes are thought to occur prior to motor dysfunction (Duff et al. 2010; Hahn-Barma et al 1998). Hahn-Barma and colleagues (1998) reported that cognitive deficits are present in people that are at risk for developing HD, despite a total lack of motor and psychiatric symptoms. Therefore, detecting early cognitive impairments might constitute sensitive markers of the disease. Sexual dysfunction, weight loss, dysphagia and drug abuse are also observed in HD (Aziz et al. 2010).
Treatments or drugs that can halt the disease progression or heal patients have not been discovered yet. As illustrated in Fig. 4, the discovery of the defective gene in 1993 has led to an increase in preclinical and clinical publications on HD. Extensive investigations have been made in patients and non-human model organisms to understand the pathogenic mechanisms underlying disease progression and to find rational therapeutic treatments. The following paragraphs will focus on animal models, especially those which have given insight into the pathology, and tasks that can be of translational value.

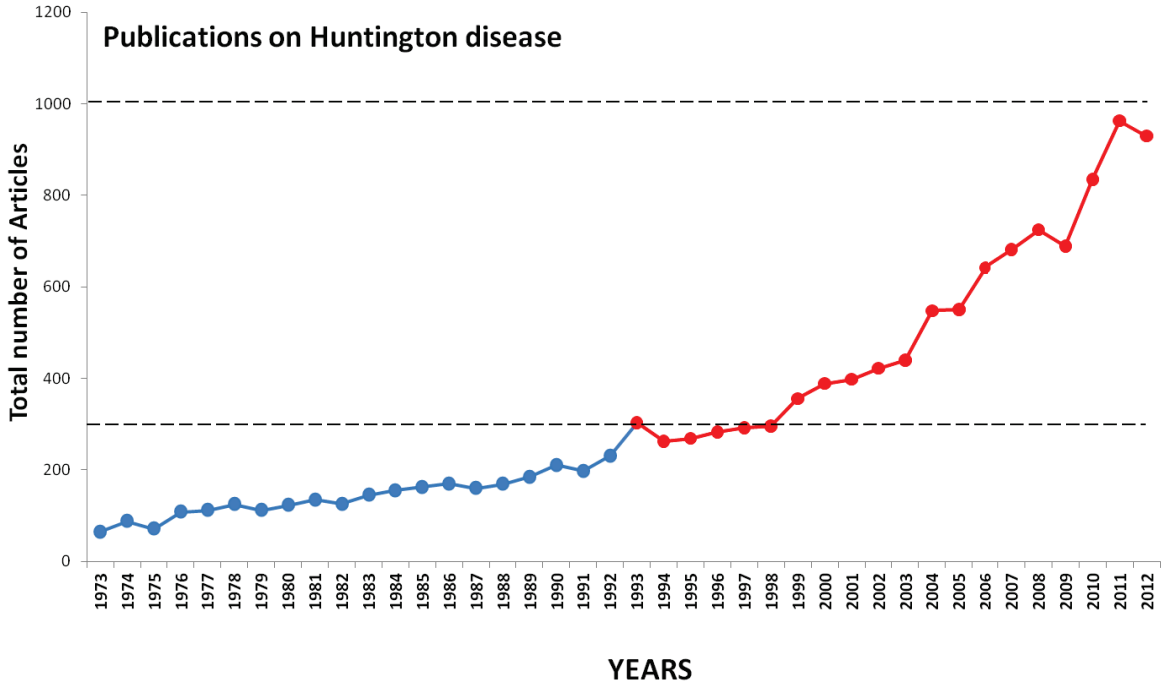


**Fig. 4** Publications on Huntington disease. For each year, the total number of publications with the words "Huntington's disease" obtained from the *US National Library of Medicine National Institutes of Health* website (www.ncbi.nlm.nih.gov/pubmed) database is depicted. A significant increase (red line) is observed since the HUNTINGTIN gene discovery in 1993 (blue line).

## *B. Animal models for HD research*

It is beyond the aim of this introduction to provide a comprehensive overview of all models. Instead, the focus is on mammalians, especially rodents as these are widely used for modelling human neurodegenerative disorders (Li and Li, 2012; Ramaswamy et al. 2007).

- *Toxin-induced models*

Toxin-induced models were especially useful before the discovery of the HTT gene and the subsequent generation of genetic rodent models. Structural and neurochemical changes were found in HD post-mortem striatum (Mann and Chiu 1978). Since striatal

neuronal loss is one of the hallmarks of the disease, the first animal models were based on the use of toxins that could more or less selectively destroy striatal MSN by deregulating mitochondrial function, synaptic transmission or excitotoxicity-induced cell death. Kainic acid, quinolinic acid and 3-nitropropionic acid (3NP) models that replicate some of the HD neuropathology were generated in mice and rats, as well as in non-human primate (Sanberg et al. 1989; Brouillet et al. 1995, 1998; Fernagut et al 2002; Schwarcz et al. 2010). However, these models are limited because they do not replicate the *progressive* degenerative processes such as production and accumulation of mutant *htt;* also, non-differential vulnerability of striatal projection neurons might make therapeutic approaches (neuroprotection or neurorestoration) targeting specific neurons more challenging (Ouary et al. 2000; Sun et al. 2002). These drawbacks make them inefficient to study the progressive nature of HD.

- *Genetic models*

The aim of (genetic) animal models is to recapitulate, at least in part, some of the phenotypes and pathogenic mechanisms that occur in patients. Numerous models of HD have been generated in the mouse (Crawley, 2008) and based on their genetic construct they can be grouped in 3 categories (Table 2):

*Fragment models*

The rationale of these models is based on the fact that the CAG repeats in human HD patients occur only in exon 1 (see Fig. 1).

In these animals, only the N-terminal portion of HTT, either human or chimeric, is expressed. The R6/2 model, for example, was one of the first mouse models expressing the mutant HTT (mHTT) exon 1 with 150 CAG repeats under the control of the human HTT promoter (Mangiarini et al. 1996). The R6/2 mice exhibit motor disturbances as early as 4.5 weeks of age, with moderate neurodegeneration (12 weeks of age) and a variety of cognitive impairments similar to those in patients (Bolivar et al. 2003; Carter et al. 1999; Lione et al. 1999; Stack et al. 2005).

*Full length models*

This class of animal models consists of transgenic animals expressing the full length mutated human HTT gene (fl-mHTT) with expanded CAG repeats. This category is engineered by using yeast or bacterial artificial chromosome (YAC, BAC). YAC 128 and BACHD mice for example, express the fl-mHTT with 128 CAG and 97 CAA/CAG repeats respectively (Gray et al. 2008; Slow et al. 2003). Comparing the genetic construct between both models, the BAC model has higher stability and minimal chimerism (Yang et al. 1997). At the protein level, the YAC128 line expresses the human mhtt at 75% while the BACHD fl-mHTT line expression is 150-200% of the endogenous murine HD homolog (*Hdh*). The relationship between CAG length repeats and behavioural deficits in BACHD mice appears to be similar to that of HD patients (Table 2). Also, these mice show a more pronounced phenotype in a number of behavioural tests than YAC 128 mice (Menalled et al. 2009).

A general concern with the fragment and the full length models is that the gene is randomly inserted in to the animal genome and the characteristic expanded CAG codon mutation is ectopically expressed. Therefore more effort and control of the breeding process is required to avoid genetic drift.

*Knock-In models*

The pathological CAG repeats replace the corresponding sequences in the mouse endogenous HTT gene (Hdh-KI). Knock-in models could be considered as the most precise genetic models because the mutant huntingtin is under the control of endogenous regulatory elements.

Compared to transgenic fragment mice, knock-in models have a normal life span and exhibit slow progression of the behavioural phenotype ($HDh^{Q111}$ and $HDh^{(CAG)150}$ models for example). However, they do not present overt neuropathology or robust markers, such as brain atrophy or neural loss, which characterize the human disease (Lin et al. 2001, Shelbourne et al. 1999; Wheeler et al. 2000). The lack of solid neuropathology findings makes it difficult to study effects of therapeutic interventions.

| Types of model | Strain | Line | Background | Construct | CAG repeat length | Life span | Motor symptom onset | Neuronal loss | Mutant htt inclusions / aggregates |
|---|---|---|---|---|---|---|---|---|---|
| Fragment | Mouse | R6/2 | C57 BL/6 x CBA | Human exon 1 HTT gene under control of human HD promoter | ~ 150 | ~ 13 weeks | 6 to 8 weeks | striatum | 4 weeks |
| | | N171-82Q | C57 BL/6 x C3H/He | First 171 amino-acid of human HTT | 82 | 16 - 22 weeks | 3 months | Cortex, striatum | 5 months |
| | Rat | tgHD51 | Sprague Dawley (SD) | Endogenous HTT gene with expanded CAG insertion | 51 | Normal | 10 months | - | 12 months |
| Full length | Mouse | YAC128 | FVB/N | Full length Human HTT gene under control of human HD promoter | 128 | Normal | 6 months | striatum | 2 months |
| | | BACHD | FVB/N | | 97 (mixed)* | Normal | 2 months | Dark degenerating neurons | 12 months |
| | Rat | BACHD | Sprague Dawley (SD) | | 97 (mixed) | Normal | 1 month | Dark degenerating neurons | > 9 months |
| Knock-in | Mouse | Hdh $^{(CAG)150}$ | Mixed 129 Ola x C57 BL/6 | Endogenous HTT gene with expanded CAG insertion | 150 | Normal | 25 months | striatum | 6 months |
| | | Hdh$^{Q111}$ | Mixed 129 Sv x C57 BL/6 | Endogenous murine HTT gene, chimeric human/mouse exon1 | 109 | Normal | 24 months | Striatal dark degenerating neurons | 10 months |

**Table2. Types of transgenic mice and rats models of HD.** Presented are brief characteristics of some commonly studied models of HD. (*) These HD animal models have a mixed trinucleotide CAA / CAG repetition in the mutated gene, both coding for glutamine. (Sources : Crook and Housman, 2011; Figiel et al. 2012; Meade et al. 2002; von Horsten et al. 2003; William Yang and Gray, 2011 ; Yu-Taeger et al. 2012).

## C. Motor and cognitive assessment in genetic animal models

As discussed above, HD is associated with progressive manifestation of motor, cognitive and psychiatric symptoms. In order to evaluate the face validity of the animal models for HD, a broad range of tests has been developed.

- *Motor assessment*

In animal models, neuromuscular strength can be measured with a grip strength test; whereas ataxia and tremor can be evaluated with a beam walking test. Other tests, such as the open field and the rotarod test have shown robust effects across laboratories. The open-field apparatus usually consists of a Plexiglas box arrayed with infrared beams. Locomotion and general exploration of animals is recorded as total beam breaks when the animal walks around in the arena. This test can be used to assess spontaneous locomotor activity in HD models. The rotarod test evaluates motor coordination and balance in animals by scoring the latency to fall from an automated rotating rod. This test has proven to be sensitive and efficient for assessing motor impairment produced by brain injury (Hamm et al. 1994). The rotarod has been used in genetic models and the test has proven to be sensitive in detecting motor phenotypes (Crook and Housman 2011).

For the detection of subtle gait abnormalities, the ink-footprints (or paw-print) test method allows analysis of static parameters such as stride length and width. For example, variable stride length and wide front-base have been detected in R6/2 mice (Carter et al. 1999; Menalled et al. 2009). However the reliability of this method is limited because animal speed cannot be assessed. Therefore, additional tests have been developed, that include measurement of dynamic parameters. Digigait is an automated treadmill video capture system used to record animal footsteps. This test has been used in R6/2 mice (Pallier et al. 2009). However, Digigait has raised a concern due to the forced nature of the task. The Catwalk is an automated gait analysis test which can measure both static and dynamic changes during an animal's walking cycle. The Catwalk has proven to be sensitive in detecting gait abnormalities in models for spinal cord injuries (contusion and transection), Parkinson's Disease (PD) and HD (tgHD rats) (Chuang et al. 2010; Hamers et al. 2001; Deumens et al. 2007; Vandeputte et al. 2010). One distinct disadvantage of the Catwalk system is the requirement that the animals voluntarily move across the walkway. Especially in animals with severe motor impairments this may pose problems.

- *Cognitive assessment*

Tasks that enable a comprehensive evaluation of cognitive abilities in rodents exist. A spatial navigation task for learning and memory is the Morris Water Maze (MWM). In this task, learning is based on environmental landmarks: the rat or mouse swim to an escape platform located in a quadrant of the circular maze. Memory of the learned place is confirmed during a probe trial in which the escape platform is removed. The time spent by the animal around the former platform location is indicative of the state of its spatial memory. One limitation of this test might be that spatial learning in a MWM is traditionally linked to hippocampal functioning; whereas the predominant pathology in HD is linked to the cortical striatal pathway. Also, from an ethological point of view, mice are land-based animals and become severely stressed when placed in a swimming tank; therefore, tasks such as cross-maze, T and Y mazes are designed to differentiate between spatial or non-spatial learning in animals. Moreover such tasks can be used to investigate cognitive flexibility and reversal learning. Using some of these tasks in R6/2 and YAC128 mice, specific and progressive learning and memory deficits similar to those observed in HD have been reported (Brooks et al 2012; Lione et al. 1999).

Sensory motor gating impairment could indicate attentional processing dysfunctioning. A deficit in PPI was reported in HD patients as well as in rats, R6/2 mice, YAC 128 and BACHD mice (Braff et al. 2001; Menalled et al. 2009; Pouladi et al. 2012; Swerdlow and Geyer, 1998 and 2001). Hence this measure of sensorimotor gating has translational value and can be reliably measured across species, including rodents.

Another test with potential translational value is the recognition memory task. Its design is close to the clinical pattern and spatial recognition test. In the object recognition test (ORT), animals explore a familiar versus a novel object during a probe test. In the object location test (OLT), animals explore objects at the same or at different locations of the arena. Deficits in ORT and OLT have been demonstrated in 16 months old tgHD rats (Zeef et al. 2012a). Therefore, deficits found in the ORT and OLT in tgHD rats are consistent with those reported in HD individuals in pattern and spatial recognition tasks and call for further investigation in other models as well (Lawrence 1996, 1998, 2000).

## D. Aims of the thesis and synopsis

In order to develop preventive treatment in HD it is important to determine early manifestations of the disease prior to traditional clinical diagnosis. Findings from the Predict-HD[2] study suggest that the commencement of detectable and measurable changes appear one to two decades before the predicted time of clinical diagnosis (Duff et al. 2010; Paulsen et al. 2008). Cognitive deficits are one of the triad of HD symptoms and includes impairments in attention, verbal fluency, executive and visuospatial functioning (Ho et al. 2003). Reports in pre-manifest HD or at-risk carriers have showed progressive cognitive impairment 2 to 12 years before the development of manifest motor disease (Foroud et al. 1995; Jason et al. 1988; Paulsen et al. 2001). These findings suggest that detecting robust cognitive deficits early on in animal models for HD may be valuable to help finding potential prophylactic drugs. Although many studies have been conducted in animal models for HD, most of these have focussed on motor symptoms rather than cognitive deficits, possibly because motor symptoms are easier to assess in rodents. In order to optimize our chances of developing meaningful therapies, some aspects need to be taken into consideration: (1) because HD is a time-dependent disorder, it is important to use species that model these aspects of timing. Fragment rodent models exhibit striatal atrophy and rapid onset phenotype; but they do not express the full length huntingtin protein that should replicate the human condition and some of the models (most notably the R6/2 model) exhibit such rapid symptom development that it becomes almost impossible to study presymptomatic animals. Given the generally short life-span of rodents (typically around 2 years), one might suggest other genetic models such as pigs, sheep and monkey could more optimally mimic the pathological features seen in patients (Jacobsen et al. 2010; Matsuyama et al. 2000; Yang et al. 2008). However, these models have other limitations especially in terms of practical issues (laboratory space, pricing) and the fact that few cognitive tests have specifically been developed for such large animals. In contrast to the above

[2] Predict-HD is an observational study foundation that uses genetic, neurobiological and refined clinical markers to understand the early progression of HD in both presymptomatic gene-positive and gene negative individuals. For more detail about their goals and actions, refer to the web page: https://www.predict-hd.net/

mentioned species, rat and mouse requirements are minimum; their genome is well documented and resemble that of human (Brudno et al. 2004; National Institutes of Health News, March 2004). Therefore, it is sensible to measure the progressive deficits in genetic full length rodent models for HD. (2) In order to evaluate effectively the progressive cognitive impairments, robust and reproducible assays are indispensable. We have previously described some tests that can provide a longitudinal measurement and they can be use in a routine screening.

*Aims of the thesis*

The goal of this thesis is to provide an in depth characterization of the development of the motor, cognitive and psychiatric symptoms in BACHD mice and BACHD rats model for HD. These models express the full length of the human m-HTT and initial reports demonstrated similarities in neuropathology between these models and HD patients (Gray et al. 2008; Yu-Taeger et al. 2012).

Subtle cognitive impairments are considered as an early indicator for the disease. In our longitudinal characterisation of BACHD mice and BACHD rats, we want to address specifically the followings: (1) to what extend cognitive deficits occurred prior to motor impairment? (2) Is the disease progression similar/dissimilar in 2 species with identical genetical constructs? (3) Which experimental assays are sensitive and of value for drug screening?

**Synopsis**

The following studies have investigated behavioural-like symptoms in transgenic BACHD mice and rats models for HD.

***Study 1***

Reports from different studies showed that BACHD mice have progressive and pronounced behavioral deficits when assessed in several tasks such as rotarod, open field, paw print, and sensorimotor gating tests (Gray et al. 2008; Menalled et al. 2009). Many studies in mice models have been largely limited to assessing motor deficits. Our first study aims to provide a more comprehensive behavioral analysis by assessing the full triad of symptoms, motor, emotional *and* cognitive behavior, in adult BACHD mice.

Motor deficits were tested in a rotarod test, a robust and quantitative test that is generally used across laboratories. We hypothesized that transgenic mice may present coordination imbalances on the rotarod test as previously described (Gray et al. 2008; Menalled et al. 2009). Gait abnormalities were investigated in the catwalk, an automated system that can detect static and dynamic parameters during walking. In contrast to the paw print test, the Catwalk provides a better insight into subtle motor impairments that may occur in transgenic mice as described previously for HD patients (Koller and Trimble, 1985). Finally, emotional disturbances and cognitive deficits were evaluated in elevated zero maze, fear conditioning and cross maze tests respectively. We expected that transgenic mice may present anxiety and difficulties in shifting from one set of learning to another as reported in HD patients (Lawrence et al. 1998, 1999).
The results have replicated some of the motor deficits reported in this model and we gain new insight in to gait abnormalities with the catwalk test. We have demonstrated that BACHD mice have difficulties on a cognitive task (cross maze) and present psychiatric like symptom as found in patients.

***Study 2***

A novel BACHD transgenic rat model for HD has recently been established. Like the BACHD mouse model, transgenic rats carry the human fl-mHTT with 97 polyglutamine repeats (mixed CAG/CAA) with all the regulatory elements (Yu-Taeger et al. 2012). They exhibit some of the futures of the disease like progressive motor deficits on a rotarod starting at 1 month of age, and gait abnormalities in footprint test at 14 months of age. A decreased in anxiety-like behaviour was observed in an elevated plus maze test. Neuropathological markers such as reduced dopamine level and presence of huntingtin aggregates have also been reported in these rats. Although preliminary data generated by Yu-Taeger and colleagues are of value, little is known about this novel model. The rationale was to determine the potential validity of this model. Therefore, we longitudinally characterise the progressive emergence of motor, sensorimotor and cognitive deficits in BACHD transgenic rat model for HD.
Exploratory behaviour, motor coordination and gait deficits were assessed with an open field, rotarod and catwalk test, respectively. We expected to replicate – in part - the motor deficits reported on a rotarod test, and to gain a better understanding of gait

parameters that are sensitive to changes at the early stage of the disease. Swerdlow and colleagues (1995) have demonstrated sensorimotor deficits in HD patients. We hypothesized that transgenic rat might present acoustic startle reflex deficits in sensorimotor tests. Recognition memory is impaired in manifest HD subjects (Montoya et al. 2006). We investigated transgenic rats in ORT, a task similar to the pattern and spatial recognition task (Lawrence et al. 1996). We expected that transgenic rats will show deficits in ORT.

The results have demonstrated robust motor phenotype in transgenic rats, but sensorimotor deficits yield little and any recognition memory impairment was detected up to 12 months. Further investigations are indispensable to demonstrate face validity in BACHD rats.

***Study 3***

In lights of the results of the previous study, we aimed to expand our understanding of progressive cognitive deficits in BACHD transgenic rats by evaluating reversal learning and associative memory in fear conditioning and cross-maze tests, respectively. One advantage of using fear conditioning is that the neuroanatomical circuitry and neurochemical basis underlying fear & anxiety and learning & memory, are well described and conserved in rodents and humans (Fendt and Fanselow, 1999; Knight et al., 2004). The cross-maze is a spatial alternation task that allows evaluating the strategy that a rodent adopts during learning (Packard and McGaugh 1996; Packard, 1999). We used similar models and experimental conditions as in the previous mouse study to allow a direct comparison between the phenotypes of the BACHD mouse and rat models.

The results have demonstrated progressive cognitive deficits in transgenic rats and were informative on the time course of emergence. Like the BACHD mice, BACHD rats are useful for preclinical drugs evaluation.

# II. Studies

# Study 1

# Motor, Emotional and Cognitive deficits in adult BACHD mice: A model for Huntington disease

Yah-se ABADA, Rudy SCHREIBER, Bart ELLENBROEK

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**Abstract**

*Rationale*

Huntington's disease (HD) is characterized by progressive motor dysfunction, emotional disturbances and cognitive deficits. It is a genetic disease caused by an elongation of the polyglutamine repeats in the huntingtin gene. Whereas HD is a complex disorder, previous studies in mice models have largely been confined to assessing motor deficits.

*Objectives*

The aim of the present studies is a comprehensive phenotypical assessment of not only motor and gait deficits, but also of emotional and cognitive deficits in adult BACHD mice.

*Material and methods*

46 male BACHD mice between 9 and 10 months of age were used. Wild type (+/+) and transgenic (+/T) mice were tested for motor deficits on a Rotarod and Catwalk system. Emotional deficits were assessed with the zero-maze and fear conditioning tests. Cognitive deficits in a strategy shifting task were evaluated in a cross-maze test.

*Results*

Comparing +/T and +/+ mice, we replicated the motor deficits in the transgenic mice that were previously described in the Rotarod test. For the first time, motor coordination imbalances in +/T animals are described in the Catwalk gait analysis system. +/T mice showed more anxiety-like behavior in the zero-maze test and a higher freezing response in the fear conditioning test. Reversal and strategy shifting impairments were demonstrated in the cross-maze, indicative of a disturbed prefrontal-striatal pathway.

*Conclusion*

The results suggest that BACHD mice represent an animal model with a high degree of face validity for HD and may be very useful for testing novel therapeutic strategies.

## 1. Introduction

Huntington's disease (HD) is one of the 9 neurodegenerative diseases which are characterized by unstable Glutamine (CAG) repeats expansion in their target gene. The huntingtin (*htt*) gene is widely expressed in the brain and in non-neuronal tissues. The pathology in HD is caused by an elongation of the polyglutamine (polyQ) repeats (> 35 CAG) in the *htt* gene, on chromosome 4 (Huntington's disease collaborative research group, 1993). Many studies have suggested that *htt* plays a role as a scaffolding protein, essential for intracellular and synaptic vesicular trafficking, required during development and normal brain functioning; however, its precise role in cell apoptosis mechanisms for example are not yet well-understood (Caviston and Holzbaur, 2009; Cisbani and Cicchetti, 2012; Trushina et al. 2004; Zheng and Diamond, 2012). What is known in HD is that the accumulation of elongated polyQ-containing proteins in the nucleus and the subsequent formation of nuclear inclusions lead to a widespread degeneration of neurons, especially in striatal and cortical regions of the brain (Douaud et al. 2006; Jones and Hughes, 2011; Vonsattel et al. 1985). Medium spiny striatal neurons are the most sensitive to neuronal degeneration. Despite recent findings, molecular mechanisms underlying the selective vulnerability of these neurons in HD remain unclear (Bezprozyanny and Hayden, 2004; Kovalenko et al. 2012).

Several rodent models have been designed to reproduce the pathogenesis of the disease with the ultimate goal of developing potential novel drugs for clinical therapies (Crook and Housman, 2011). A good animal model should recapitulate as many of the hallmark pathology and symptoms of the disease as possible (Bowles et al. 2012). A particularly interesting model is the BACHD transgenic mouse in which the full length human mutant huntingtin (fl-*mhtt*) gene with 97 CAA-CAG mix repeats under control of the human HD promoter is expressed (Gray et al. 2008). The neuropathology in this model closely resembles that of HD patients, including nuclear accumulation of aggregated *mhtt* and reduction in cortical BDNF mRNA expression levels, an important neurotrophic factor in the regulation of neuronal activity and survival (Zuccato and Cattaneo, 2009).

Functional impairments in HD patients worsen throughout the course of their disease ultimately leading to an inability to perform normal daily living activities (Hamilton et al. 2003). This progression is paralleled in animal models by an increased motor deficit as observed using the Rotarod test and foot-print test. For example, R6/2 mice, YAC 128 mice and BACHD mice show marked, progressive and phenotypic motor deficits starting already at 2 months of age (Carter et al. 1999; Kordasiewicz et al. 2012; Menalled et al. 2009; Southwell et al. 2009). However, dynamic gait characteristics that appear more relevant for HD, such as velocity and cadence, cannot be assessed using the Rotarod or the foot-print test. Koller and Trimble (1985) have revealed specific gait deficits in HD patients using video recordings and gait analyses. Indeed, movement disorders become more pronounced as the disease progresses, and contribute to clinical diagnosis of HD. Therefore, one of the aims of the present study was to perform a detailed gait analysis of BACHD mice using a Catwalk system. The Catwalk is an automated video-computer based system that detects and measures a variety of spatial and temporal aspects of inter-limb coordination in rodents (Hamers et al. 2001). The Catwalk has shown to be efficient in detecting both static and dynamic gait parameters deficits after adult rat sciatic nerve lesion (Deumens et al. 2007), and behavioral recovery from sciatic nerve crush injury (Bozkurt et al. 2008).

In addition to the motor deficits, neuropsychiatric symptoms such as obsessions, apathy, depressed mood, irritability and anxiety contribute to the functional decline in HD patients and often constitute reasons for hospitalization (Hamilton et al. 2003; Van Duijn et al. 2007). Emotional symptoms have a significant impact on the daily life of patients. Lack of emotional control (Paradiso et al. 2008) and lower fear ratings in response to fear stimuli (Eddy et al. 2011) have been described for the clinical and preclinical stage of the disease in patients. Few reports on emotional deficits have been published in HD mouse models. Bolivar (Bolivar et al. 2003) has reported variable anxiety-like behavior in R6/2 mice in an activity monitor test, while Hickey (Hickey et al. 2008) showed in 4 months old CAG 140 knock-in (KI) mice increased anxiety in a dark light box test and a fear conditioning paradigm. In BACHD mice, anxiety-like behavior was observed as early as 4 weeks of age in a dark-light choice test, in an open field test, and in elevated plus-maze test (Carter et al. 1999; Kordasiewicz et al. 2012;

Menalled et al. 2009; Southwell et al. 2009). Since all these represent similar conflict paradigm (i.e. the conflict between exploration and anxiety), we planned to extend our understanding of emotional reactions related to anxiety in BACHD mice by additionally testing them in the fear conditioning paradigm and the zero-maze. In comparison with the widely used elevated plus maze for the assessment of anxiety-like behaviour, the design of the zero maze (annular runway), has been optimized to decrease ambiguity in interpretation of the behavior of mice in the centre compartment (Shepherd et al. 1994). As compared with the above-mentioned anxiety tests, the neural circuits involved in fear conditioning paradigm have been well described and are highly conserved between rodents and humans (Kim et al. 2011; Pezze and Feldon, 2004). The test depends much less on intact motor capacity than the conflict tests discussed above.

Cognitive deficits have been described in the early stages of HD and symptoms worsen throughout the course of the illness. Patients with HD are impaired in their visuospatial memory, reversal learning and executive functioning. In visual discrimination learning and attentional set shifting tasks, patients perseverate in the selection of a previous reinforced stimulus (Lawrence et al. 1996, 1998). In general, tasks for measuring cognitive deficits in rodent have been mostly based on spatial learning and memory. Visuospatial learning and strategy shifting memory have not been well described in BACHD mice (Southwell et al. 2009). Cognitive and visuospatial deficits were found in 4 week old R6/2 mice in a Morris Water Maze (MWM) task, a two choice swim task and a T-maze task (Ciamei and Morton, 2009; Lione et al. 1999). A drawback of these tasks is that they do not probe the functioning of some of the core cognitive domains that are impaired in patients. There are also concerns that mice testing in water mazes may lead to confounds due to the stress associated from being immersed in water. Therefore, in the present paper, we used a ‘land-based’ cross-maze task to specifically assess cognitive functioning in BACHD mice. The cross-maze is a closed elevated four arms at 90 degree to each other. A T-maze configuration is made by closing one arm and spatial alternation (acquisition and reversal learning) can be investigated. In addition, the cross-maze allows us to assess the strategy (spatial vs. non-spatial) used by each animal. Finally, since HD patients show a deficit in extra dimensional shift learning in a

Wisconsin Card Sorting Test (Brandt et al. 2008; Lawrence et al. 1996, 1998), it is of interest to assess if transgenic mice also present deficits in shifting.

The aim of this study was to investigate motor, emotional and cognitive deficits in adult BACHD mice. The results from the present study showed that in addition to replicating the well-known motor deficits in BACHD mice, we found that these animals showed an increase in anxiety-like behavior as well as cognitive deficits in the cross-maze.

## 2. Material and methods

### Husbandry and genotyping

A total of 46 male BACHD mice, aged between 9 to 10 months were used at EVOTEC AG (Hamburg, Germany). Mice were obtained from the CHDI's breeding colony (Universität klinikum Hamburg Eppendorf, Germany), maintained on the FVB/N background. Transgenic mice expressed the full length human *htt* with 97 mix CAA-CAG repeats [13]. Mice were group-housed 3 to 5 per cage with wood shavings and a filter top. The environment was enriched with a play tunnel, wooden sticks and shredded paper. Homozygous wild type (+/+) and heterozygous transgenic (+/T) mice were maintained in climate controlled housing, with a 12-h reversed dark/light cycle (light on 17:00h and off at 07:00h). Mice had free access to food and water except during experiments, which took place during their active phase (dark cycle). Animals were held and all experiments performed in accordance with the German animal welfare act and the EU legislation (council directive 86/609/EEC).

All the animals were tail tipped at weaning. Genotyping was performed using a validated protocol. Briefly, Genomic DNA was prepared from tail biopsies using proteinase K digestion, followed by phenol/chloroform extraction (Qiagen DNeasy Tissue kit). Primers flanking the polyQ repeat in exon 1 were designed (EVOTEC-AG, Germany) to recognize both endogenous and exogenous gene (htt, mhtt), and were used to PCR amplify the polyQ regions [Q3: 5' – AGG TCG GTG CAG AGG CTC CTC - 3' and Q5: 5' – ATG GCG ACC CTG GAA AAG CTG - 3']. The PCR product was run on an

automated apparatus PTC-200 (Peltier Thermal Gradient Cycler) and the Agilent 2100 Bioanalyser (Agilent technologies) was used to determine the fragments size.

Behavioral testing

All the behavioral tests were performed during the dark phase and conducted over a 6 week period. Only male mice were used for this study to avoid any effects of changes in the female estrus cycle on behavioral responses. Mice were weighed twice a week during the testing phase. For the following tests, the reader is referred to the experimental time course in Fig. 1a.

- *Rotarod*

Motor coordination and strength were assessed with a Rotarod apparatus (Ugo Basile, Italy). Mice were trained to walk against the motion of a rotating drum. First, a training session of 5 min was done at a constant speed (4 R.P.M.; rounds per minute). Then, 1 h later, mice were tested 3 times in 5-min trials at an accelerating speed (from 0 to 40 RPM over 5 min). The mean latency to fall off the Rotarod was recorded and mice remaining on the rod for more than 300 s were removed and their time scored as 300 s. The test was repeated once a week for a total of four times.

- *Catwalk system*

The gait analysis system Catwalk 7.1 (Noldus IT, the Netherlands) consisted of an enclosed walkway made with a glass plate and a speed camera. Assessment of gait performance was performed with a recording and analysis software. On the first day, the mice were habituated to the apparatus for 5 min with the goal to cross the walkway. The 3 following days, free runs across the walkway were recorded. From among the correct runs, one run per animal per day was selected randomly for analysis. A correct run was defined as one complete (60 cm) crossing of the walkway without interruption. For one crossing, a mouse needs a minimum of 9 step sequences patterns. In addition, runs with step sequence categories related to exploration, Rotate Ra (RF-LF-LF-RH)

and Rotate Rb (LF-RF-RH-LH) were not analyzed (RF= right forelimb; RH= right hindlimb; LF= left forelimb; LH= left hindlimb).

The following parameters were evaluated: walking speed (measured as the average of strides in cm/s); the normal step sequence patterns (NSSP, i.e. the order in which the four paws were placed); base-of-support (B.O.S, i.e. distance between two hind or fore paws, as measured perpendicular to the walking direction); stride length (i.e. distance between the placement of a hind or fore limb and the subsequent placement of the same limb); print position (space relation between a fore and a hind paw of the same side in mm); regularity index [RI, i.e. an index for the degree of interlimb coordination during gait, as measured by the NSSP, multiplied by four (number of paws), divided by the number of limbs placements, and multiplied by 100%]; stance (i.e. time of contact of the hind or fore limbs with the glass floor); swing (i.e. time that the hind or fore limbs are not in contact with the glass plate); Phase of dispersion [i.e. the timing relationships between paw placements. It is expressed as percentage of time of initial contact of one paw (the target) related to the stride length of another paw, the anchor]; max contact (at) (i.e. the point where the breaking phase turns into propulsion phase); intensity (measured as the mean brightness of all pixels of the print at maximum paw contact ranging from 0 to 255 arbitrary units). A full description of the apparatus, method, parameters and analysis is provided by Hamers (2001).

- *Zero maze*

The zero maze consisted of an elevated annular runway (diameter 46 cm, width 5.5 cm, 40 cm above the floor) made from grey plastic. It was divided into four equal sectors: two opposing 90° sectors of the runway were protected by an inner and outer wall of grey polyvinyl-chloride (height 16 cm). The two remaining sectors were unprotected. The maze was exposed to diffuse room light (70 lux).

Each test started by placing the mouse in the protected area. A video camera was suspended above the maze and a tracking system (Ethovision, Noldus IT, the Netherlands) was used for 5 min recordings. We analyzed the time spent, the distance moved, the frequency of entrance in each quadrant, and the velocity.

- *Fear conditioning*

The apparatus (Med Associates Inc., Italy) consisted of a sound-attenuated box with wire stainless steel rods on the floor. On the first day, mice were given 5 min habituation session in the apparatus. On day 2, after 2 min of acclimatization, mice received 6 pairings (120 s inter trial time) of a 30-s tone (80 dB) with a 0.45-mA, 2-s foot shock. The foot shock terminated at the same time as the tone. On day 3, mice were tested for contextual freezing in the conditioning chamber for 3 min, in the absence of tone or foot shock. One hour later, a new context was generated in the conditioning chamber by applying a white polyvinyl chloride material. Mice were tested 3 min without tone to assess freezing in the new context and 3 min with the tone in absence of foot shock.

- *Strategy shifting*

For the strategy shifting test a standard dual-solution task (Tolman et al. 1946) was used to assess the respective contributions of response (or egocentric) and place (or allocentric) learning strategies on memory. It determines the relative involvement of these 2 strategies during the course of learning.
Spatial alternation was assessed using a modified version of the standard cross-maze; the home made maze consists of 4 identical arms (40 cm x 8 cm x 20 cm) at 90 degrees to each other. The maze was made with clear Plexiglas, elevated 45 cm above the floor, and a T-maze was created by closing one arm (north, N) with a guillotine door. The T-maze configuration was as follow: 2 arms [east (E) and west (W)] are at 180 degrees to each other and the last arm (south, S) was perpendicular to these arms. Two holes were present: one at the end of the E and W arms each, and spatial cues were placed on a black curtain which surrounded the maze. The home cage was put at one end of the arms (E or W) to motivate the animals to explore the maze and find the exit into their home cage (where they were additionally rewarded with food pellets). One week prior to the test, mice received small sucrose food pellets in addition to their normal diet. One day prior to the start of the experiments, all mice received a 5-min habituation session in the apparatus. During that period, food was not available.

The next day, the acquisition sessions started and the mouse was placed in the S arm. Its home cage containing sucrose food pellets was placed under the hole in the W arm. The mouse had to guide itself in the maze and reach the home cage. During acquisition, mice received one trial per day for 8 days. During the first 2 days, the goal arm (i.e. arm giving access to the home cage) was baited with small sucrose food pellets. The same training procedure was run during the reversal session except that the mouse had to reach this time the home cage placed underneath the E arm (opposite of the previously learn arm). When a mouse made a wrong choice (entrance into the arm without home cage), it was allowed to trace back to the goal arm. If the mouse failed to reach the home cage within 2 min, the mouse was gently guided manually to the goal arm and the trial ended 30 s later.

Two probe tests were performed at day 9 and day 18, at the end of the acquisition and reversal training sessions, respectively. During the probe trials the S arm was closed and the N arm was used as the new start arm; the strategy (place or response) that the animal used to reach the goal arm was assessed. If the animal during the acquisition training had learned to use a place strategy, it would select the W arm. However, if the animal had used a response strategy (i.e. learned to turn left), it would select the E arm.

### Statistical Analysis

All data are shown as the mean ± SEM. *Graph Pad* statistics software was used to perform all analyses. Differences between groups were assessed with Student's t-test or mixed ANOVA with repeated measures, with the factor genotype as between subject and time as within subject variable. When significance was found, a Bonferroni - post hoc analysis was performed. In the Strategy shifting test, Chi-square ($\chi 2$) analyses were computed on animal's choice during Acquisition and Reversal learning in order to determine discrepancies between groups and to determine potential changes in strategies between both probe trials. The significance level was set for all analysis at 0.05.

## 3. Results

- Experimental timeline and gross phenotype (Fig. 1)

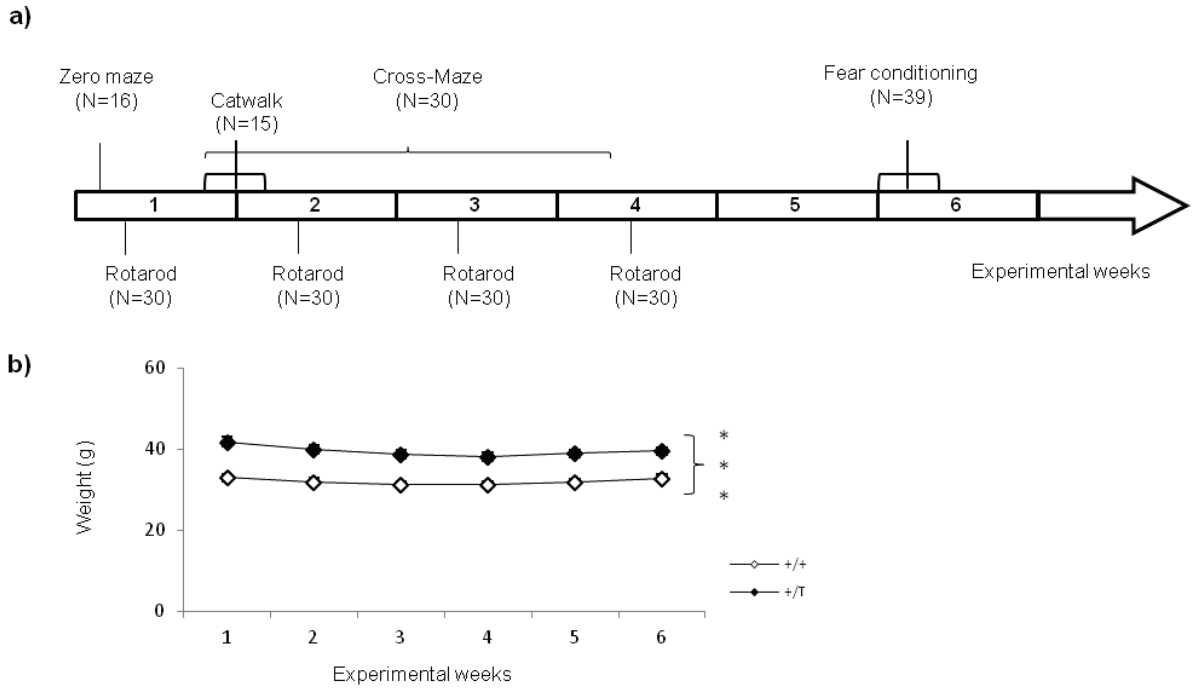


**Fig. 1** (a) Experimental time course (b) Body weight**.** Results are expressed as Mean ± SEM. Transgenic mice (+/T) are heavier than control (+/+) mice. Asterisks indicate significant difference between control and transgenic mice (***p < 0.001).

One week prior to the beginning of the experiments, a general inspection of mice did not show any gross abnormality in the phenotype and all animals looked healthy. During the course of the experiments, 3 mice (one transgenic and two wild types) showed severe circling behavior and were euthanized.

Transgenic mice had a significantly higher weight than their wild type littermate controls (Fig. 1b; results from 2-way ANOVA for the main factors GENOTYPE: $F(1, 43) = 27.85$, $P < 0.0001$ and TIME: $F(5, 215) = 5.70$, $P < 0.0001$). The difference in body weight was maintained and no significant interaction between the main factors GENOTYPE and TIME was found.

- Motor behavior in a Rotarod test and a Catwalk gait analysis system (Fig. 2)

- *Rotarod test*

The latency to fall from the rod was shorter in transgenic mice. Transgenic and wild type mice showed statistically significant differences in the overall latency to fall (Fig. 2a; GENOTYPE: $F(1, 28) = 17.78$, $P< 0.001$; TIME: $F(3, 84) = 9.79$, $P< 0.0001$). The absence of a significant GENOTYPE X TIME interaction suggests that the difference in latency to fall was maintained over time. However, although visual inspection of Fig. 2a suggests a difference between both groups at week four, this was not confirmed by post hoc analysis ($t = 2.276$ and $P < 0.05$).

- *Catwalk gait analysis system*

We analyzed static and dynamic gait parameters changes in BACHD mice with the Catwalk system. Because velocity can significantly influence gait parameters (Cendelin et al. 2010), we analyzed walking speed as well. As shown in Fig. 2b, there were no significant differences in walking speed over the days for both transgenics and wild types. Therefore the data were pooled.

For the static parameters, significant differences were seen in the order in which the four paws were placed [NSSP, Fig. 2c: *cruciate a* (sequence = RF-LF-RH-LH, $t= 2.727$ and $P< 0.05$), *cruciate b* (sequence = LF-RF-LH-RH, $t= 2.628$ and $P< 0.05$) and *alternate Ab* (sequence = LF-RH-RF-LH, $t= 3.643$ and $P< 0.01$)]. The degree of coordination, measured by the regularity index (RI), was not different. With respect to the base of support (B.O.S), the transgenic group presented a larger distance between forelimbs placements (Fig. 2d; $t= 2.786$, $P< 0.05$) but not between hindlimbs placements. Also, no significant difference between groups was observed for front and hind-limbs intensity (Fig. 2e), stride length and print positions (data not shown).

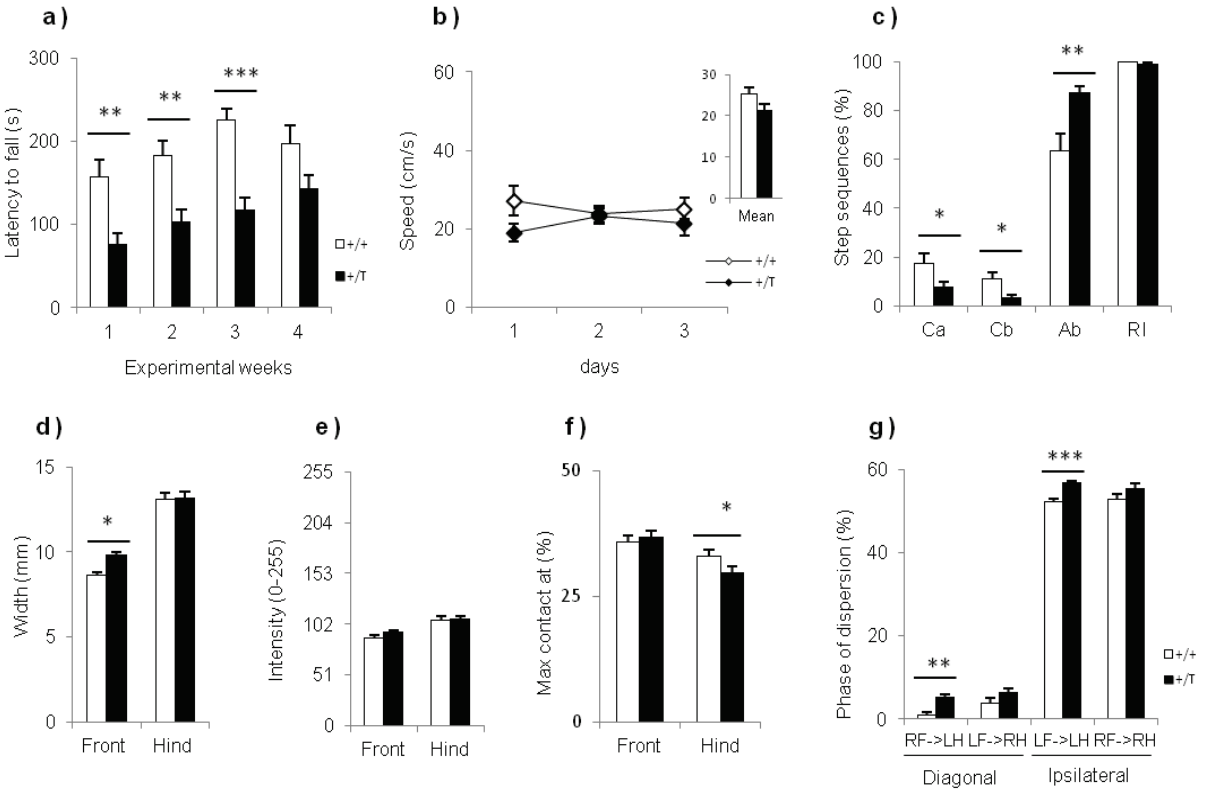


**Fig. 2** Results are expressed as Mean ± SEM. (a) Rotarod performance. Presented is the latency to fall on the accelerating (4 - 40 r.p.m) rod. Although there was no significant difference between groups on day 4, the overall genotype deficit was significant. [b-g] Catwalk gait analysis: (b) Walking speed. There was no significant difference between transgenic and wild type in the crossing time. Significant differences were found in static parameters (c) NSSP; Cruciate a; Cruciate b, Alternate Ab (d) Base of support (B.O.S) of the front paws width, (e) Intensity, and dynamic parameters: (f) Max.contact at of the hind paws, and (g) Phase of dispersion; Diagonal RF → LH and Ipsilateral LF→ LH. See Methods for a full description of these parameters. Asterisks indicate significant differences between control (+/+) and transgenic (+/T) mice (*p < 0.05; **p < 0.01 and ***p < 0.001). Note: RF, right forelimb; RH, right hindlimb; LF, left forelimb; LH, left hindlimb; R.index, Regularity index.

With respect to the dynamic parameters, no significant difference in stance and swing phases for both front and hind-limbs were found (data not shown). Transgenics had a shorter stand/propulsion time with their hindlimbs (Fig. 2f; max contact at (%), t= 2.245, P< 0.05). There was no significant difference for the forelimbs. We further analyzed the parameter which describes the temporal relationship between placements of two paws within a step cycle, that is, the phase dispersions. Transgenic mice show a higher

percentage of dispersions for the diagonal RF-LH (t= 3.316, P< 0.01) and ipsilateral LF-LH (t= 4.578, P< 0.001) phases (Fig. 2g). There was no significant difference in the girdle paws pairs dispersions phases or in the diagonal LF-RH and ipsilateral RF-RH.

- Anxiety-like behavior in a zero-maze test (Fig. 3a-c)

We first investigated the behavior of the animals in the elevated zero maze which represents a conflict test: on the one hand rodents have a natural tendency to explore a novel environment; on the other hand they prefer to remain in confined spaces. Transgenic BACHD mice spent significantly more time in the closed sectors (t= 2.852, P< 0.05) and less time in the open sectors (t= 2.871, P< 0.05) than wild type mice (Fig. 3a). Also, there was a significant difference between wild type and transgenic BACHD mice in the distance moved in open sectors (Fig. 3b; t= 2.459, P< 0.05). There was no significant difference between groups in frequency of entrance in open and closed arms (Fig. 3c).

- Freezing expression in a fear conditioning test (Fig. 3d)

Next, we moved on to a different kind of anxiety test, not based on the conflict paradigm: fear conditioning. There was no significant difference in freezing response during habituation (base line) and new context. Transgenic mice showed a significantly stronger freezing response to a tone stimulus (Fig. 3d; t=3.486, P< 0.01 Bonferroni post test). Two-way ANOVA revealed a significant effect for the factors GENOTYPE (F= 7.61, P< 0.01) and TEST (F= 10.47, P< 0.0001). There was no statistically significant interaction (GENOTYPE X TEST). Although visual inspection of Fig.3d suggests that transgenics present higher freezing to the context, this effect was not statistically significant (t = 1.767 and P > 0.05 Bonferroni post hoc test).

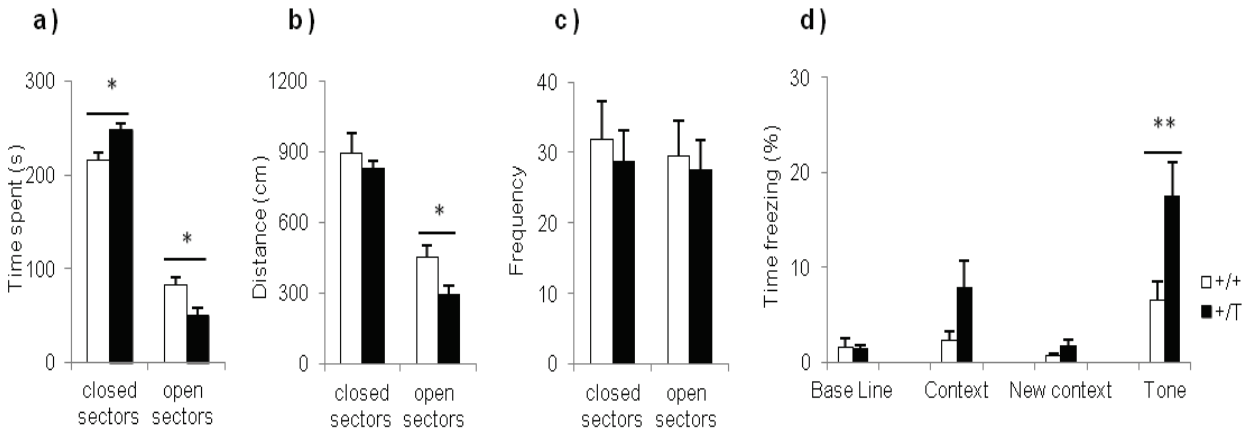


**Fig. 3** Results are expressed as Mean ± SEM. Zero maze test: (a) time spent, (b) distance moved and (c) frequency of entrance in closed and open sectors of the maze. Transgenic mice (+/T) spend less time and moved less in the open sectors compared to their wild type (+/+). However, no significant difference was seen in frequency of entrance in both sectors. (d) Fear conditioning. Transgenic mice spent significant more time freezing to the tone (cue) compared with the wild type littermates (+/+). A tendency for increased freezing to the context is observed in transgenic mice. Asterisks indicate significant differences between +/+ and +/T mice (*$p < 0.05$, **$p < 0.01$).

- Learning and memory response in a strategy shifting task: the cross-maze test (Fig. 4)

Data from acquisition and reversal training sessions at the first 2 days were not analyzed because the goal arm was baited with sucrose food pellets to guide mice to their home cage.
A schematic representation of the cross-maze task is presented in Fig. 4a.

- *Learning index* (Fig. 4b)

The learning index is calculated as the ratio of the mean number of correct choices over trials per animal. During acquisition, transgenic mice displayed improved learning as compared to the wild type littermates. During reversal, transgenic mice showed a lower

learning index; however, the difference between wild type and transgenic mice did not reach statistical significance.

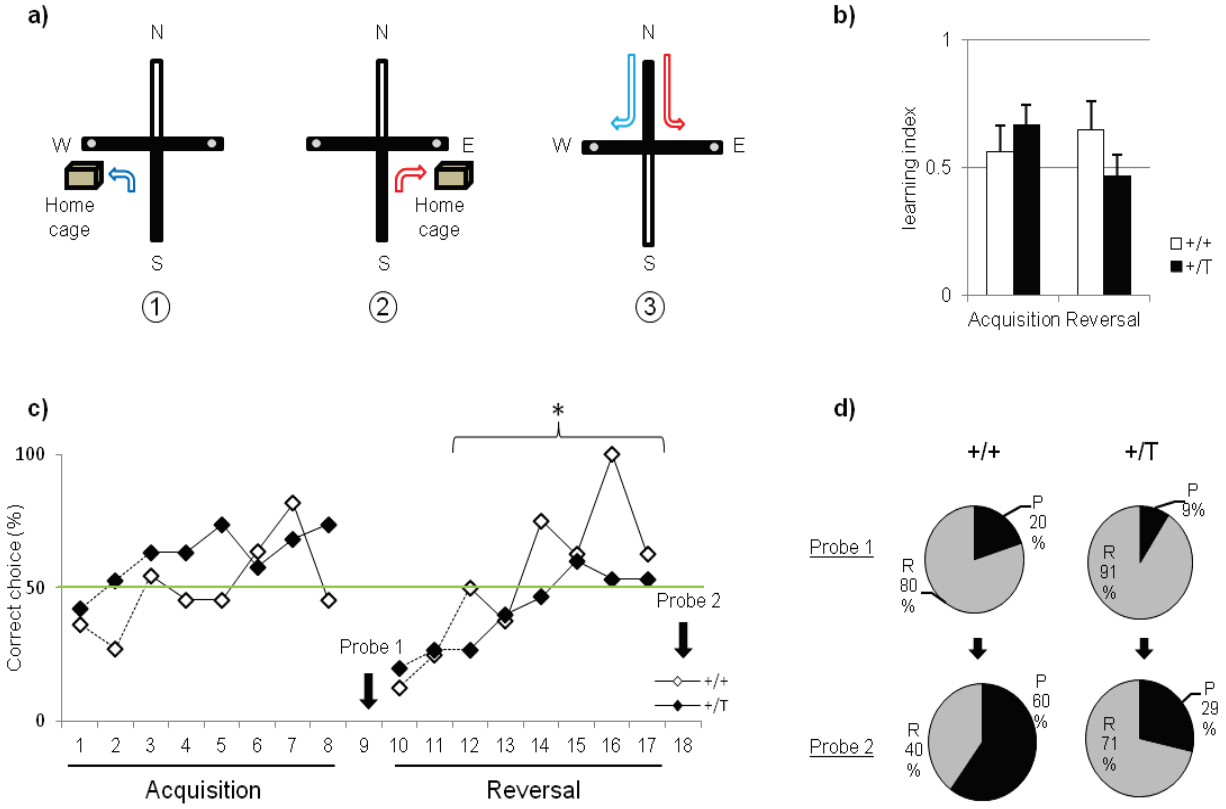


**Fig. 4** Strategy shifting task. (a) Schematic representation of the cross-maze task. The north (N) arm is closed. The mouse starts training in the south arm (S) and reaches the home cage through the hole located in the west arm (w, (1) acquisition) or east arm (E, (2) reversal). During (3) probe trial days (9 and 18), the (S) arm is closed and the mouse starts in the (N) arm. Mice reaching the usual home cage arm are Place learners, while those reaching the other arm are Response learners. (b) The learning index. (c) Acquisition and reversal training. Percentage of correct choice made during trainings is depicted. There was no difference in acquisition training. Transgenic mice (+/T) differed significantly from wild type (+/+) during the reversal training. (d) Strategy shifting on Probe trials. Wild type mice (+/+) tended to shifted from a Response learning (R) (probe 1, R = 80%) to a Place learning (P) (probe 2, P = 60%) strategy, while transgenic BACHD mice (+/T) persevered in the same strategy of response (R) (probe 1, R = 91% and probe 2, R = 71%). Asterisks indicate significant difference between transgenic (+/T) and control (+/+) mice during reversal learning (*$p < 0.05$, between day 12 and day 17).

- *Acquisition and reversal training* (Fig. 4c)

The effect of acquisition and reversal learning in both groups was investigated by analyzing the number of correct choices made. There was no significant difference in acquisition learning. For reversal learning, only animals with learning index higher than 0.5 during acquisition were analyzed. That is, animals that actually learned the task. Wild type – but not transgenic - animals made significant more correct choices over the course of reversal learning ($\chi 2 = 6.153$, $P < 0.05$). Transgenic mice made fewer correct choices during reversal training as compared to acquisition training ($\chi 2 = 8.251$, $P < 0.01$). Wild type mice did not show differences in their correct response choice during acquisition and reversal trainings.

- *Strategy* (Fig. 4d)

On days 9 and 18, a probe trial was done to assess which strategy mice used to solve the task. The new start arm was now at the opposite site of the arm that the mice were initially trained on. Mice entering the same arm as during training sessions were designated place learners (allocentric learning) and mice entering the opposite arm were designated response learners (egocentric learning). Data were only analyzed for animals that made correct arm choices and had a learning index interval greater than 0.5 during each training session.

At the first probe trial (day 9), both wild type and transgenic mice exhibited a preference for response learning. At the second probe trial (day 18), transgenic mice maintained a response learning preference (71% of mice). Although wild type mice shifted more towards a place learning strategy (60% of mice), the strategy shift between both probe sessions was not statistically significant.

## 4. Discussion

We compared the BACHD transgenic mice to their wild type littermate controls in a series of standard and novel behavioral tests assessing sensory-motor, emotional and cognitive functions. BACHD mice showed a clear motor imbalance and gait coordination problems in both static and dynamic walking sequences. Zero maze and fear conditioning results demonstrated that transgenic mice are more anxious and present a higher freezing response to stressful stimuli. In the cross maze, we found cognitive impairment that may be related to striatal-frontal dysfunctioning in transgenic mice, especially in reversal learning.

- *Motor deficits*

At an early stage of the disease, assessment of motor dysfunction is an important component in the diagnosis of HD (Harper, 1996). Measurement of motor function in BACHD mice was therefore a logical starting point for our comprehensive behavioral phenotyping effort. The shorter latency to fall off the Rotarod is consistent with previous findings and indicates that BACHD mice have difficulties in balancing (Gray et al. 2008; Menalled et al. 2009). The lack of significant difference between both groups at week four could be due to an outlier and does not alter the general conclusion that, the Rotarod deficit persists for up to 12 months in BACHD mice. The subsequent slight increase in latency upon repeated testing suggests a learning process in both transgenic and wild type mice (Pouladi et al. 2012).

Deficits in gait were found during Catwalk testing for both static and dynamic parameters. These deficits are unlikely to be confounded by differences in weight and/or the health status of the mice. Weight bearing changes in limbs can be objectively evaluated with the intensity parameter, a measure of the mean brightness of all pixels at maximum paw contact with the glass plate (Masocha and Parvathy, 2009). We did not find a difference in measured intensity, notwithstanding the fact that transgenic mice are

heavier than wild type mice. These data suggest that BACHD mice have no arthritis or abnormal nociceptive activity in weight load changes during walking.

Static gait analysis revealed variability in step sequences in transgenic mice and a large width of their front limbs B.O.S. Dynamic gait analysis showed deficits in phase of dispersion and at the point where the breaking phase turns into propulsion phase.

Other labs have used different equipment for the assessment of gait deficits. For example, using an ink-footprint test in R6/2 transgenic mice, Carter (1999) found that the front base was significantly broader and more splayed, starting at 8-9 weeks of age. Similar observations were also reported in R6/2 and BACHD transgenic mice with shorter/longer splay, wider base and sensory-motor gaiting deficits (Menalled et al. 2009; Pouladi et al. 2012). Amende (2005) looked at motor behavior in a treadmill in a striatal neurodegeneration mouse model for HD using the mitochondrial toxin, 3-nitropropionic acid (3-NP). Mice repeatedly administered with a cumulative dose of 50 mg/kg of 3-NP showed a higher stance width variability in their forelimbs and a *greater* stance duration spent in propulsion (stance/propulsion) phases in their hind limbs. We observed *shorter* stance/propulsion duration in the hindlimbs of BACHD mice. This discrepancy may involve differences in methods such as voluntary vs. forced walking, and the HD animal models that were used. In contrast to the Catwalk, mice submitted to a motorized treadmill are 'forced' to walk and a deficit in walking could have affected their gait. The mechanism by which 3-NP induce striatal neurodegeneration in animals is not yet well elucidated and this model does not mimic specific morphological and neurochemical features of the progressive neurodegeneration seen in the early stage of HD (Tunez et al. 2010).

An attractive feature of gait analysis with the Catwalk model is that similar parameters can be measured in humans. Indeed, similar deficits in gait parameters were reported in HD patients (Koller and Trimble, 1985). The larger distance between forelimbs (B.O.S) and the ipsilateral phase of dispersion (LF-LH) in BACHD mice may correspond to the wide base station and the lateral swaying in patients (Tian et al. 1992).

A more profound understanding of the functional neurocircuitry that underlies the motor behavior assessed by Rotarod and Catwalk testing would greatly add to the translation of the mouse findings to humans. Available evidence supports, perhaps not

surprisingly, a key role for striatal areas. A bilateral projection from motor cortex to striatum is well-documented in rat (McGeorge and Faull, 1989). Rats chronically treated with 3-NP showed neurodegeneration in the dorsolateral striatum but not in the hippocampus, thalamus, cerebral cortex and cerebellum (Guyot et al. 1997). They develop dystonia and bradykinesia as well as gait abnormalities such as decreased tangential velocity and higher peak lateral velocity (parameters related to position of the animal's centroid as a function of time during the board cross). A study using 2- deoxy-D-[14C] glucose autoradiography in rats, revealed activation of different areas in the striatum during stimulation of fore/hind limbs (Brown, 1992). In addition, an EMG study on the gastrocnemius muscle/soleus muscle using intrastriatal injections of drugs affecting the dopamine, acetylcholine and gamma-aminobutyric acid (GABA) neurotransmission suggested a functional flow of information from the rostral to the intermediate part of the striatum (Ellenbroek et al. 1986). Thus, the striatum is not only important for the actual execution and control of motor behavior but has also a role that overlaps with that of the primary motor cortex.

In a next step, abnormalities in static and dynamic phases of gait that we observed in transgenic BACHD mice should be correlated to striatal dysfunction. For example, using imaging technologies such as fMRI in awake BACHD mice it is possible to reconstruct distributed, integrated neural circuits or 'fingerprints' of brain activity (Ferris et al. 2011). Interestingly, a marked motor phenotype is observed notwithstanding a lack of huntingtin aggregates and no loss of medium spiny neurons at 9 and 10 months of age in BACHD mice (Gray et al. 2008; Kordasiewicz et al. 2012; Pouladi et al. 2012; Southwell et al. 2009). This is apparently not unique for rodent models as there are also reports of HD patients that show (chorea) motor abnormalities in the absence of neural loss (Myers et al 1988). These findings support the suggestion that an animal model of HD may not only provide relevant genetic features but may also develop progressive neurological phenotype as seen in early HD. This facilitates comparisons between species and the validation of animal models and molecular drug targets.

- *Emotional deficits*

Transgenic mice spend less time in the open, “unsecure”, arms and this is unlikely to involve a deficit in locomotor activity because the groups did not differ in the total frequency of entries into open or close arms. The results from the zero-maze are consistent with the anxiety-like phenotype reported in a light-dark choice test (Kordasiewicz et al. 2012; Menalled et al. 2009).

In the fear conditioning test, the tone stimulus elicited a higher level of freezing in BACHD than wild type mice. Again, confound by motor deficits seems unlikely since the mice did not show a difference in percentage of time freezing during habituation (base line) and when presented with a new context (Fig. 3d). Although visual inspection of Figure 3D suggests that the conditioned fear response to the context was higher in the transgenic mice, this difference was not statistically significant. It cannot be excluded that this is confounded by the low intensity of the unconditioned stimulus (foot shock) as the overall mean percentage of freezing was very low (15% to 20 %). Results obtained in other HD models have been mixed: A *deficit* in contextual fear-conditioning was shown in R6/2 mice whereas an *enhanced* fear response was reported in CAG140 Knock-In (KI) mice (Bolivar et al. 2003; Hickey et al. 2008). The reasons for the different findings are not well understood but may involve differences in fear conditioning methods and progression of the disease in different models (Blanchard et al. 2003; Fendt and Fanselow, 1999). This illustrates the challenge of replicating fear conditioning results across laboratories. A comparative study using the same protocols for the different HD models in the same lab would be the preferred way to reach a definite conclusion on the fear conditioning phenotype in HD models.

Nonetheless, our data are consistent with the high anxiety rate described with the Neuropsychiatric inventory (NPI) in HD patients (Paulsen et al. 2001b), suggesting that our fear conditioning protocol in BACHD mice may have translational value.

- *Cognitive deficits*

Navigation in complex environments involves the use of different memory strategies. In the cross-maze task, subjects can either make a body turn response (egocentric learning), or use spatial cues (allocentric learning) to locate their home cage at the end of a dedicated arm. All mice acquired the task and used an egocentric learning strategy during the first probe trial. Results from other studies found that the allocentric strategy is normally adopted by rodents during acquisition learning (Botreau and Gisquet-Verrier, 2010; Restle, 1957; Tolman et al. 1946). It is not clear why our mice used an egocentric strategy during acquisition. Maybe the navigational cues (polystyrene expanse foam cross, small plastic container, Christmas tree star and cardboard cube) that we used were not strong enough, despite their validation for the MWM. However, Espina-Marchant and colleagues (Espina-Marchant et al. 2009) has demonstrated in a cross maze paradigm that A x C rats (phenotypically similar to wild rats) injected with saline solution in Neostriatum presented egocentric learning strategy during acquisition and extend training (probe days 11 and 19), whereas Long-Evans rats shifted from allocentric to response learning strategy. These findings indicate that a specific learning strategy in the cross-maze could be strain-dependent.

Transgenic mice made more errors during reversal learning which is not surprising as reversal learning is more demanding than acquisition learning. The former requires a) memory of the previous task learned, b) suppression of this acquired learning and c) learning of the new task. Our data are in line with reports in YAC 128 mice; they displayed reversal learning deficit in a water T-maze task from 27 weeks of age (Brooks et al. 2012; Van Raamsdonk et al. 2005). The perseverative strategy response we observed in BACHD transgenic mice during probe trials are similar to that reported by Lione and colleagues (1999) in R6/2 mice during a spatial alternation T-maze test; they have demonstrated that transgenic mice, in contrast to control turned in the same direction in the maze irrespective of their start position and this deficit was present as early as 5 weeks of age.

Clinical investigation in early HD patients has revealed impairment in the shifting but not the formation or maintenance of a learned response set (Lawrence et al. 1996). The

perseverative responding strategy during reversal training in transgenic mice is reminiscent of the cognitive deficits seen in HD patients. HD patients show deficits in the extradimensional shift (EDS) test and in the Wisconsin Card Sorting Test (WCST), when attention has to be shifted from one perceptual dimension to another. This cognitive set shifting behaviour is mediated by the prefrontal cortex (Josiassen et al. 1983; Lawrence et al. 1999). The mosaic organisation of striatum suggests that its dorsal regions receive input primarily from dorsal prefrontal cortex, and the ventral regions of the caudate to receive input from limbic-related areas (Gerfen et al. 1992). Examination of neostriatal tissue from autopsy cases of early HD revealed a progressive wave of neural loss. In fact, immunocytochemical stains showed that this neuronal loss extended from the dorsal to the ventral regions of striatum in later stages of the disease (Hedreen and Folstein, 1995). Thus, reversal and strategy shift deficits could be attributed to disruption of the prefrontal-striatal activity.

Different brain regions, such as the medial temporal lobe, the prefrontal cortex, the basal ganglia or the amygdala complex, are important for the consolidation of different memory domains and memory flexibility. Devan (1999) has demonstrated after different lesions to the rat striatum that the medial part may underlie allocentric learning. In addition, it has been reported that the hippocampus and the striatum selectively mediate expression of allocentric and egocentric learning strategies, respectively. A shift from a place to a response strategy can occur in the course of extended training (Chang and Gold, 2004; Packard, 1999; Packard and McGaugh, 1996; Pych et al. 2005; Yin and Knowlton, 2004). These findings suggest that memory functions are mediated by distributed networks in the brain where key neuroanatomical substrates such as the hippocampus, the striatum and the prefrontal cortex interact with each other.

## 5. Conclusion

Our study replicates and extends published results in adult BACHD mice. We showed that BACHD mice have motor imbalance and gait coordination problems in both static and dynamic walking sequences. The results of the zero maze and of the fear conditioning tests, demonstrated that transgenic mice are more anxious and present a higher freezing response to stressful situations. Finally, using the cross maze, we could demonstrate cognitive impairment related to a striatal-frontal dysfunctioning in transgenic mice especially in reversal learning. In the future the same study would be interesting to address in female BACHD mice. Nevertheless, our data indicate that BACHD mice show several of the symptoms found in HD patients. The results suggest that these animals are suited to investigate the developmental time course of these deficits in relation to motor deficits and for the assessment of potential novel therapeutics for the treatment of HD.

# Study 2

# Assessment of motor function, sensory motor gating and recognition memory in a novel BACHD transgenic rat model for Huntington disease

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**Abstract**

*Rationale*

Huntington disease (HD) is frequently first diagnosed by the appearance of motor symptoms; the diagnosis is subsequently confirmed by the presence of expanded CAG repeats (> 35) in the *HUNTINGTIN* (*HTT*) gene. A BACHD rat model for HD carrying the human full length mutated *HTT* with 97 CAG-CAA repeats has been established recently. Behavioral phenotyping of BACHD rats will help to determine the validity of this model and its potential use in preclinical drug discovery studies.

*Objectives*

The present study seeks to characterize the progressive emergence of motor, sensorimotor and cognitive deficits in BACHD rats.

*Material and methods*

Wild type and transgenic rats were tested from 1 till 12 months of age. Motor tests were selected to measure spontaneous locomotor activity (open field) and gait coordination. Sensorimotor gating was assessed in acoustic startle response paradigms and recognition memory was evaluated in an object recognition test.

*Results*

Transgenic rats showed *hyper*activity at 1 month and *hypo*activity starting at 4 months of age. Motor coordination imbalance in a Rotarod test was present at 2 months and gait abnormalities were seen in a Catwalk test at 12 months. Subtle sensorimotor changes were observed, whereas object recognition was unimpaired in BACHD rats up to 12 months of age.

*Conclusion*

The current BACHD rat model recapitulates certain symptoms from HD patients, especially the marked motor deficits. A subtle neuropsychological phenotype was found and further studies are needed to fully address the sensorimotor phenotype and the potential use of BACHD rats for drug discovery purposes.

## 1. Introduction

Huntington disease (HD) is a progressive neurodegenerative disorder that is associated with widespread degeneration of cortical neurons and striatal medium spiny neurons (MSN) (Douaud et al. 2006; Jones and Hughes, 2011; Vonsattel et al. 1985). The neuronal loss is caused by an expanded polyglutamine tract (> 35 CAG) in the *HUNTINGTIN* (*HTT*) gene on chromosome 4 (Huntington's Disease Collaborative Research Group, 1993). Huntingtin (htt) has many functions in cells and is essential for development (Bezprozvanny et al. 2004; Caviston and Holzbaur, 2009; Cisbani and Cicchetti, 2012; Trushina et al. 2004). It is not well-known why MSN neurons are selectively vulnerable in HD (Zheng and Diamond 2012). A wide variety of motor, cognitive and neuropsychiatric symptoms have been observed in HD patients (Di Maio et al. 1993; Myers et al. 1985). As the disease progresses, patients become completely dependent and eventually require full-time care. To date, clinically proven treatments that can cure or halt the disease's progression have not yet been discovered.

Important insights into the pathogenic mechanisms in HD were gained by the development and use of multiple transgenic murine models. At present, a number of knock-in and transgenic mouse models, expressing the N-terminal fragment of HD exon 1 (e.g. R6/2 mice, (Mangiarini et al. 1996) or the full-length endogenous / human mutant htt (such as the BACHD mice, (Gray et al. 2008) provide ample opportunities to study the chronically progressing phenotype of the disease (Crook and Housman, 2011). A good animal model should reflect as many of the neuropathological and clinical symptoms of the human disease as possible (Bowles et al. 2012). The BACHD mouse model of HD, for example, recapitulates several aspects of the neuropathology and symptoms, including formation of mutant htt positive aggregates, reduction in cortical BDNF mRNA expression levels, cognitive, emotional, motor and sensory gating deficits (Abada et al. 2013; Gray et al. 2008; Kordasiewicz et al. 2012; Menalled et al. 2009; Pouladi et al. 2012; Southwell et al. 2009). Notwithstanding the value of existing mouse HD models, certain behavioral processes related to learning and memory and pharmacological validation are typically more challenging to evaluate in mice (Tecott

and Nestler, 2004). This is one of the reasons why the availability of HD models in other species is important. Certain aspects of the behavioral repertoire of rats are more adequate and/or accessible for assessment in specific tasks, such as in learning and memory models. A BACHD rat model of HD has recently been established. This rat model is of particular interest since it expresses, like the BACHD mouse, the full length human mutant huntingtin (fl-*mhtt*) with 97 CAG-CAA mix repeats under control of the human HD promoter gene (Yu-Taeger et al. 2012). This model has not been fully characterized yet and this was the aim of the present studies that are described below.

We first wanted to get a detailed description of possible gait abnormalities in BACHD rats because HD patients have motor impairments such as gait deficits, imbalance, clumsiness and unsteadiness (Di Maio et al. 1993). In this study, we used the Catwalk, an automated video-computer based system that detects and measures a range of spatial and temporal aspects of rodent's inter-limb coordination during free walking (Hamers et al. 2001). Also, we wanted to replicate motor imbalance reported in BACHD rats with a Rotarod test (Hamm et al. 1994; Yu-Taeger et al. 2012). The Rotarod has become an essential tool for assessment of motor coordination and balance in rodent models of HD (Carter et al.1999; Gray et al. 2008; Menalled et al. 2009; Von Horsten et al. 2003; Woodman et al. 2007). Finally, we employed an Actimot apparatus to evaluate exploratory locomotor activity of BACHD rats in an open field.

The progressive degeneration of striatal MSN in HD patients may result in deficits in sensorimotor gating inhibition. This 'gating' inhibition can be measured in patients by exposing them to startle stimuli. The startle reflex response elicited by either tactile or acoustic stimuli is typically inhibited when it is preceded by a weak prepulse. Interestingly, Swerdlow and colleagues (1995) have reported prepulse inhibition (PPI) deficits in HD patients. In animals, startle response and PPI can be derived from the assessment of whole body movement following exposure to auditory or visual stimuli (Menalled et al. 2009; Pouladi et al. 2012; Swerdlow et al. 1992). Since the PPI test is of particular interest for its 'translational' value, we investigated the acoustic startle response (ASR) and prepulse inhibition in BACHD rats.

Neuropsychological assessments found that, in general, cognitive impairments start prior to the emergence of motor deficits, although these are generally diagnosed later

(Hahn-Barma et al. 1998; Lawrence et al.1998). Episodic memory – that is, the memory for events which is described as a spatio-temporal record of a subject's experience - is impaired in HD patients. Some of the most common tests used to measure episodic memory are based on auditory and verbal learning [Rey auditory verbal learning test (RAVLT), California verbal learning test (CVLT), Wechsler memory scale (WMS)], or pattern and spatial recognition. Results from recognition memory performance in patients have led to mixed results and seem to depend on the task that is used. For example, verbal recall memory impairment was reported in the CVLT and WMS tests (Rosenberg et al. 1995); whereas performance in spatial recognition memory was intact (Lawrence et al. 1998). A meta-analysis conducted on a computer-based search in presymptomatic, symptomatic and control subjects has revealed deficit in recall-recognition memory in HD (Montoya et al. 2006). In addition, investigations made in a tgHD rat model have showed spatial and recognition memory deficits at 16 months of age (Zeef et al. 2012a). Although there seems to be a clear impairment in recall-recognition memory in HD patients and tgHD rats, there is a further need to clarify recognition memory performance. Therefore we evaluated the recognition memory in BACHD rats in a novel object recognition task (ORT).

## 2. Materials and methods

- Ethics statement

The study was carried out in strict accordance with the German animal welfare act and the EU legislation (EU directive 2010/63/EU). The protocol was approved by the local ethics committee *Behörde für Gesundheit und Verbraucherschutz* (BGV, Hamburg).

- Husbandry and genotyping

Wild type (+/+, WT) and transgenic (+/T, TG) male BACHD rats carrying the mutant human huntingtin gene, under the control of the human huntingtin promoter and its regulatory elements were used. The transgene contains 97 CAG-CAA mix repeats, and

additional 20 kb upstream and 50 kb downstream sequences ensure stability of the repeat length (Yu-Taeger et al. 2012). Two transgenic males (TG5 line) were supplied from the original BACHD colony of the Universitätsklinikum Tübingen (Tuebingen, Germany) and an in-house breeding colony was established and maintained at EVOTEC AG (Hamburg, Germany) by cross-breeding these males with wild type female rats. BACHD animals were maintained on a Sprague-Dawley background. All the animals at weaning were group-housed 2 to 4 per cage with wood shavings and a filter top. The environment was enriched with a play tunnel and shredded paper. BACHD rats were maintained in climate controlled housing, with a 12-h reversed dark/light cycle (light from 19:00 to 07:00). Rats had free access to food and water except during experiments.

Ear punches were collected from the litters at weaning in order to determine the rats genotype. Genotyping was performed before and after all the studies using a validated protocol. Briefly, genomic DNA was prepared from ear biopsy tissue using proteinase K digestion, followed by phenol/chloroform extraction (Qiagen DNeasy Tissue kit). Primers flanking the polyQ repeat in exon 1 were designed to recognize whether or not the rat carried at least one copy of the mutation, and were used to PCR amplify the polyQ regions [Q3: 5' – AGG TCG GTG CAG AGG CTC CTC - 3' and Q5: 5' – ATG GCG ACC CTG GAA AAG CTG - 3']. Gene status was confirmed in parallel by using designed primers from Tuebingen [exon 1: FW 5'-ATG GCG ACC CTG GAA AAG CTG- 3' and RV: 5' -AGG TCG GTG CAG AGG CTC CTC- 3'; exon 67: FW 5'-TGT GAT TAA TTT GGT TGT CAA GTT TT- 3' and RV: 5' –AGC TGG AAA CAT CAC CTA CAT AGA CT-3']. The PCR product was run on an automated apparatus PTC-200 (Peltier Thermal Gradient Cycler) and the Agilent 2100 Bioanalyser (Agilent technologies) was used to determine the fragments' size.

- Behavior testing

All the behavioral tests were performed during the dark phase and male BACHD rats were weighed twice per month. Acoustic startle experiments and object recognition tests were undertaken independently on all animals of a cohort at a specific age. For other tests, subgroups of either one or more cohorts were allocated for longitudinal

testing. Before each behavioral test, animals were given a 1h minimum habituation period to the testing room.

*Exploratory behavior*

Spontaneous locomotor activity was evaluated with the automated Actimot system (TSE system, Germany). The apparatus consists of a square shape frame equipped with a transparent cage (50 $cm^3$) and two pairs of light-beam strips. Each strip is equipped with 32 infrared sensors and their height can be adjusted. Thus, the coordinates of the animal can be determined in three dimensions (X-Y-Z axis). The apparatus was cleaned and dried with a 10% ethanol solution before each use. Each rat was placed in the center of the box and the number of beam breaks recorded during free walking for a 1 hour testing period. We examined and analyzed the total activity (X+Y beam breaks) directly from the data collected by the system software.

*Rotarod*

Motor coordination and balance was assessed using a rotarod apparatus (Med Associates, Italy). All rats underwent a 3-day training program by which time a steady baseline level of performance was attained. During that period, rats were trained to walk against the motion of a rotating drum at a constant speed of 12 R.P.M (rotations per minute) for a maximum of 2 min. In total, four training trials per day with an interval trial time of one hour were performed. Rats falling off during a training trial were put back on the rotating drum. Following the training days, a one day test of three trials was performed using an accelerating speed levels (4 to 40 R.P.M) mode of the apparatus over 5-min. The apparatus was wiped with a 70% ethanol solution and dried before each trial. The mean latency to fall off the rotarod was recorded and rats remaining on the drum for more than 300 s were removed and their time scored as 300 s.

*Catwalk system*

The gait analysis system Catwalk 7.1 (Noldus IT, the Netherlands) consisted of an enclosed walkway with a glass plate and a speed video recording camera. Gait performance was assessed and recorded using the catwalk analysis software. The glass plate was cleaned and dried with a 70% ethanol solution before each use. On the first day, rats were habituated to the apparatus for 300 s with the goal to cross the walkway. The following day, free runs across the walkway were recorded. From among the correct runs, three runs per animal were selected randomly for analysis. A correct run was defined as one complete (60 cm) crossing of the walkway without interruption. For one crossing, a rat needs a minimum of 4 to 5 step sequences patterns. Runs with step sequence categories related to exploration, Rotate Ra (RF-LF-LF-RH) and Rotate Rb (LF-RF-RH-LH) were not analyzed (RF= right forelimb; RH= right hindlimb; LF= left forelimb; LH= left hindlimb).

The Catwalk parameters are: walking speed (measured as the average of strides in cm/s); the normal step sequences patterns (NSSP, i.e. the order in which the four paws were placed); base-of-support (B.O.S, i.e. distance between two hind or fore paws, as measured perpendicular to the walking direction); stride length (i.e. distance between the placement of a hind or fore limb and the subsequent placement of the same limb); print position (space relation between a fore and a hind paw of the same side in mm); regularity index [RI, i.e. an index for the degree of interlimb coordination during gait, as measured by the NSSP, multiplied by four (number of paws), divided by the number of limb placements, and multiplied by 100%]; stance (i.e. time of contact of the hind or fore limbs with the glass floor); swing (i.e. time that the hind or fore limbs are not in contact with the glass plate); phase of dispersion [i.e. the timing relationships between paw placements. It is expressed as percentage of time of initial contact of one paw (the target) related to the stride length of another paw, the anchor]; max contact (at) (i.e. the point where the breaking phase turns into propulsion phase); intensity (measured as the mean brightness of all pixels of the print at maximum paw contact ranging from 0 to 255 arbitrary units).

Based on the previous study in BACHD mice (Abada et al. 2013), the hypothesis was to investigate gait abnormalities in the rat model with the same construct. Therefore we focused on the same parameters we identified in the BACHD mice study. Some of the selected parameters were: the normal step pattern sequences, the width between forelimbs placements, stand/propulsion time with their limbs and timing relationships between paw placements. A full description of the apparatus, parameters and analysis is provided by Hamers and colleagues (Hamers et al. 2001).

*Object Recognition Test (ORT)*

Our ORT protocol was similar to a previously described (Zeef et al. 2012a). The apparatus consisted of a circular arena, which was 80 cm in diameter. The floor of the arena and the 35 cm high wall was made of grey polyvinyl chloride.
During testing periods, the experimental room was homogenously illuminated (~ 20 lux). Two objects were placed in a symmetrical position about 20 cm away from the wall. In each trial the objects were placed on the exact same location. Two different sets of objects were used, which had no natural significance or possible association for the rats. The different sets used were: (1) three food cans made of metal painted in blue (diameter 7 cm, height 11 cm), (2) three metal tee caddies cubes (8.5 cm × 8.5 cm × 11 cm, coffee background color) with different shape and size letterings. The objects were secured to the floor to prevent rats from displacing them. On the first day, rats were habituated to the arena without objects for 5 min. In the period preceding the testing, rats were adapted to the arena and all objects 5 min/day for two days.
An ORT testing session (day 4) comprised of two trials (T1 and T2) and the duration of each trial was 3 min. During T1 the arena contained two identical objects. A rat was always placed in the arena facing the wall. After the first exploration period (T1) the rat was returned to its home cage. Subsequently, after a retention interval of 90 min, the rat was put back into the arena containing a familiar (a replicate of the object) and a novel object (T2). The time spent exploring each object during T1 and T2 was recorded with the help of a video tracking system (Ethovision, Noldus). The retention interval time of 90 min was chosen to ensure a reliable recognition memory in WT rat. Exploration of an object was defined as directing the nose towards the object at a distance of no more

than 2 cm and/or touching the object with the nose. Sitting on or leaning on an object was not considered as exploratory behavior. In order to obtain reliable results in this task, sufficient exploration time of the objects is critical. The cut-off point for sufficient exploration time was set at 7.5 s total exploration time (both objects) per trial. Since rodents can discriminate between objects based on olfactory cues, the objects were thoroughly cleaned and dried after each trial with a 10% ethanol solution. All combinations and locations (left and right) of the objects were used in a balanced manner in order to prevent potential bias due to preferences for particular locations or objects.

The following variables were calculated:

- (e1) is the amount of the time (in s) spent in exploring both identical objects (a1 and a2) during T1: e1 = a1 + a2
- (e2) is the amount of the time (in s) spent in exploring both the familiar (a3) and new object (b) during T2: e2 = a3 + b
- d1 and d2 correspond to the ability to discriminate between the old and new object during T2: d1 = b − a3, and
  d2 = (b − a3) / e2 (which is d1 corrected for the total exploration time during T2).

A negative d2 indicates a preference for the familiar object; a value of zero indicates no preference, and a positive d2 indicates a preference for the novel object.

*Acoustic Startle Response (ASR)*

The experiments took place in standard “Prime” isolation cabinets from San Diego Instruments (SD Instruments, California). Each ventilated chamber contains a loudspeaker at the top and a cylindrical animal enclosure (10 cm diameter and 20 cm length) made with Plexiglas and mounted on a plastic frame. A piezoelectric accelerometer was mounted under the plastic frame to record and transduce the motion of the tube. All chambers were cleaned with a 70% ethanol solution and dried before each use. Rats were individually placed in the startle enclosure and the resulting movement of the rat was measured during 100 milliseconds (ms) after startle stimulus onset. The response was rectified, amplified and digitized into a computer which calculated the maximal response over the 100 ms period. All rats were habituated for 5-

min during which a 70 decibels (dB) background white noise was present. After this habituation period, one of three different experiments was performed similarly to established protocols on independent cohorts: (Chabot and Taylor, 1992; Ellenbroek et al. 2004).

- Prepulse Inhibition (PPI)

Rats underwent a test session of 51 trials consisting of: eighteen startle pulse, twenty-four prepulse and nine no stimulus trials where no acoustic pulses were delivered. The startle pulse (P120) consisted of a single 120 dB [A] white noise burst lasting 40 ms. Five pulse-alone (P120) stimuli were presented at the beginning and at the end of the test session. Three types of auditory prepulse at 3, 6, 12 dB above the background noise (70 dB) were presented in random order, followed by a single P120. Each prepulse lasted 20 ms and the prepulse-pulse interval time was 80ms. An average of 16 s (ranging from 10 to 20 s) separated consecutive trials and the total session was approximately 17 min. PPI was calculated as the percent decrease of the ASR in pulse-alone trials compared to the ASR in prepulse-pulse trials [100× ((pulse-alone trial) – (prepulse-pulse trial))/pulse-alone trial].

- Startle Habituation

Rats were subjected to 50 startle trials with an intertrial interval varying between 10 s and 20 s. The startle trial (P120) consisted of a single 120 dB [A] white noise burst lasting 40 ms. Data were analyzed as mean of 10 trials in 5 blocks (BL).

- Startle Threshold

Differences in ASR magnitude for different stimulus intensities (dB) were assessed. Rats received 60 startle trials with varying dB levels ranging from 80 to 120 dB [A] in 10-dB increments, and a no stimulus-free trial (70 dB background noise) per 10 trials. These stimuli lasting 40 ms were randomly assigned to trials within each set of 10 trials.

The mean response for all trials of a given dB level, including trials for the 70 dB background white noise for each individual rat was calculated.

*Statistical Analysis*

All data are shown as the mean ± SEM. *Graph Pad* and *InvivoStat* software were used to perform all analyses. Differences between groups were assessed with Student's t-test or mix ANOVA with repeated measures, with the factor GENOTYPE as between subject and TIME, AGE, INTENSITY or TRIAL as within subject variable. When significance was found, a Bonferroni - post hoc analysis was performed where appropriate. In the ORT test, to evaluate the preference of each genotype, we performed a one sample t-test on the discrimination index. The hypothetical value was set at zero. The significance level was set at 0.05.

## 3. Results

Gross inspection of each BACHD rat prior to the experiments did not reveal any visible differences in phenotype between wild type (WT) and transgenic (TG) animals: all animals appeared healthy. Only male rats were used to avoid any potential effects of changes in the female estrus cycle on behavioral responses. For each test, dedicated cohorts and their size are reported in Table 1.

- *Body weight* (Fig. 1a)

Both, TG and WT littermate control rats body weight increased over time. A repeated measure ANOVA did not reveal any significant GENOTYPE effect ($F(1,276) = 2657.06$, $P > 0.1$), but, as expected, a significant AGE effect was found ($F(6,276) = 717.67$, $P < 0.001$). A GENOTYPE x AGE interaction was not found.

| BACHD rats | AGE (months) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Behavioral tests** | 1 | 1.5 | 2 | 3 | 4 | 6 | 9 | 12 |
| Weight | (25:15)* | | (29:15) | (15:15) | (30:20) | (25:16) | (27:13) | (31:12) |
| Locomotor Activity | (17:7) | (16:16) | (29:15) | (30:20) | (13:13) | (37:22) | (28:13) | (31:12) |
| Catwalk | | | | | | (14:15) | | (14:5) |
| Rotarod | (8:9) | | (24:27) | (19:18) | (12:13) | (15:16) | (20:12) | |
| Object recognition | | | | | (15:15) | | | (14:6) |
| Prepulse inhibition | (17:17) | | | | (13:13) | | (14:15) | (28:12) |
| Startle habituation | | | | | | (15:15) | | |
| Startle threshold | | | | | | | (13:15) | |

**Table 1.** Summary of BACHD rat cohort sizes for each behavioral experiment. (*) indicates group size (WT:TG).

- *Locomotor activity* (Fig. 1b)

In comparison to WT, TG rats showed *hyper*activity at one month of age (t= 2.978, P < 0.01) followed by *hypo*activity starting at 4 months of age (t= 4.415, P < 0.001). This hypoactivity in TG rats persisted till 12 months of age [6 months (t= 3.586, p < 0.001), 9 months (t= 2.784, p < 0.01) and 12 months (t= 2.176, p < 0.05)]. No statistical difference between WT and TG rats was present in 1.5, 2 and 3 months old BACHD rats. A repeated measure ANOVA revealed significant GENOTYPE (F (1,331) = 26.97, P <0.001), AGE (F (7,331) = 10.54, P <0.001) and GENOTYPE x AGE (P < 0.01) effects.

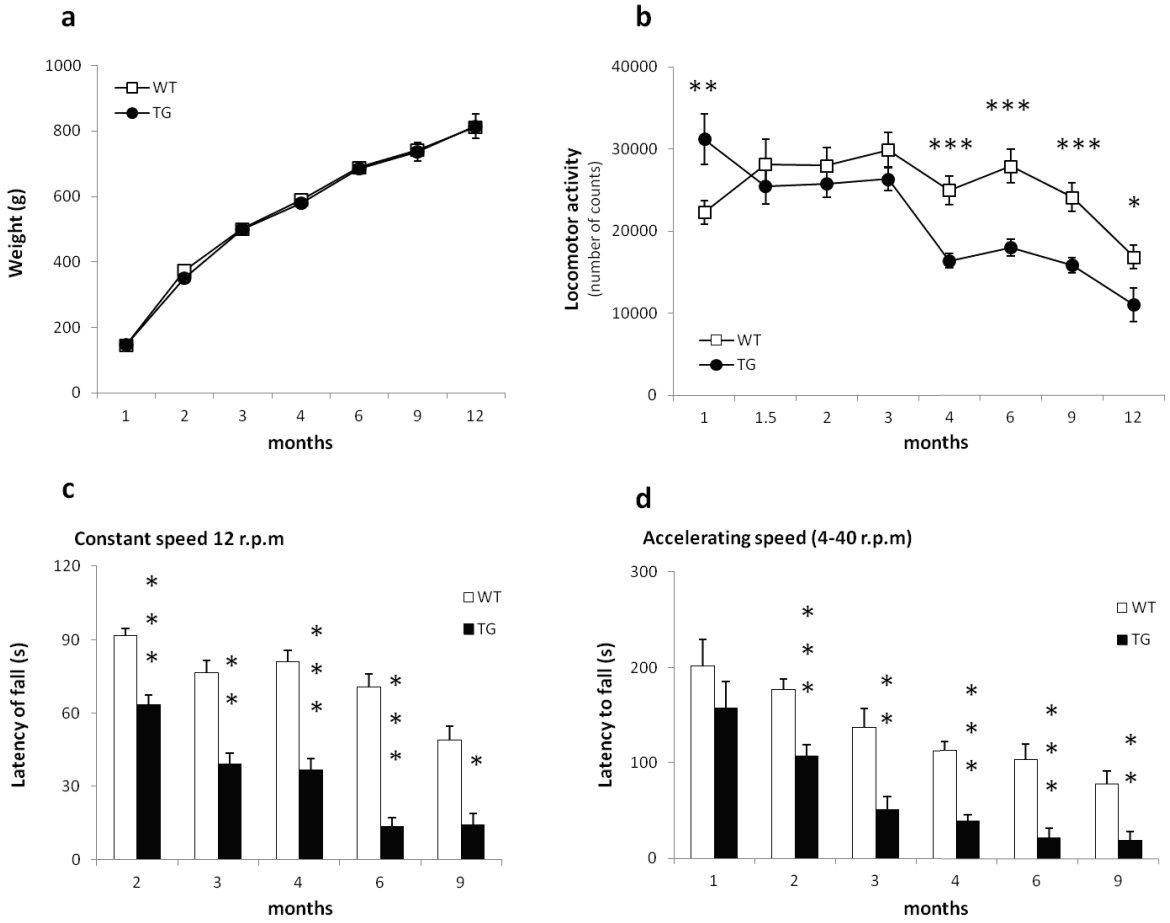



**Figure 1.** Phenotype. Results are expressed as Mean ± SEM. (a) Body weight. There is no significant difference between TG and WT control rats. (b) Locomotor activity. Compared to WT, TG rats have a higher total activity at one month followed by a lower activity starting at 4 months of age. (c-d) Rotarod. Presented are the latency to fall off the rod during (c) constant speed (12 r.p.m) and (d) accelerating speed (4 - 40 r.p.m). A significant difference between groups was present already at 2 months of age [constant speed (2 months: t= 3.373, P< 0.001; 3 months: t= 3.53, P< 0.01; 4 months: t= 4.798; P< 0.001; 6 months: t=5.433; p< 0.001 and 9 months: t= 2.742, P< 0.05); accelerating speed (1 month: t= 1.066, P> 0. 1; 2 months: t= 4.172, P< 0.001; 3 months: t= 3.549, P< 0.01; 4 months: t= 6.493, p< 0.001; 6 months: t= 4.263, P< 0.001 and 9 months: t= 3.015, P< 0.01)]. Asterisks indicate significant differences between WT and TG rats (*p < 0.05; **p < 0.01 and ***p < 0.001).

- *Rotarod* (Fig. 1c-d)

Differences in latency to fall off the rotating beam between TG and WT rats were found for training trials at constant speed (Fig. 1c, 12 r.p.m) and for testing trials at accelerating speed (Fig. 1d, 4 to 40 r.p.m); For technical reasons, results from training at constant speed mode for 1 month old rats cannot be presented. The 12 months old cohort could not be tested because the animals were simply too big to stand on the beam of the rotarod apparatus that we used. Visual inspection of Fig. 1(c-d) indicates a progressive decline in rotarod performance for all animals during training at constant speed and test trials at accelerating speed. In fact, TG fell off the rotarod faster than WT rats. This motor coordination deficit and imbalance started at 2 months of age and persisted across time. A repeated measure ANOVA revealed significant GENOTYPE and AGE effects [constant speed: GENOTYPE, $F(1,167) = 75.47$, $P < 0.001$ and AGE, $F(4,167) = 14.53$, $P < 0.001$; accelerating speed: GENOTYPE, $F(1,188) = 58.34$, $P < 0.001$ and AGE, $F(5,188) = 17.08$, $P < 0.001$]. No GENOTYPE x AGE interaction was found.

- *Catwalk gait analysis system* (Fig. 2)

We investigated gait deficits in BACHD rats with a Catwalk system. Results from rats of 2 independent cohorts that had made 3 good runs, aged 6 months (WT, n= 6; TG, n= 7) and 12 months (WT, n= 7; TG, n= 3) were analyzed. There was no significant difference in general walking speed at 6 and 12 months. No significant differences in static and dynamic parameters were observed between WT and TG rats at 6 months (data not shown). However, at 12 months, TG rats exhibited a shorter stride length (front limbs, $t= 2.194$ and $p<0.05$; hind limbs, $t= 2.355$ and $P< 0.05$), shorter stand (the duration contact with the glass plate; front limbs, $t= 2.107$ and $p<0.05$; hind limbs, $t= 2.690$ and $P< 0.05$) and shorter front limbs swing ($t= 2.871$ and $p< 0.01$). There was a statistical trend for a difference in hind limb swing (the duration of no contact with the glass plate; $t= 1.819$ and $p = 0.079$). No significant differences were found for the other parameters (data not shown).

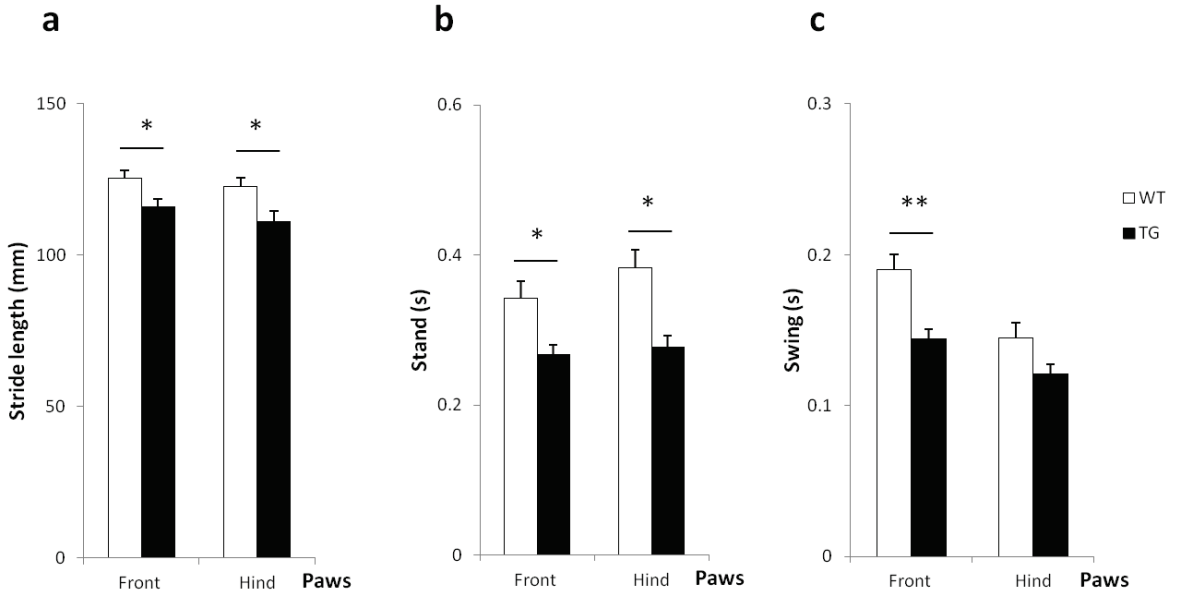



**Figure 2.** Catwalk gait analysis in 12 months old BACHD rats. Results are expressed as Mean ± SEM. There was no significant difference between TG and WT control rats in walking speed (data not shown). (a) Static parameters: Stride length. Compared to WT, TG rats had a significant shorter stride length for both front and hind paws during walking. (b-c) Dynamic parameters: presented are the Stand (c) and the Swing (d). TG rats had a significant shorter time of stand (or Stance) for front and hind paws and a shorter time in front swing than WT rats. Asterisks indicate significant differences between WT and TG rats (*$p < 0.05$ and **$p < 0.01$).

- *Object Recognition test (ORT)* (Fig. 3)

Three WT rats of the 4 months old group and one WT rat from the 12 months old group were excluded from the analysis as they did not explore the objects. Visual inspection of Fig. 3a indicates that TG rats had a higher total exploration time (e2). Indeed, during T1 and T2, significant GENOTYPE and TIME effects were found in four months old [2-way ANOVA: GENOTYPE, $F(1,25) = 4.75$, $P< 0.05$; TIME, $F(1,25) = 9.45$, $P< 0.01$] and 12 months old rats (GENOTYPE, $F(1,18) = 4.92$, $P< 0.05$; TIME, $F(1,18) = 4.87$, $P< 0.05$). Bonferroni post hoc testing revealed significant differences only for (e2) exploration time in 12 months old rats ($t = 2.612$, $P < 0.05$). No interaction between GENOTYPE and TIME was found.

Analysis of the recognition performance during T2 (Fig.3b) revealed a TIME effect at 4 months of age [2-way ANOVA: GENOTYPE, F(1,25) = 3.482, P> 0.05; TIME, F (1,25) = 16.62, p< 0.001] whereas a GENOTYPE and TIME effects were found at 12 months of age (GENOTYPE, F (1,18) = 6.589, P< 0.05; TIME, F (1,18) = 39.34, p< 0.001). In all, WT and TG had a significantly higher exploration time for novel objects than familiar objects at both ages (4 months: WT, t = 2.389, P< 0.05 and TG, t = 3.444, P< 0.01; 12 months: WT, t = 4.218, P< 0.01 and TG, t = 4.735, P< 0.001).

The relative discrimination index (d2) was positive in both age groups (Fig. 3c). A one sample t-test revealed a statistically significant difference from zero for both 4 months old (WT, t = 2.8, P< 0.05; TG, t = 4.1, P< 0.01) and 12 months old (WT, t = 5.38, P< 0,001; TG, t = 6.18, P< 0.01) rats.

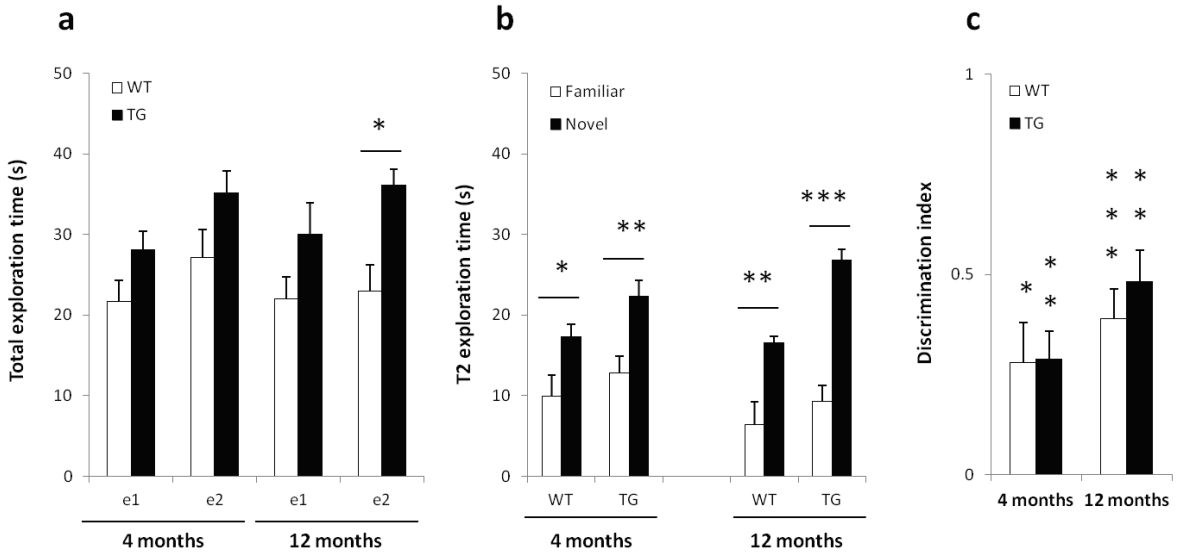
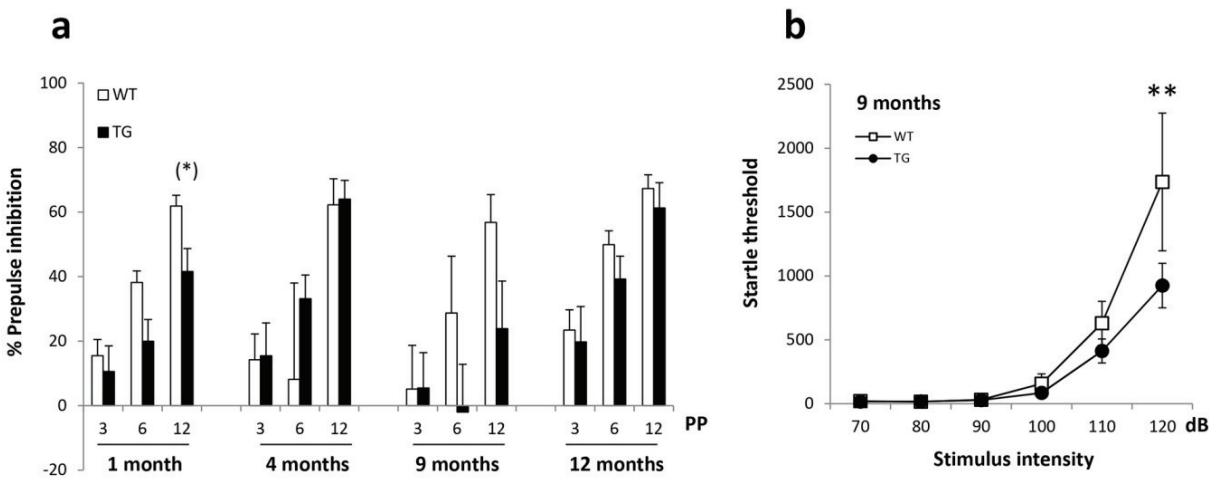



**Figure 3.** Object Recognition Test (ORT). Results are expressed as Mean ± SEM. Three WT rats of the 4 months old group and one WT rat from the 12 months old group were excluded from the analysis as they did not explore the objects (a) Exploration time (e1) and (e2) during T1 and T2 respectively. TG rats showed a significantly higher exploration time during e2 at 12 months of age. (b) Recognition testing during T2: 4 months and 12 months old BACHD rats had a significantly higher exploration time to the novel object than the familiar object. (c) The discrimination index (d2) for both groups are above zero and were significant at each age. Asterisks indicate statistical significance (*p < 0.05; **p < 0.01 and ***p < 0.001).

- *Acoustic Startle Response (ASR)* (Fig. 4)

We evaluated sensorimotor gating in different BACHD rat cohorts. Prepulse inhibition (PPI) was assessed in 1, 4, 9 and 12 months old rats, whereas startle habituation and startle threshold were evaluated in 6 and 9 months old rats, respectively. A first look to the results yields very little with large standard errors in PPI and startle threshold (Data S1). Given the fact that we have rigorously executed the experiments with fairly good large size of animals per test and time points, possible confounding factors could be attributed to outliers. Therefore, we performed a Grubb's test analysis and as a result removed the outliers, which indeed significantly reduced the variability of the groups.




**Data S1.** Startle testing. Results are expressed as Mean ± SEM. (a) Prepulse inhibition and (b) Startle threshold amplitude in BACHD rats. 2 way ANOVA: Prepulse inhibition [GENOTYPE: (1 month, F (1,32) = 3.84, P = 0.0587; 4 months, F (1,24)= 0.376, P= 0.545; 9 months F (1,27)= 1.447, P= 0.23 and 12 months, F (1,38)= 0.311, P= 0.58); TRIAL: (1 month, F (2,64) = 68.22, P = 0.0492 with PP 12, t= 2.438 P= 0.0498 ; 4 months, F (2,48)= 10.74, P= 0.0001, 9 months F (2,54)= 13.22, P< 0.0001 and 12 months, F (2,76)= 16.25, P< 0.0001) ]; Startle threshold [9 months, GENOTYPE: F (1.26) = 2.109, P= 0.158); INTENSITY: F(5,130)= 23.73, P< 0.0001 with 120 dB, t = 3.473 and P = 0.004]. The general observation of data indicated that no statistical differences in over all GENOTYPE might potentially be due to some outliers. The results without outliers are presented in figure 4.

- *Prepulse Inhibition (PPI)* (Fig. 4a)

For the PPI experiments, prepulse intensities (PP) of 3, 6, 12 dB above background were used. One month old (17WT:16TG), 4 months old (12WT:13TG), 9 months old (13WT:14TG) and 12 months old (25WT:11TG) BACHD rats data were analyzed. A 2-way ANOVA analysis with GENOTYPE as a between subjects factor and TRIAL (PP3, PP6, PP12) as a within subjects factor revealed a significant GENOTYPE and TRIAL effect in 9 months old rats [GENOTYPE: ($F(2,25) = 5.42$, $P< 0.05$; TRIAL: $F(2,50) = 12.96$, $P< 0.0001$) with PP6, $t = 2.78$, $P< 0.05$ and PP12, $t= 2.76$, $P< 0.05$ Bonferroni post hoc test)]. An interaction between GENOTYPE and TRIAL was detected ($F(2,50)= 4.589$, $P< 0.05$). No significant GENOTYPE effect was found at 1, 4 and 12 months of age; however a significant TRIAL effect was detected in all groups [(1 month, $F(2,62)= 65.02$, $P< 0.0001$; 4 months, $F(2,46)= 53.14$, $P< 0.0001$ and 12 months, $F(2,68)= 41.27$, $P< 0.0001$)]. There was no interaction between GENOTYPE and TRIAL. A close inspection of the data for the 1 month old rats suggests a difference between WT and TG rats at PP6 and PP12. A post hoc analysis of 1 month old rats revealed a trend to significance at PP12 (PP6, $t = 2.026$, $P = 0.136$; PP12, $t = 2.206$ and $P = 0.089$). This suggests that 1 month old transgenic rats have mildly impaired sensorimotor gating. Together with 4 and 12 months old rats from Fig.4 data, the deficits were too subtle to reveal a significant, overall, genotype difference between WT and TG rats at any of the other prepulse intensities following post hoc analysis. Finally, analysis of startle amplitude on P120 alone trials in BACHD rats did not show differences between groups (data not shown).

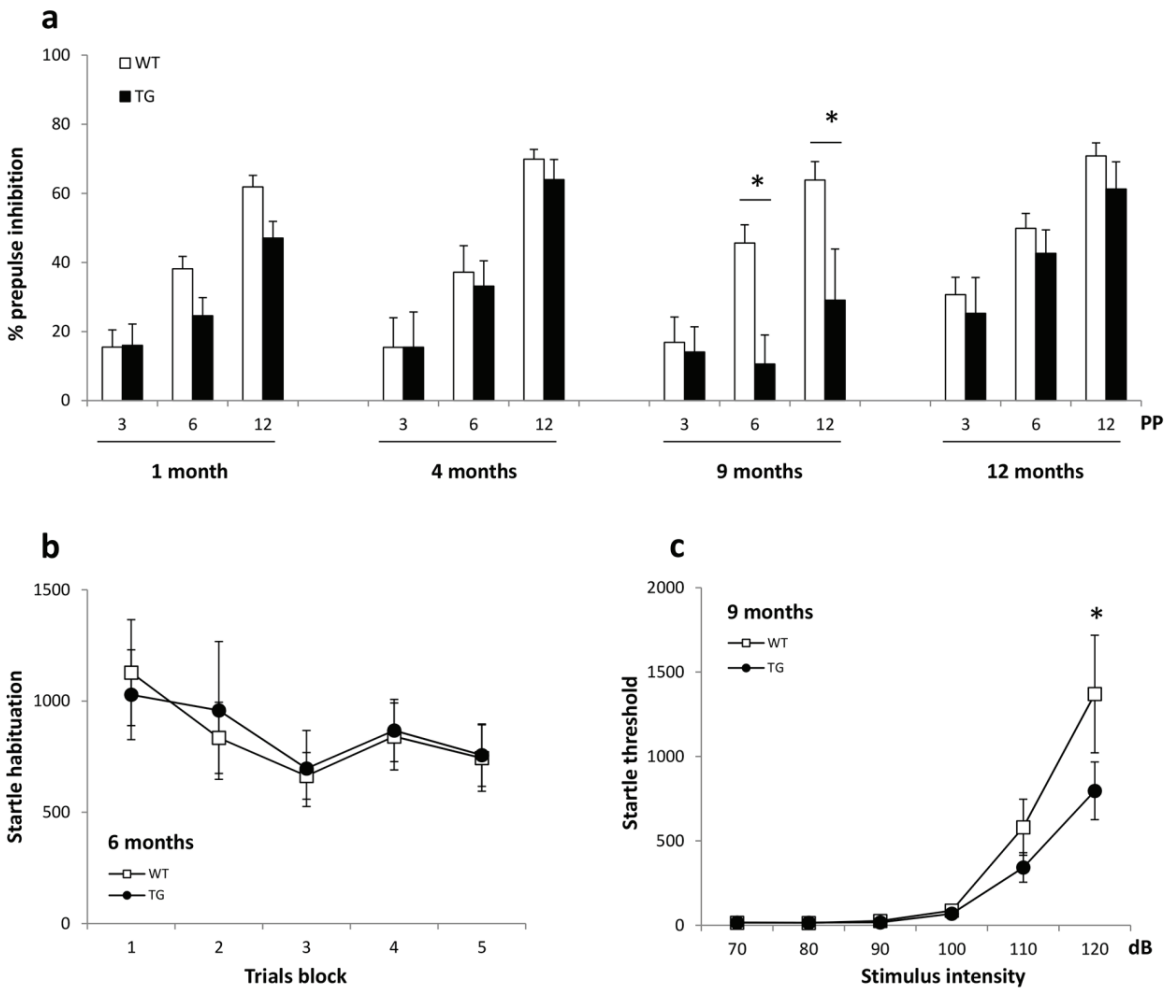



**Figure 4.** Startle testing. Results are expressed as Mean ± SEM. (a) Prepulse inhibition. A 2 way-ANOVA revealed a GENOTYPE difference in 9 months old BACHD rats especially at PP 6 and PP 12; however any significant differences were found in 1, 4 and 12 months old rats. (b) Startle habituation amplitude in 6 months old rats. Each trial consisted of 10 blocks of 120 dB startle stimuli. WT and TG rats presented a normal startle habituation. (c) Startle threshold. Amplitude to varying startling stimulus intensities in 9 months old rats. WT and TG response amplitude increased with higher stimulus intensities. No GENOTYPE effect was observed. However, a significant difference was detected at 120 dB. Asterisks indicate statistical significance in BACHD rats (*$p < 0.05$).

- *Startle Habituation (Fig. 4b)*

To further evaluate startle habituation in BACHD rats, six months old rats were presented 50 trials of a 120 dB (P120) startle stimulus. The results are presented in 5 blocks of 10 trials. Visual inspection of Fig. 4b suggests a general decrease in startle amplitude. This was confirmed by a 2-way ANOVA with a significant TRIAL effect ($F(4,112) = 3.36$, $P < 0.05$). However, no statistically significant GENOTYPE effect or GENOTYPE and TRIAL interaction was found.

- *Startle Threshold (Fig. 4c)*

We evaluated 9 months old BACHD rat's response magnitude to different stimulus intensities; five startle intensities (80dB to 120 dB), 10 trials each, were presented. Ten WT and 12 TG BACHD rats' data were analyzed. WT and TG startle amplitude increased with the startle stimulus intensity. A 2-way ANOVA analysis revealed a significant INTENSITY effect at 120 dB [INTENSITY, $F(5,100) = 26.80$, $P < 0.0001$ and 120 dB, $t = 2.965$ and $P < 0.05$]. However, there was no over-all GENOTYPE effect and no interaction between GENOTYPE and INTENSITY was found.

## 4. Discussion

We evaluated transgenic BACHD rats and wild type littermate control in a series of standard behavioral tests assessing motor, sensory-motor and cognitive function. TG rats showed a clear motor coordination imbalance on a Rotarod, as well as gait coordination deficits in both static and dynamic free walking sequences as measured with a catwalk system. These data demonstrate a progressive motor impairment over time as seen in patients. Rats showed intact recognition memory as measured in an object recognition test. A clear deficit in sensory-motor paradigms (startle habituation, and startle threshold) was absent, although a subtle impairment in PPI was found. Whereas we confirmed the motor phenotype as described previously (Yu-Taeger et al.

2012), we expected a more profound cognitive and sensorimotor phenotype. Additional studies will be needed to provide further insights into the validity of the BACHD rat model for HD and how this model compares with other rodent models for HD.

*Motor Behavior*

The first clinical reports on HD predominantly addressed motor symptoms, including progressive involuntary movements named “chorea” (George Huntington, 1972). Therefore, we started first with the assessment of motor behavior in our comprehensive behavioral phenotyping approach of BACHD rats.

A first report on ambulatory activity in BACHD rats found hypoactivity at 3 months of age (Yu-Taeger et al. 2012). In the present study, TG rats showed *hyper*activity in an open field environment at 1 month followed by a progressive and long-lasting *hypo*activity starting at 4 months. Similar observations have been reported in YAC 128 mice with a hyperkinetic phenotype at 3 months followed by hypokinetic phenotype at 6 months of age (Slow et al. 2003). However, results from other rodent models for HD have been variable. For example, *hyper*activity was found in transgenic tgHD rats carrying 51 CAG-repeats, with higher exploratory distance travelled in an open field test at 6, 7, 8 and 10 months. A progressive *hypo*activity phenotype was found in transgenic BACHD mice, starting at 7 months (Menalled et al. 2009; Zeef et al. 2012b).
The *hype*ractivity detected in BACHD rats at an early age (4 weeks) was only expressed for a very short time window as it had disappeared at 6 weeks. Yu-Taeger and colleagues have probably missed this phenotype in BACHD rats as they started testing at 3 months of age. However, the robustness of this phenotype needs to be addressed in follow-up studies with a higher number of animals (there were only 7 rats in the one month old transgenic cohort). Nevertheless, it is encouraging that the biphasic activity pattern in our rats appears to mimic more closely some of the clinical neuropsychiatric symptoms reported in pre-manifest and manifest HD patients (Paulsen et al. 2001; Van Duijin et al. 2007).

Measurement of motor coordination and balance on a rotarod showed a clear deficit in TG rats as they have a shorter latency to fall off the rotating rod during constant speed training trials and during accelerating speed trials. It is interesting to note that the performance of WT rats on the rotarod declines over time. Indeed, weight gain in rats can impact rotarod performance; however the results demonstrate that the difference we observed between WT and TG BACHD rats is unlikely to be influenced by animal's weight. The same observations were made by Yu-Taeger and colleagues (2012), with TG rats having difficulties maintaining balance on the rod at higher rotation speeds. The progressive imbalance in rats persists over time and is consistent with data obtained from BACHD mice and tgHD rats (Abada et al. 2013; Kordasiewicz et al. 2012; Menalled et al. 2009; Nguyen et al.2006; Pouladi et al. 2012; Von Horsten et al. 2003). Taken together, these data prove the reliability of using the Rotarod test for motor coordination assessment across laboratories.

Gait abnormalities were found in both static and dynamic parameters during Catwalk testing in 12 months old rats. TG rats had a shorter stride length and decreased stand duration of front and hind paws. A decrease in swing duration was observed for the front paws. Although velocity can significantly influence catwalk gait parameters (Koopmans et al. 2007), the deficits in BACHD rats are unlikely to be confounded by this parameter as TG and WT rats did not differ in walking speed. Footprints of TG rats have been previously investigated for gait abnormality at 14 months of age. Shorter steps for limbs, increased stride width and reduced overlap between forelimbs and hindlimbs placement were found (Yu-Taeger et al. 2012). In accordance with our findings, similar results have been reported in other HD rodent models. For example, tgHD rats also showed decreases in stand and swing duration in a Catwalk test (Vandeputte et al. 2010). R6/2 transgenic mice displayed a significantly shorter stride length by 8-9 weeks of age in an ink-footprint test, and shorter stance time for front and hind limbs at 17 weeks in a Digigait system during treadmill locomotion (Carter et al. 1999; Pallier et al. 2009). Finally, HD patients show gait abnormalities like mean decrease in velocity, stride length and cadence (Koller and Trimble, 1985).

Based on the early and profound coordination deficits in the Rotarod, we did not expect the late occurrence of relative mild gait abnormalities in TG rats. However, consistent

with the late onset in BACHD rats, we found gait abnormalities at an advanced age (10 months) in BACHD mice such as: differences in the NSSP cruciate and alternate, larger distance between forelimbs placements, shorter stand/propulsion time with their hindlimbs and timing relationships between paw placements (Abada et al. 2013). However, none of these deficits were found in 12 months old BACHD rats in the present study. Methodological confounds cannot be excluded but seem unlikely as the mice and rats were tested in the same laboratory, by the same experimenter, using the same equipment. This illustrates that the disease progression in 2 different species with a same construct might be different. Although gait deficits appear to be present at 14 months of age with the footprints test, it would be worthwhile investigating if major deficits in Catwalk performance are present in BACHD rats that are older than 12 months.

*Object recognition*

Recognition memory was investigated in BACHD rats in an ORT task and intact object memory was found in 4 and 12 months old rats. A close look at the recognition performance of 12 months old rats during T2 suggests a better cognitive performance in TG rats. Unfortunately, the design of the experiments (i.e. using a relatively short inter trial interval) was aimed at inducing a robust object recognition in WT rats. One way to investigate the hypothesis that TG rats actually have superior performance would be to increase the inter trial time between T1 and T2 and observe if WT rats performance decrease faster. This would, however, be beyond the scope of this study, which was signed to investigate only whether TG rats have reduced recognition memory. Given that both transgenic and control groups exhibit a significant positive discrimination index, we can conclude that their recognition memory is intact. Our results contrast with findings in another rat model for HD. tgHD rats show deficits in ORT and OLT (object location test) at 16 months of age (Zeef et al. 2012a) and cognitive deficits have been reported as early as 9 months of age with different tasks assessing visual-spatial learning and memory processing (Brooks et al. 2009; Fink et al. 2012; Kirch et al. 2013). It cannot be ruled out that we might have missed a recognition memory deficit as BACHD rats show no clear *htt* aggregates or neurodegeneration before 12 months of

age (Yu-Taeger et al. 2012). Therefore, we assume that in contrast to motor behavior, the circuitry involved in ORT may become dysfunctional only if *htt* aggregates have formed. Further studies need to be done in animals and humans to better understand if and how object memory is impaired in HD. Object memory seems to be impaired in HD patients. In a pattern recognition task (a task similar to the rat ORT), subjects had to remember and touch the abstract patterns they were shown during training and that were paired with a novel pattern during testing. Early HD patients and clinically symptomatic subjects performed significantly worse than control subjects (Lawrence et al. 1996, 2000), whereas in at-risk gene carriers, no difference in recognition memory was found (Lawrence et al. 1998). Irrespectively, this is the first study to report intact object memory in BACHD rat and further studies in older cohorts may shed further light on a potential recognition memory deficit.

*Acoustic Startle Response*

Nine months old BACHD transgenic rats had a significant prepulse inhibition deficit at 6 and 12 dB prepulse (PP) intensities. Closer inspection of 1 month old rats' data suggests subtle deficits at PP 6 and 12 dB. However, no genotype differences were found in 1, 4 and 12 months old rats. Likewise, no statistical difference was detected in startle responding and startle habituation.

PPI deficits have been demonstrated in BACHD mice and YAC 128 mice models for HD at later ages (Menalled et al. 2009; Pouladi et al. 2012; Van Raamsdonk et al. 2005). Impaired PPI has been shown in the neurotoxic rat model after systemic administration of 3-Nitropropionic acid (Kodsi and Swerdlow, 1997; Seaman, 2000). Also, PPI has been extensively investigated in several neuropsychiatric disorders (Castellanos et al. 1996; Esteban et al. 1981; Grillon et al. 1992). In HD patients, only Swerdlow and colleagues (1995) have reported reduced PPI in both acoustic and tactile startle reflex whereas no impairment was observed in startle amplitude, habituation or prepulse latency facilitation paradigms. In view of 1) the correlations between PPI impairments and degenerative changes in the striatum of animal models and HD patients; and 2) the absence of clear neurodegeneration or striatal *htt* aggregates in caudate putamen (CPu) before 12 months of age (Yu-Taeger et al. 2012), it cannot be ruled out that a PPI

deficit develops after 12 months of age. Unfortunately, we could not test older rats since they were too big for the standard PPI rat enclosures. Although this technical issue is certainly not insurmountable, an interesting alternative would be to test PPI in a non-human primate model for HD (Yang et al. 2008). Testing in a different species would help to shed more light on the robustness of a sensorimotor gating deficit in HD models.

## 5. Conclusion

In the present time course study, we investigated motor, sensorimotor and cognitive symptoms in BACHD rats. Transgenic rats showed motor coordination imbalance on a Rotarod and subtle gait deficits in a Catwalk system. These rats had an intact object recognition memory and a subtle deficit in prepulse inhibition of acoustic startle. In contrast to the symptoms progression in patients, BACHD rats may not show object memory impairment until after motor deficits occurred. Further assessment of other cognitive functions, such as reversal learning and associative memory, may shed further light on the comparative time courses for the emergence of motor and cognitive deficits.

If deficits in sensorimotor and cognition functions are linked to *htt* aggregation, it is possible that our BACHD rats were too young (12 months) to show robust deficits. Yu-Taeger and colleagues (2012) revealed that nuclear accumulation of N-terminal *htt* appeared in cortex after 9 months of age and few aggregates were present in the dorsolateral caudate putamen from 12 months old rats and increases thereafter. However, the relationship between mutant huntingtin (m*htt*) aggregation and MSN loss, motor and cognitive deficits in BACHD rodent models for HD appears complex. Sometimes symptoms occur before the neuropathology like in BACHD mice, where few m*htt* aggregates are present at 12 months of age in cortical and striatal regions (Gray et al. 2008; Kordasiewicz et al. 2012; Pouladi et al. 2012; Southwell et al. 2009). Further studies need to be performed to better understand the molecular and cellular mechanisms underpinning motor and cognitive symptoms in BACHD rats.

# Study 3

# Reversal learning and associative memory impairments in a BACHD rat model for Huntington disease

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**Abstract**

*Rationale*

Chorea and psychiatric symptoms are hallmarks of Huntington disease (HD), a neurodegenerative disorder, genetically characterized by the presence of expanded CAG repeats (> 35) in the *HUNTINGTIN* (*HTT*) gene. HD patients present psychiatric symptoms prior to the onset of motor symptoms and we recently found a similar emergence of non motor and motor deficits in BACHD rats carrying the human full length mutated *HTT* (97 CAG-CAA repeats).

*Objectives*

We aimed to replicate and expand our initial findings and evaluated cognitive performance in reversal learning and associative memory tests in different age cohorts of BACHD rats. *Material and methods*

Male wild type (WT) and transgenic (TG) rats between 2 and 12 months of age were tested. Learning and strategy shifting were assessed in a cross-maze test. Associative memory was evaluated in different fear conditioning paradigms (context, delay and trace). The possible confound of a fear conditioning phenotype by altered sensitivity to a 'painful' stimulus was assessed in a flinch-jump test.

*Results*

In the cross maze, 6 months old TG rats showed a mild impairment in reversal learning. In the fear conditioning tasks, 4, 6 and 12 months old TG rats showed a marked reduction in contextual fear conditioning. In addition, TG rats showed impaired delay conditioning (9 months) and trace fear conditioning (3 months). This phenotype was unlikely to be affected by a change in 'pain' sensitivity as WT and TG rats showed no difference in their threshold response in the flinch-jump test.

*Conclusion*

Our results suggest that BACHD rats have a profound associative memory deficit and, possibly, a deficit in reversal learning as assessed in a cross maze task. The time course for the emergence of these symptoms (i.e., before the occurrence of motor symptoms) in this rat model for HD appears similar to the time course in patients. These data suggest that BACHD rats may be a useful model for preclinical drug discovery.

## 1. Introduction

Huntington disease (HD) is one of the neurodegenerative disorders where the origin has been unequivocally identified, that is, an elongation of polyglutamine (>35 CAG repeats) in the *HUNTINGTIN* (*HTT*) gene on chromosome 4 (Huntington's Disease Collaborative Research Group, 1993). Patients carrying the mutation present a combination of motor symptoms such as chorea, psychiatric symptoms, and cognitive changes (George H 1872; Myers et al. 1988). The disease is associated with degeneration of neurons in the striatum (especially the Medium Spiny Neurons, MSN) and cortex (Douaud et al. 2006; Jones and Hughes, 2011; Vonsattel et al. 1985). Treatments to delay HD onset or inhibit the mechanisms by which neural loss occurs are still lacking (Jackson et al. 1998; Pidgeon and Rickards, 2013), and therefore there is a continuing need for improved animal models to support drug discovery efforts.

During the last decades, many animal models for HD have been generated, from insects (*Drosophilae melanogaster*), to nonhuman primates (*Macaca mulatta*), including several rodents models (Crook and Housman, 2011; Jackson et al. 1998; Vlamings et al. 2012; Yan et al. 2008). The availability of such a wide range of models increases the potential opportunities to understand the disease progression and to find a cure. Besides selection of a reliable and valid animal model, the timing of drug treatment is of critical importance for HD drug discovery studies. One plausible explanation for the recent failure of monoclonal antibodies against the beta-Amyloid protein to reverse symptoms in patients with advanced Alzheimer's disease in Phase III studies, has been that therapeutic intervention is needed at a time point when the disease has not yet caused too much neurodegeneration for treatments to be effective(Mullard, 2012). Accordingly, it is increasingly recognized that identification and validation of prodromal symptoms and biomarkers is critical. For HD, cognitive impairments may consist of prodromal symptoms that could be used as clinical endpoints in drug discovery. HD patients present several impairments in executive and visuospatial mnemonic functions (Lawrence et al. 1996, 1998, 1999). Cognitive impairment appears to occur *before* the emergence of motor symptoms. For example, patients exhibited impairments in the

California verbal learning test (CVLT) and the Wechsler memory scale (WMS)] in the absence of motor disturbances (Hahn-Barma et al. 1998). An important aim for future animal model development is to identify, characterize and validate cognitive symptoms that occur *before* the onset of motor symptoms. It is not yet clear to what extent the occurrence of cognitive and motor symptoms are adequately reflected in the current rodent HD models. In fact, several studies reported cognitive impairments *after* the appearance of motors deficits. For example, in a tgHD rat model of HD that carry the human mutation with 51 CAG repeats (von Hörsten et al. 2003), Fielding and colleagues (Fielding et al. 2012) have not found significant impairment in object recognition, set shifting, and operant tests, although motor deficits were present at 13 months of age. Deficits were shown at 12 months of age in radial maze and at later ages (15 to 20 months old rats) in choice reaction time tasks, spatial, and location recognition memory tests, whereas in the R6/2 mouse model for HD, selective deficits in spatial, visual and reversal discrimination were observed before or during subtle motor deficits (3.5-5.5 weeks and 7-8 weeks respectively) (Cao et al. 2006; Kantor et al.2006; Lione et al. 1999; Mangiarini et al. 1996; Nguyen et al. 2006; Zeef et al. 2012b).

Herein, we used BACHD rat, a novel model for HD that has been recently established (Yu-Taeger et al. 2012). Like the mouse BACHD model (Gray et al. 2008), the rat model carries the full length human mutant *HUNTINGTIN* (fl-*mHTT*) with 97 CAG-CAA mix repeats under control of the human HD promoter gene. An advantage of the rat model is that behavioral processes related to learning and memory and pharmacological validation have been well described for this species. We have previously found progressive motor deficits during rotarod testing, starting as early as 2 months of age; a decrease in spontaneous locomotor activity, as well as, gait deficits in a catwalk test. However, we were unable to show a significant cognitive impairment in an object recognition task or robust sensorimotor deficits in a prepulse inhibition test (Abada et al. 2013b). These results were somewhat unexpected in light of the cognitive deficits reported in patients. Therefore, we decided to perform a more profound evaluation of the cognitive phenotype of BACHD rats. The cognitive performance of different age cohorts of BACHD rats was assessed in reversal learning and associative memory tests. The cross-maze and the fear conditioning paradigms tests (contextual, delay and

trace conditioning) were selected, because they have proven to be efficient for cognitive assessment in rodents. An important consideration for selection of the rat fear conditioning paradigms was that the neural circuitry for fear conditioning has been well described in rodents and humans and that these neural circuits are well conserved (Fendt and Fanselow, 1999; Knight et al. 2004). This offers a potentially powerful translational approach as fear conditioning studies in tandem with functional brain imaging studies in both species could be used for future drug discovery studies.

## 2. Materials and methods

- Ethics statement

The study was carried out in strict accordance with the German animal welfare act and the EU legislation (EU directive 2010/63/EU). The protocol was approved by the local ethics committee *Behörde für Gesundheit und Verbraucherschutz* (BGV, Hamburg).

- Husbandry and genotyping

Wild type (WT) and transgenic (TG) BACHD rats, carrying the mutant human HTT gene, under the control of the human huntingtin promoter and its regulatory elements were used. The transgene contains 97 CAG-CAA mix repeats, which produces a particular stability of the repeat length, and additional 20 kb upstream and 50 kb downstream sequences that reduce its position effect [25]. Two transgenic males were supplied from the original BACHD colony of the Universitäts Klinikum Tübingen (UKT, Germany) and an in-house breeding colony was preserved and maintained at EVOTEC AG (Hamburg, Germany) by cross-breeding these males with wild type female rats. BACHD animals were maintained on a Sprague-Dawley background. All the animals at weaning were group-housed 2 to 4 per cage with wood shavings and a filter top. The environment was enriched with a play tunnel and shredded paper. BACHD rats were maintained in climate controlled housing, with a 12-h reversed dark/light cycle (light from 19:00 to 07:00). Rats had free access to food and water except during experiments.

Ear punches were taken at weaning to determine their genotype. Genotyping was performed before and after all the studies using a validated protocol. Briefly, Genomic DNA was prepared from ear biopsy tissue using proteinase K digestion, followed by phenol/chloroform extraction (Qiagen DNeasy Tissue kit). Primers flanking the polyQ repeat in exon 1 were designed to recognize whether or not the rat carried at least one copy of the mutation, and were used to PCR amplify the polyQ regions [Q3: 5' – AGG TCG GTG CAG AGG CTC CTC - 3' and Q5: 5' – ATG GCG ACC CTG GAA AAG CTG - 3']. Gene status was confirmed in parallel by using designed primers from UKT [exon 1: FW 5'-ATG GCG ACC CTG GAA AAG CTG- 3' and RV: 5' -AGG TCG GTG CAG AGG CTC CTC- 3'; exon 67: FW 5'-TGT GAT TAA TTT GGT TGT CAA GTT TT- 3' and RV: 5' –AGC TGG AAA CAT CAC CTA CAT AGA CT- 3']. The PCR product was run on an automated apparatus PTC-200 (Peltier Thermal Gradient Cycler) and the Agilent 2100 Bioanalyser (Agilent technologies) was used to determine the fragments size.

Our concern in this longitudinal study was to reduce as much as possible potential confounds that hamper the interpretation or extrapolation of the results. Therefore only male rats were used in the cognitive tests as the female estrus cycle may influence experimental outcomes (Farr et al. 1995; Pearson et Lewis, 2005).

- Strategy and shifting (Cross-Maze)

The strategy shifting test is a standard dual-solution task which was used to assess the respective contributions of response (or egocentric) and place (or allocentric) learning strategies on memory (Tolman et al. 1946). It determines the relative involvement of these 2 strategies during the course of learning. We essentially used the same method as has been described for testing the BACHD mice (Abada et al. 2013a).
Spatial alternation was assessed using a modified version of the standard cross-maze; the home made maze consists of 4 identical arms (50 cm x 12 cm x 20 cm) at 90 degrees to each other. The maze was made with clear Plexiglas, elevated 45 cm above the floor, and a T-maze was created by closing one arm (north, N) with a guillotine door. The T-maze configuration was as follow: 2 arms [east (E) and west (W)] are at 180 degrees to each other, and the last arm (south, S) was perpendicular to these arms.

Two holes were present: one at the end of the E and W arms each, and spatial cues were placed on a black curtain which surrounded the maze. A home cage was put at one end of the arms (E or W) to motivate the animals to explore the maze and find the exit into this home cage (where they were additionally rewarded with food pellets). One week prior to the test, rats received small sucrose food pellets in addition to their normal diet. One day prior to the start of the experiments, all rats received a 5-min habituation session in the apparatus. During that period, food was not available.

The next day, the acquisition sessions started and a rat was placed in the S arm. The home cage containing sucrose food pellets was placed under the hole in the W arm. The rat had to guide itself in the maze and reach the home cage. During acquisition, rats received one trial per day for 7 days. During the first 2 days, the goal arm (i.e. arm giving access to the home cage) was baited with small sucrose food pellets. The same training procedure was run during reversal and extended reversal training sessions except that the rats had to reach this time the home cage placed underneath the E arm (opposite of the previously learned arm). When a rat made a wrong choice (entrance into the arm without home cage), it was allowed to trace back to the goal arm. If the rat failed to reach the home cage within 2 min, the rat was gently guided manually to the goal arm and the trial ended 20 s later. The maze was cleaned after each animal crossing with a 10% ethanol solution to avoid any bias related to odor.

Three probe trials were performed at days 8, 16 and 22, at the end of the acquisition, reversal and extended reversal training sessions, respectively. During the probe trials the S arm was closed and the N arm was used as the new start arm; the strategy (place or response) that the animal used to reach the goal arm was assessed. If the animal during the acquisition training had learned to use a place strategy, it would select the W arm. However, if the animal had used a response strategy (i.e. learned to turn left), it would select the E arm.

- Fear conditioning

Classical fear conditioning (FC) is a form of associative learning in which subjects express fear responses to a neutral conditioned stimulus (CS) after it has been paired with an aversive, unconditioned stimulus (US). The tests were run in an apparatus (Med

Associates Inc., Italy) consisting of a ventilated sound-attenuated box and a rectangular testing chamber (30 x 26 x 25 cm) with stainless steel rod floor. Measurements were accomplished through a front digital video recording camera, connected to a computer with video freeze software. All rats received 5 min acclimatization one day prior to training and testing days. The chambers were wiped with a 70% ethanol solution and were dried prior to each rat testing. Three different tasks were used: contextual, delay and trace fear conditioning.

- *Contextual fear conditioning*

The training session consisted of a 5 min acclimatization followed by 6 pairings (1 min inter trial time) of a 0.6-mA, 1-s foot shock. Animals were returned to their home cage 3 min after receiving the last foot-shock. On the next day, conditioned freezing was assessed by placing rats in the conditioning chambers for 5 min, in the absence of foot shock. For the evaluation of long term memory (LTM), animals were re-exposed one and 2 months later during a 5-min sessions to the conditioning chambers.

- *Delay conditioning*

The testing protocol is similar to the contextual fear paradigm, except that on the training day, after 3 min acclimatization, rats received 6 pairings (120 s inter trial time) of a 30-s tone (85 dB) with a 0.6-mA, 2-s foot shock. The foot shock terminated at the same time as the tone and rats were removed from the testing chambers 60s after the last pairing. On the testing day, rats were tested for contextual freezing in the conditioning chambers for 3 min, in the absence of tone or foot shock. One hour later, an altered context was generated with white polyvinyl chloride materials that covered the shock-grid bars and the inside of the conditioning boxes. Freezing was assessed in the altered context without tone for 3min, followed by a 3-min tone presentation in the absence of foot shock.

- *Trace fear conditioning*

The test was adapted from an existing protocol (Blum et al. 2006). In this associative learning paradigm, rats received during the training day eight trials of a 85 dB, 10s tone (CS), followed by 20s trace period, after which a 1s – 0.6 mA foot shock (US) was delivered. Each CS-US pairing was separated by a random inter-trial interval (ITI) that varied between 60 and 120s. The random ITI time was used to prevent time between foot-shocks to be used as a cue for the US. Rats were removed from the chamber 60s after the last CS-US presentation.

Retention tests for contextual, auditory and trace fear memory were carried out 24 h after conditioning. Rats were first tested for tone and trace period in an altered context made with a white polyvinyl chloride insert to cover the shock-grid bars and the inside of the conditioning boxes. Each rat was given 2 min habituation, followed by four presentation of the CS with varied ITI, in the absence of US. Freezing behavior during the four CS presentations and trace periods were averaged for each animal. Following CS and trace retention testing periods, contextual retention test was measured by placing the animals back into the original context for 2 min during which freezing was scored, without exposure to the CS or US.

For all the paradigms, freezing behavior was defined as the lack of any movement, except respiration. The percent of time spent freezing was assessed using the linear methods of observation measures (video freeze software).

- The ‘flinch-jump’ test

The method has been described by Lehner and colleagues (2010). Rats were placed individually into the fear conditioning boxes (Med Assoc. Italy). Shocks were delivered to the grid floor of the test box through a shock generator. After a 3-min period of habituation to the test box, shock titrations continued to increase in a stepwise manner (0.05 mA, 0.05–0.6 mA range). In this way, the ‘flinch’ and ‘jump’ thresholds in mA is defined for each rat. The interval between shocks was 2 min, and each animal was tested only once at each intensity. Behavior for each rat was recorded through a front

digital video recording camera and analysis was done blind to the genotype. The 'flinch' threshold was defined as the lowest shock intensity that elicited a detectable response. The 'jump' threshold was defined as the lowest shock intensity that elicited simultaneous removal of at least three paws (including both hind paws) from the grid.

- Statistical Analysis

All data were analyzed using *GraphPad* and *InVivoStat* software. Differences between groups were assessed with Student's t-test or mix ANOVA with repeated measures, with the factor GENOTYPE as between subject and TIME or TEST as within subject variable. When significance was found, a Bonferroni - post hoc analysis was performed when appropriate. For the cross maze, the learning index is defined as the ratio of the mean number of correct choices over trials per animal. Therefore, we have generated a binary data set with 2 possible outcomes (correct choice vs. incorrect choice). The hypothetical value that results is "½" because each animal has 50% chance at every trial. The one sample t-test was used to evaluate the learning index in each population with a hypothetical value set at "½". Chi-square ($\chi 2$) analyses were computed on animal's choice during Acquisition (A), Reversal (R), and Extend Reversal (ER) learning in the cross maze test, in order to determine discrepancies between groups and to determine potential changes in strategies between both probe trials. The Chi square test assesses whether an observed frequency distribution (i.e. the number of correct choices) differs from a theoretical distribution, and if this distribution is independent (i.e. the choice is genotype dependent). Finally, a Mann Whitney U-test was used to analyze 'flinch-jump' data. The significance level was set for all analysis at 0.05.

## 3. Results

We inspected each BACHD rat cohort animals prior to the experiments and all animals looked healthy. No global differences in phenotype were observed between wild type (WT) and transgenic (TG) rats. Only male rats were used during the study. No difference in weight was found between WT and TG (data not shown).

- *Acquisition, Reversal learning and Strategy shifting in a Cross-maze* (Fig. 1)

The cross maze test was performed in independent cohorts of 2 months (n = 17 per genotype) and 6 months (n = 15 per genotype) old BACHD rats in order to avoid any bias related to recall or long term memory of the task. Data from acquisition and reversal training sessions at the first 2 days were not analyzed because the goal arm was baited with sucrose food pellets to guide BACHD rats to the home cage. A schematic representation of the cross-maze task is presented in Fig. 1a. One WT rat of 2 months was removed from the data analysis because it did not show any interest in the task and did not make a choice (turn left or right) during the experimental period.

- *Learning indices during acquisition, reversal and extended reversal learning*

The learning index is calculated as the ratio of the mean number of correct choices over trials per animal (Fig.1b). Both cohorts of 2 and 6 months old BACHD rats displayed improved learning during *acquisition* (A) (2 months: WT, $t = 5.91$ and $P < 0.0001$; TG, $t = 4.02$ and $P < 0.001$; 6 months: WT, $t = 4.35$ and $P < 0,001$; TG, $t = 5.776$ and $P < 0.0001$). During *reversal* (R), 2 months old WT and TG have a learning index above the chance level of 0.5, although both groups did only reach a statistical trend (p value between 0.05 and 0.1; 2 months: WT, $t = 2.126$ and $P = 0.0532$; TG, $t = 1.884$, and $P = 0.0805$). The 6 months old cohort (WT and TG) showed a (R) learning index below chance level (< 0.5) and did not reach statistical significance. In the *extended reversal* training (ER), both WT and TG rats of 2 months presented a statistically significant learning index (WT, $t = 3.809$ and $P < 0.01$; TG, $t = 2.874$ and $P < 0.05$). Six Months old

WT rats had a significant learning index during (ER) ($t = 3.323$, and $P < 0.01$) whereas TG did not reach statistical significance ($t = 0.743$, and $P > 0.1$); 6 months old TG learning index was around 0.5. A comparison between the (A) and (ER) learning index in WT and in TG rats showed only a difference for 6 months old TG rats (A vs. ER, $t= 2.846$, $P < 0.01$). A closer look at the 6 months rats (ER) bar graph (Fig. 1b) suggests a difference between WT and TG rats, and statistical analysis found a trend ($t= 1.737$, $P =0.0946$).

- *Correct choices during acquisition, reversal and extended reversal learning*

The progress of 2 and 6 months old BACHD rats in learning the task during training sessions for (A), (R) and (ER) is presented in Fig. 1 (c-d). We analyzed the number of correct choices that both groups made. All rats had one trial per day. There was no statistical difference during (A) in 2 and 6 months old BACHD rats. For (R) and (ER), only animals with learning index higher than 0.5 during acquisition were analyzed. That is, animals that actually learned the task. Four (WT, n= 2 and TG, n = 2) 2 months old rats and two (WT, n= 1 and TG, n = 1) 6 months old rats did not reach the criteria and therefore were not included in the analysis. Although 2 months old TG rats made correct choices during (R) and (ER) training, as did WT control rats, 6 months old TG rats made fewer correct choices. In fact, WT - but not TG - animals made more correct choices over the course of (ER) training. The difference between 6 months WT and TG rats was significant already on reversal trial 7 ($\chi2= 6.23$, $P< 0.05$) and persisted during (ER) trainings ($\chi2= 8.594$, $P< 0.01$).

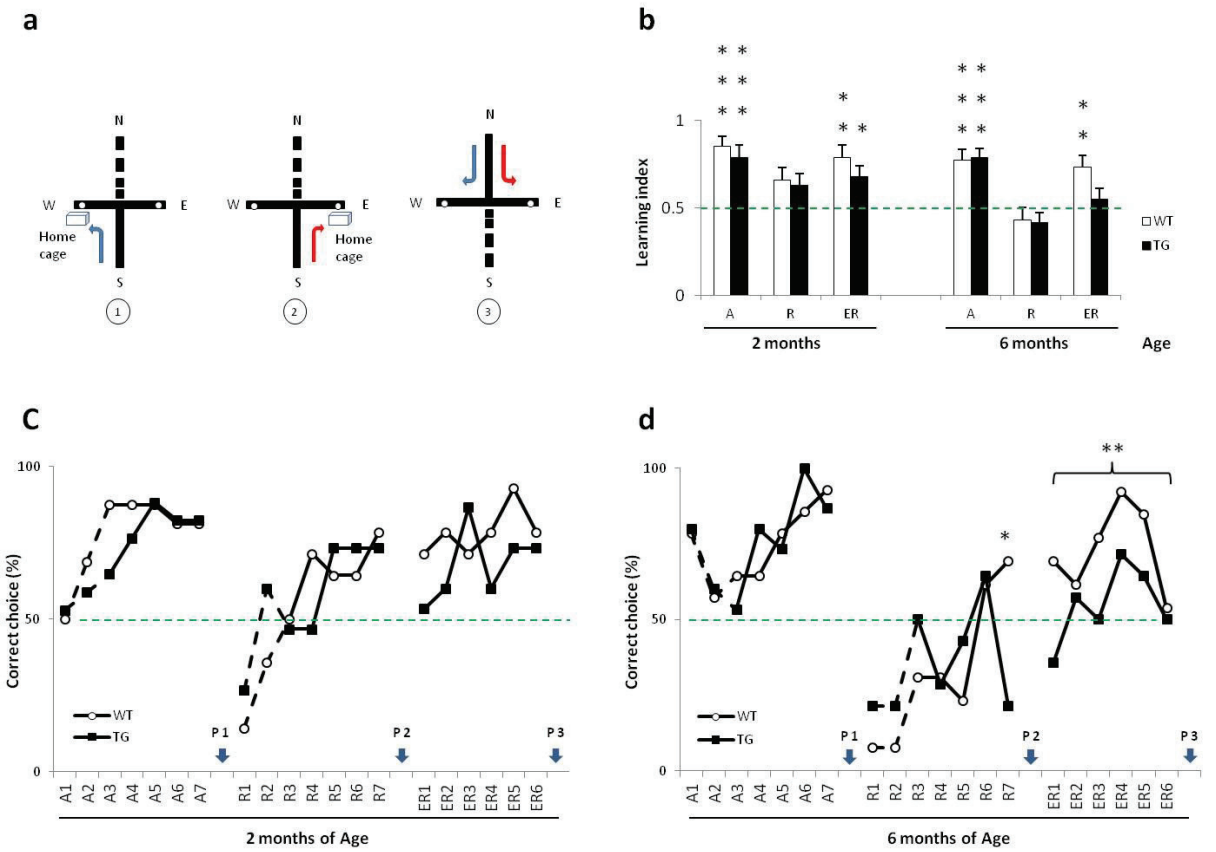


**Figure 1.** Cross-maze task. 2 months (WT n=17, TG n=17) and 6 months (WT n=15, TG n=15) BACHD rats were used. [**a**] Schematic representation of the cross-maze task. The north (N) arm is closed. The rat starts training in the south arm (S) and reaches the home cage through the hole located in the west arm (w, (1) acquisition) or east arm (E, (2) reversal). During (3) probe trial days 8 (P1), 16 (P2) and 23 (P3), the (S) arm is closed and the rat starts in the (N) arm. Rats reaching the home cage arm are Place learners, while those reaching the other arm are Response learners. [**b**] Learning index. Mean number of correct choices over acquisition (A), reversal (R) and extended reversal (ER) trials in BACHD rats. Both WT and TG rats of each age showed difficulties during (R); however, with (ER) training, 2 months old rats have improved learning whereas 6 months old TG rats have a learning index barely above chance level. Asterisks indicate significant difference from the hypothetical value (One sample test, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). [**c** and **d**] Training trials. The percentage of correct choices made during acquisition (A), reversal (R) and extended reversal (ER) training are depicted. For the first 2 days, results where the goal arm was baited with sucrose food pellets are presented by dashed lines. There was no difference in acquisition training for both age cohorts. 6 months old TG rats (d) differed significantly from WT rats during reversal trial 7 and overall extended reversal trials (ER1 to ER6). Asterisks indicate significant difference (Chi square test, *$p < 0.05$ and **$p < 0.01$).

- *Strategy shifting during probe trials* (Fig. 2)

On trial days 8, 16, and 23 a probe trial was done to assess which strategy rats used to solve the task. The north arm (N) was now the new start arm. Rats entering the same arm as during training sessions were designated place learners (allocentric learning) and rats entering the opposite arm were designated response learners (egocentric learning). Data were only analyzed for animals that made (1) correct arm choices with a learning index greater than 0.5 during each training session, and (2) were successful for the two last trials prior to the probe trial. Two months old rats exhibited a preference for response learning on P1 (*response:* WT= 73 % and TG = 77 %) and P2 (*response:* WT = 67 % and TG = 57 %). This preference for response learning was maintained on P3 (*response:* WT = 64 % and TG = 67 %). Six months old WT rats again exhibited a clear response learning during P1 (WT = 73 %) while only half of TG rats were response learners. However, during P2 and P3, WT rats have adopted a place learning strategy (WT, *place:* P2 = 66% and P3 = 57 %), whereas TG rats showed a response learning strategy (TG, *response:* P2 = 50% and P3 = 60%). Although WT rats results between both probe sessions (P1 → P3) would suggest a shifting towards a place learning ($\Delta$ = 30%), this was not statistically significant ($\chi 2$= 1.606, P> 0.05).

- *Contextual fear conditioning* (Fig. 3)

Rats of 4 months (WT, n= 13 and TG, n = 13), 6 months (WT, n= 7 and TG, n = 9) and 12 months (WT, n= 16 and TG, n = 6) of age underwent a one day training session in conditioning chambers. Baseline activity was recorded 5 min before foot shocks were given and contextual memory was measured 24h later (Fig. 3a).

As shown in Fig. 3b, there was no significant difference between WT and TG baselines at all testing ages. However, TG rats expressed a significant lower freezing behaviour when re-exposed to the conditioning context (4 months: t= 5.757, P< 0.0001; 6 months: t= 4.987, P< 0.001 and 12 months: t= 5.147, P< 0.0001).

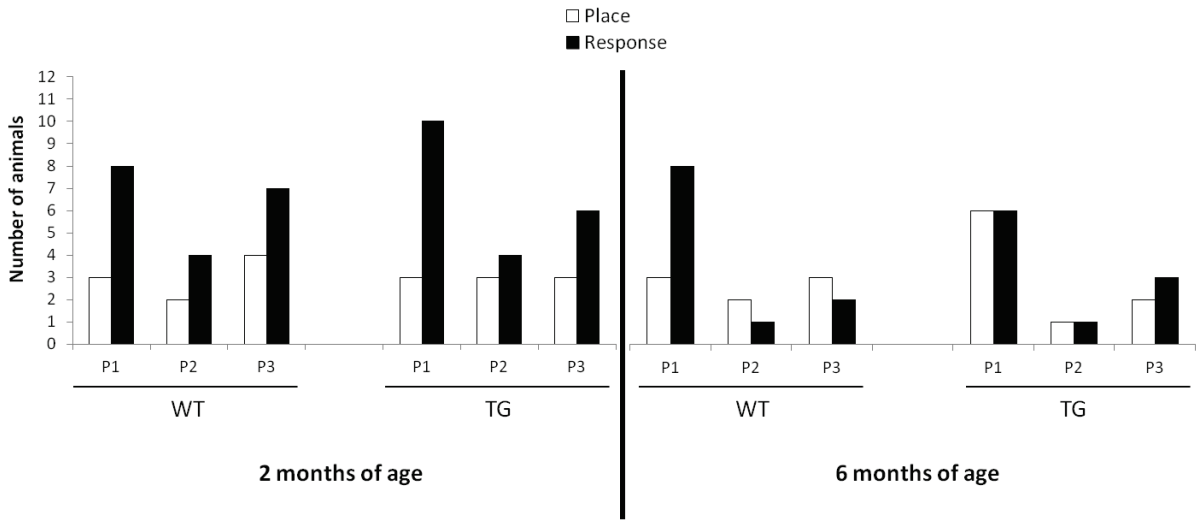


**Figure 2.** Strategy shifting. Number of rats that exhibited Place (P) or Response (R) learning strategy during each Probe trial P1, P2 and P3 (days 8, 16 and 23 respectively) are represented for WT and TG cohorts of 2 and 6 months of age. The size corresponds to animals that made (1) correct arm choices with a learning index greater than 0.5 during each training session, and (2) were successful for the two last trials prior to the probe trials in the cross maze.

Visual inspection of fig. 3b indicates a decrease in percentage of freezing between 4 months, 6 and 12 months old rats. In fact, a 2-way ANOVA analysis on Context results showed significant GENOTYPE ($F(1, 79) = 76.53$, $P < 0.001$) and AGE ($F(2, 79) = 13.42$, $P < 0.001$) effects. No interaction between GENOTYPE x AGE was found.

We next evaluated long term memory for contextual freezing in 4 months old rats by exposing them again to the conditioning chambers 1 and 2 months after the contextual test (retention tests, Fig. 3c). A progressive '*extinction*', characterized by a decrease in percentage freezing was observed in WT and TG rats (2-way ANOVA, GENOTYPE: $F(1, 72) = 82.92$, $P < 0.001$ and AGE: $F(2, 72) = 24.48$, $p < 0.001$). This trend is sustained as no interaction (GENOTYPE x AGE) was found.

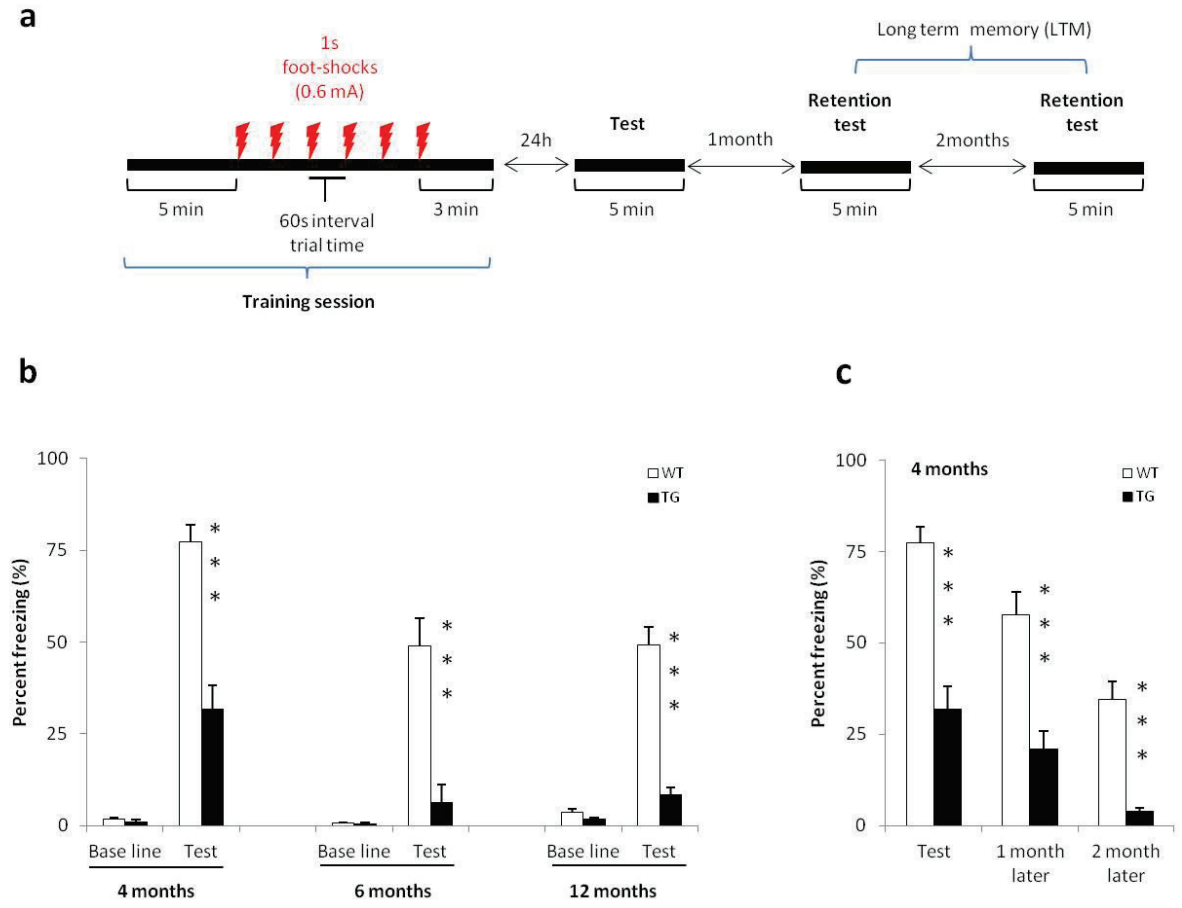


**Figure 3.** Contextual fear conditioning. [**a**] Schematic illustration of the contextual fear conditioning protocol. [**b-c**] Results are expressed as Mean ± SEM of percentage freezing. 4 months (WT n= 13, TG n = 13), 6 months (WT n= 7, TG n = 9) and 12 months (WT n= 16, TG, n = 6) BACHD rats were used. No difference in baseline responding to training was observed. TG rats showed less fear memory to the context as they freeze less in comparison with WT rats at 4, 6, and 12 months of age [**b**]. Long term memory was assessed 1 month and 2 months after retention testing were conducted in the 4 months old rats cohort (i.e. they were tested at 5 and 6 months of age respectively) [**c**]. TG compared to WT still had lower freezing to the context. A progressive freezing 'extinction' was observed. Asterisks indicate significant differences between WT and TG rats (***$p < 0.001$).

- *Delay and Trace conditioning* (Fig. 4)

The delay conditioning experiment evaluated the acquisition of a tone (85dB) fear conditioning when presented for 30s before a 2s foot-shock co-termination (Fig. 4a). Thirteen WT and fifteen TG rats of 9 months of age were given 6 trials training sessions. Baseline activity was recorded 3 min prior to the first trial and expressed as percentage freezing. WT and TG rats did not show differences in baseline freezing behavior (Fig. 4b). A 3-min retention test was performed after 24h in the conditioning context and, for the tone, in an altered context. Both WT and TG rats expressed a trend for increased freezing to the context and the tone. TG rats showed a lower percentage freezing to the context and to the tone than WT rats. A 2-way ANOVA revealed significant effects for the main factors GENOTYPE and TEST (GENOTYPE, $F(1,52) = 18.84$, $P < 0.001$; TEST, $F(2,52) = 132.04$, $P < 0.0001$) as well as a significant interaction between both factors ($F(2,52) = 5.04$, $P < 0.01$).

In the trace fear conditioning paradigm, the memory for context, tone and trace training is evaluated in 3 months old rats (Fig. 4c). The highest freezing responses were found during the trace period (Fig. 4d). No differences in baseline activity were found, but for all the stimuli (context, tone and trace), TG rats displayed a significantly lower freezing response than WT rats. A 2-way ANOVA analysis showed significant effects for the main factors GENOTYPE and TEST (GENOTYPE, $F(1,78)= 39.34$, $P < 0.0001$; TEST, $F(3,78)= 96.42$, $P < 0.0001$), as well as a significant interaction between both factors ($F(3,78)= 15.08$, $P < 0.0001$).

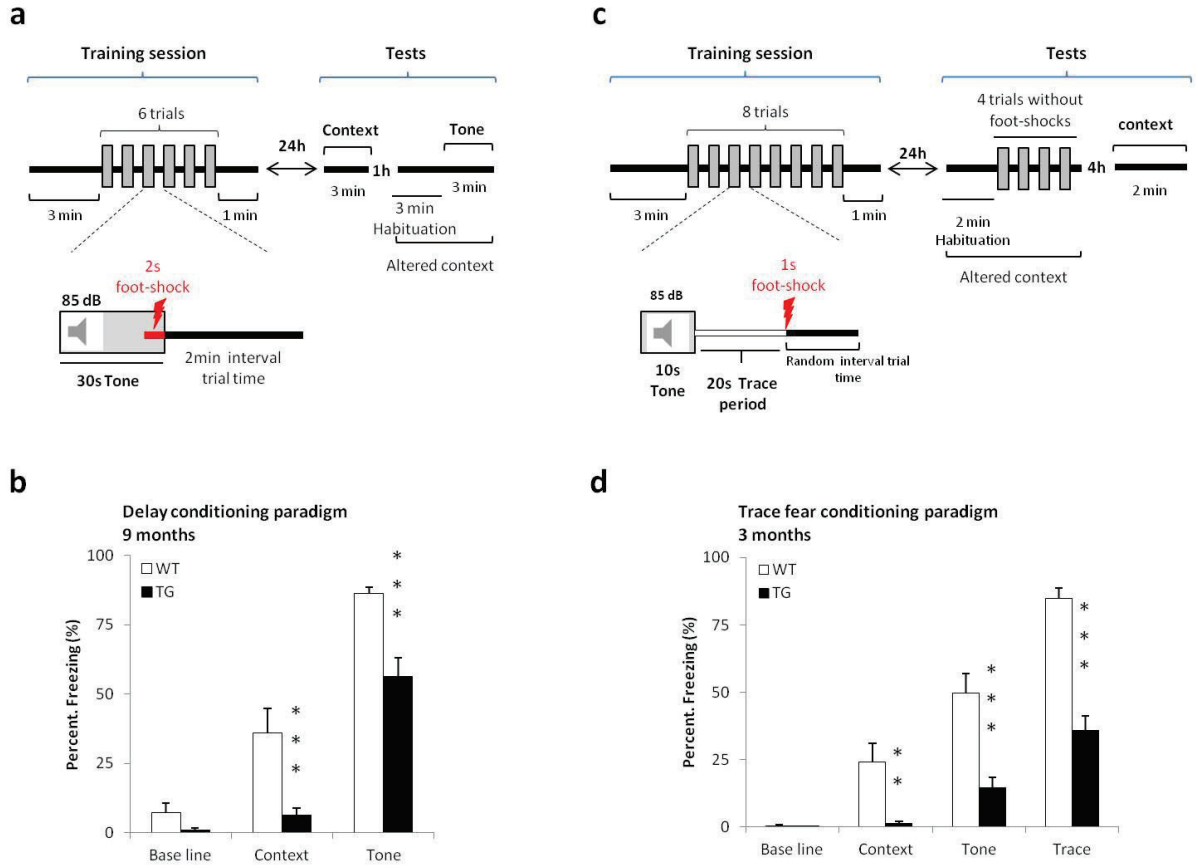


**Figure 4.** Delay and Trace fear conditioning. [**a**] Schematic illustration of the delay fear conditioning paradigm. [**b**] Results in 9 months old BACHD rats are expressed as Mean ± SEM (WT n=13, TG n= 15). TG rats presented a significant lower freezing response to the context and to the tone. [**c**] Schematic illustration of trace fear conditioning paradigm. [**d**] Results in 3 months old BACHD rats are expressed as Mean ± SEM (WT n=13, TG n= 15). TG rats, compared to WT rats, showed a significantly lower freezing response to the context, to the tone and trace period during retention tests. Asterisks indicate significant differences between WT and TG rats (**$p < 0.01$ and ***$p < 0.001$).

- *Flinch-Jump test* (Fig. 5)

Thirty BACHD rats (n=15 per genotype) of 6 months of age underwent the flinch-jump test. A flinch response was observed in all rats (Fig. 5a. WT, Mean = 0.23 ± 0.048 SEM; TG, Mean = 0.25 ± 0.042 SEM); whereas only 7 WT and 9 TG rats presented a jump response (Fig. 5b. Mean = 0.535 ± 0.037 SEM and Mean = 0.538 ± 0.048 SEM, respectively). In fact, statistical analysis did not reveal significant differences between WT and TG rats (Mann Whitney U-test: flinch, U= 85 and P= 0.23; Jump, U= 29 and P= 0.82).

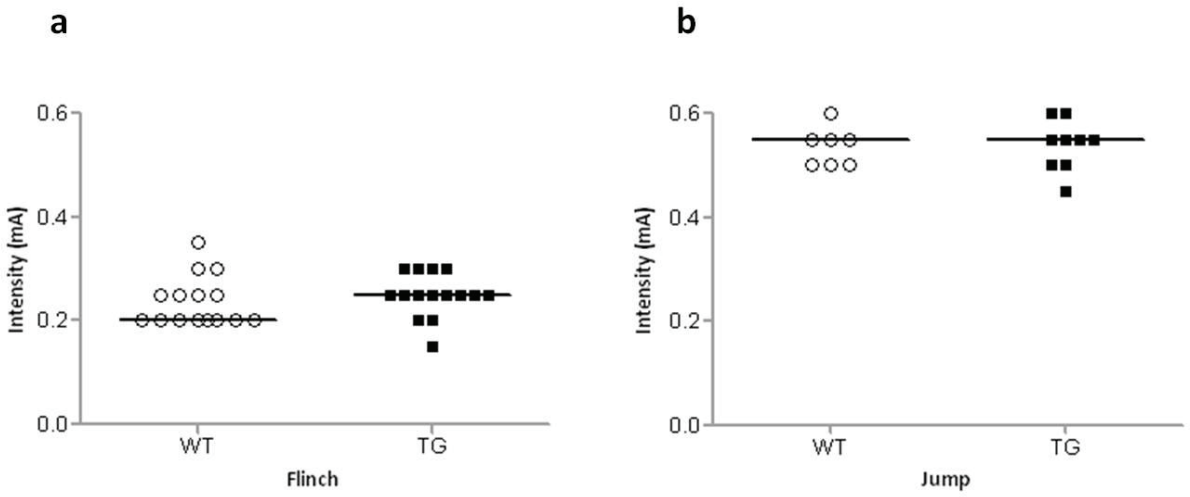


**Figure 5.** Flinch-jump test. Sensitivity of 6 months old BACHD rats to foot-shocks for [**a**] flinch and [**b**] jump (WT n=15, TG n= 15). Individual intensity response is plotted and bars indicate median values for each genotype. There was no difference between WT and TG rats in current intensities that elicited a flinch or a jump response.

## 4. Discussion

We investigated the cognitive phenotype of BACHD rats at ages 2 through 12 months. Learning deficits were found at 6 months of age in a cross-maze test. Pronounced associative memory deficits were found in context, delay (context and tone) and trace (context, tone and trace) fear conditioning. This fear conditioning phenotype is unlikely to be confounded by altered pain sensitivity, as WT and TG rats showed no differences in foot-shock intensity threshold as determined in a flinch-jump test. This is the first study to report robust and specific memory deficits in BACHD rats.

*Reversal learning deficit in cross maze test*

We have investigated BACHD rats in a spatial memory paradigm with the cross maze test. A T-maze standard dual solution task (Tolman et al. 1946) has proven to be useful in distinguishing between spatial and non-spatial learning in animals. Rats were trained over several trials from the same start arm to consistently enter the arm where a baited home-cage was located. We used a home-cage baited with sucrose pellets as an alternative to traditional motivational procedures that use water or food deprivation. Since BACHD transgenic rats show reduced food intake (Yu-Taeger et al. 2012), we felt that procedures that avoid food deprivation may be less liable to potential confounds and misinterpretation of behavioral data.

During Acquisition training (A) both TG and WT rats learned the task and showed a significant learning index (~ 0.8). This indicates that a 'return to home-cage' is an effective incentive and that a training protocol of only one trial per day is sufficient. These observations are in line with results from a study in adult B6D2$F_2$ mice (cross of C57BL/6J and DBA/2J strains) in a Lashley III maze. It was demonstrated that one training trial per day with a home-cage reward procedure led to a significant learning index (~ 0.7) after just 4 days (Blizard et al. 2006).

During Reversal training (R), the home cage was located at the end of another arm, different from the initially trained arm. The same start arm was used. *All* rats of 2 and 6 months of age initially had difficulties finding the new location. This was confirmed by a

comparison of the percentage correct choice of the last two acquisition trials (6 and 7) with the first two reversal trials (1 and 2). A difficulty in finding the new location is perhaps not surprising, as reversal learning is more challenging per se because rats have to disengage from a previous learned task in order to acquire a new task. We decided to extend the reversal training for 6 days (Extended Reversal training; ER) and all rats eventually learned the new task, although the 6 months old TG rats performed significantly worse than WT rats. We previously reported similar reversal learning deficits in adult transgenic BACHD mice of 10 months of age in a cross-maze task (Abada et al. 2013a). In 6 months old $Hdh^{(CAG)150}$ knock-in mice, cognitive impairments were shown in compound reversal of an extra-dimensional shift task (EDS) (Brooks et al. 2006). Reversal learning difficulties were also reported in a spatial operant reversal test paradigm of 9 months old tgHD rats and in 27-week old YAC128 mice in a water T-maze task (Brooks et al. 2012; Fink et al. 2012; Van Raamsdonk et al. 2005). These data are in accordance with the present findings, suggesting a progressive cognitive decline between 2 and 6 months of age.

To discover which learning strategy rats have adopted, a test trial was performed after training was finished. The new start arm was now located opposite of the arm used during training. Accordingly, rats which used spatial cues to find the correct arm would enter the same baited arm as during trainings (place learners); whereas rats that used 'body turn response' learning (stimulus response, S-R) should enter the non-baited arm (response learners). The first probe trial (P1) demonstrated that BACHD rats of 2 and 6 months of age were predominantly *response* learners. Using a similar cross maze task, this preference for the *response* strategy on (P1) was also observed in WT and TG BACHD *mice* (Abada et al. 2013a). Consistent with our findings, homozygote tgHD rats of 6 and 12 months of age were also mostly response learners in a Morris water maze task (Kirch et al. 2012). Interestingly, our results are in contrast with findings suggesting that during (A), *place* learning is typically adopted by rats in a cross-maze (Packard et McGaugh, 1996; Packard, 1999; Tolman et al. 1946). The reason why 2 months old rats maintain their preference for response learning during P3 is unclear, but may involve a developmental time scale of spatial representation. In fact, spatial memory in the cross-maze involves association of the object (landmarks) to their spatial location (home cage); we assume that the network underlying the memory of spatial location in 2

months old WT rats is slower to develop (Ainge and Langston, 2012). The preference (shift) for *place* strategy was only observed in 6 months old WT during the 2nd and 3rd probe trial (P2 and P3), whereas TG rats maintained their *response* strategy. The same strategy was seen in adult BACHD mice during reversal probe trial (Abada et al. 2013a). The reason why BACHD rats maintain response learning may probably involve altered functioning of fronto-hippocampal (place learning) vs. fronto-striatal (response learning) circuitry (Ciamei and Morton, 2009; Pych et al. 2005; Restle, 1957). Indeed, TG rats show already at 3 months of age abundant *htt* aggregates in the CA3 region of the hippocampus, whereas only few aggregates were present in the caudate-putamen (Yu-Taeger et al. 2012). Therefore, the striatal-based 'body turn response' might prevail during learning and subsequent probe testing in TG rats.

The rodent data are consistent with human data. Similar impairments in reversal learning and strategy, when attention has to be shifted from one perceptual dimension to another, have been demonstrated in early and advanced-stage HD patients in an extradimensional shift (EDS) test and in a Wisconsin Card Sorting Test (WCST). These patients made perseverative errors suggesting memory inflexibility (Josiassen et al. 1983; Lawrence et al. 1996, 1998, 1999). Finally, cognitive set shifting ability in EDS and WCST involves cortical and basal ganglia circuitry system, especially the prefrontal cortex and the caudate nucleus (Rogers et al. 2000).

*Associative learning deficits in fear conditioning test*

We performed an extensive characterization of BACHD rats in various fear conditioning paradigms and found very robust deficits across all age cohorts and under all experimental conditions. A technical challenge that was successfully mastered was the selection of an appropriate current intensity. One that was not too high - high intensities would lead to a generalized freezing response – or too low – low intensities would lead to large variability in freezing and inconsistent fear conditioning (Baldi et al. 2004). A confound of the BACHD fear conditioning phenotype by motor deficits seems unlikely since rats did not show any difference in percent of time freezing during habituation. In order to address if altered sensitivity to foot shocks (US) may have confounded the fear conditioning phenotype, we employed a flinch-jump test and found no differences

between WT and TG rats. We used a relatively low shock intensity (0.6 mA) which may explain that not all rats showed a 'jump' reflex. Our data are consistent with findings in Wistar rats where no correlation was found between pain sensitivity, conditioned and novelty-evoked fear responses in 'flinch-jump', 'tail flick' and 'contextual' fear tests (Lehner et al. 2010). Together, these data suggest that the deficit in conditioned fear responses in BACHD rats are not confounded by motor deficits or altered sensitivity to foot shocks.

What are the mechanisms underlying the fear conditioning deficits in BACHD rats? Learning in contextual fear conditioning is thought to involve association of stimuli present in the conditioned chamber (texture, shape, dimensions) with the (US) itself. More complex stimuli may put a higher demand on effective learning and memory, and thus may affect subjects with impaired activity in fear conditioning circuitry to a larger extent than unaffected subjects (Rescorla, 1972). The amygdala and hippocampus are involved in complex stimuli learning and BACHD rats show *htt* aggregates in both brain areas (Phillips and LeDoux, 1992; Yu-Taeger et al. 2012). Therefore, TG rat's lower freezing response to the context could be associated to a hippocampal-amygdala dysfunction. Such a conclusion is consistent with our findings from delay and trace fear conditioning testing. As expected from a stimulus with a higher salience, all rats showed higher levels of fear conditioning to the tone than to the context. The BACHD rats showed again a fear conditioning impairment. During trace fear conditioning, the Tone and shock (US) are separated by a time interval and the Trace period becomes predictive of the (US). Using these more complex stimuli, BACHD rats showed a clear deficit in fear conditioning. Impairments in fear conditioning have also been reported in mouse models for HD. For example, 5 weeks old R6/2 mice showed less contextual freezing than their wild-type control, although no difference was observed in tone conditioning (Bolivar et al. 2003). In addition, a reduced fear expression during extinction retrieval and a reinstatement of a fear conditioning in R6/2 mice was not associated with a weakness in CS-US, but with neuronal *hypoactivation* in the prelimbic cortex, a subregion region of the prefrontal cortex (Walker et al. 2011). Four months old CAG140 Knock-In (KI) mice have shown an increased freezing response during training, but, again displayed no deficit in recall tone fear conditioning (Hickey et al.

2008). We reported that adult transgenic BACHD mice present *higher* freezing rates to the context and tone during retention testing, and attributed this impairment to emotional deficits (Abada et al. 2013a). The difference in fear conditioning phenotypes between BACHD mice and rats is surprising. However, in view of the robustness of the rat phenotype and the fact that we are eventually interested in the translation of these findings to humans, it would be more sensible to perform fear conditioning studies in a non-human primate model for HD, rather than undertaking an effort to further characterize the mouse fear conditioning phenotype (Yang et al. 2008).

Interestingly, reversal learning impairments in a cross-maze appeared at 6 months of age, whereas associative learning and memory deficits in fear conditioning tasks were already present at 3 months of age. Matching the different onset of these deficits with the emergence of *htt* aggregates in brain areas involved in the circuitry underlying cross maze behavior and fear conditioning will be helpful to translate the findings from rodents to humans. Especially for fear conditioning the functional neuroanatomy has been well described (Fendt and Fanselow, 1999). Rodent data support a role for the amygdala in the acquisition of conditioned fear, whereas the hippocampus and the medial prefrontal cortex (mPFC) are required for consolidation of long-term memory (Faure et al. 2011; LeDoux, 2000; Gilmartin and Helmstetter, 2010, 2012). Human functional magnetic resonance imaging (fMRI) studies in delay and trace fear conditioning, have demonstrated a role of the hippocampus and other brain regions that support working memory processes in encoding temporal information and maintaining the associative representation CS-US during trace intervals (Knight et al. 2004). Wide spread *htt* aggregates have been observed in brain areas involved in fear conditioning such as the neocortex, hippocampus, and the amygdala of BACHD rats (Yu-Taeger et al. 2012). However, the behavioral effects occurred at an earlier age than the *htt* aggregates (12 months). It is possible that more subtle molecular and cellular deficits in the cortex, hippocampus and amygdala contribute to the early deficits in fear conditioning. Further studies should address the developmental mechanisms underlying the disease progression in BACHD rats.

## 5. Conclusion

Our study is the first to provide evidence of progressive cognitive deficits in a transgenic BACHD rat model for HD. TG animals showed difficulties in associative learning at 3 months of age in a fear conditioning test, and impairments in spatial memory at 6 months of age, mainly in reversal training where attention has to be shifted from one set of learning to another. BACHD rats recapitulate some of the cognitive impairments seen in HD patients. The precise time course for development of the cognitive symptoms requires further studies in additional age cohorts. As fear conditioning deficits appeared already in the youngest cohort tested, animals of 1 and 2 months of age need to be tested to determine if the onset of the fear conditioning is similar to the onset of, for example, rotarod deficits that occur at 2 months of age (Abada et al. 2013b). Emergence of cognitive deficits before motor deficits might more closely mimic the time course in HD patients (Hahn-Barma et al. 1998). In conclusion: the robust fear conditioning phenotype offers a firm foundation for future studies aimed to further characterize the time course for the associative memory deficit and its underlying neural circuitry. In addition, this functional readout can be validated for drug discovery approaches that target *htt* aggregates, using, for example, adenovirus-based viral transfection methods against htt (Ramaswamy and Kordower, 2012).

# III. General Discussion & Conclusion

# General Discussion

One of the most important milestones in HD research since the discovery of the gene itself has been the generation of different genetic animal models. Although clinical reports have shown evidence of progressive cognitive impairments to occur in gene carriers before motor symptoms are diagnosed, results from animal models have been less clear (Foroud et al. 1995; Jason et al. 1988; Paulsen et al. 2001; Höhn et al. 2011; Fielding et al. 2012). The studies presented in this thesis evaluated the behavioral phenotype of BACHD mice and rats that have the human fl-mHTT with mixed 97 CAA/CAG repeats expressed in their genome (Gray et al. 2008; Yu-Taeger et al. 2012). The results from these studies increase our understanding of the disease progression in transgenic BACHD rodent models for HD (Table 1). The general discussion will mainly focus on the results from our studies.

## 1. Gross phenotype

BACHD mice and BACHD rats grow normally with a general increase of their body weight. However, a significant weight gain was reported in transgenic BACHD mice starting at 2 months of age and this weight gain was maintained (Gray 2008; Menalled 2009). On the other hand, no difference in body weight was reported in the BACHD rat model, despite a reduction in food consumption during the dark phase (Yu-Taeger et al. 2012). Our studies replicated these findings. We found a sustained difference in the body weight of adult BACHD mice but no difference in BACHD rats compared to their WT counterparts.

There are several physiological mechanisms that could mediate the body weight gain in BACHD mice: 1) reduced metabolic rate; 2) an increase in food intake; 3) a reduction in total activity, or 4) a combination of these 3 factors. For example, in one report, transgenic mice displayed higher food intake and reduced metabolic rate, but no reduction in activity in an open field at an age when they were already significantly heavier than WT mice (Hult et al. 2011). With regard to the molecular basis for the

increase in body weight, changes in the expression levels of HTT may represent a potential biological mechanism. Heterozygous mice (Hdh +/-) expressing 50% of the htt protein compared to WT mice, were *lighter* than WT mice. YAC 128 mice line B60 and line 212 over expressing WT htt at about 2 to 3 times endogenous levels were physically larger and *heavier* than WT mice (Van Raamsdonk et al. 2006). BACHD animals express 2 copies of the endogenous full length WT HTT (gene) as well as 1 or more copies of the human fl-mHTT in their genome. Therefore, reports mentioned above suggest that BACHD mice's body weight phenotype is due to an increase of the mutated huntingtin protein, or a combination of both endogenous and mutated HTT total expression. Van Raamsdonk and colleagues (2006) demonstrated with western blot techniques that endogenous htt levels in transgenics were comparable to that of WT in YAC128 mice, suggesting that the increased body weight is due to an increase in mutated rather than normal htt. Thus, we can postulate that, in BACHD mice, the "gain" in body weight also results from increased fl-mHTT expression.

From a molecular point of view, body weight changes in YAC 128 and BACHD mice have been associated with IGF-1 (insulin-like growth factor 1) levels and HAP-1 (huntingtin associated protein 1) expression, both playing important roles in mediating organs growth and food intake (Li et al. 1995; Li et al 2003; Pouladi et al. 2010). Finally, a recent study by Hult et al. (2011) point to the importance of the hypothalamus mHTT's role in regulating body weight. In this experiment, the authors used the cre-recombinase technique to selectively rescue the mHTT production in the hypothalamus in BACHD mice. As a result, these mice showed a normal body weight. Interestingly, the serum levels of IGF-1 were still significantly higher in these animals compared to WT mice, emphasing the complex regulation of body weight.

If changes in HTT level expressions may explain, at least in part, the "gain" in body weight phenotype of BACHD mice, the "normal" body weight phenotype in BACHD rats is unexpected, especially since both species have the same construct. One possible explanation for this discrepancy may involve species-specific genetic differences. In fact, as previously reported by Hult et al. (2011), the relationship between IGF-1/HAP-1 and body weight is a complex one. It is possible that mHTT expression in BACHD rats' brain may affect metabolic pathways in hypothalamus circuits, including corticotrophin-

releasing hormone (CRH) and the neuropeptide orexin (Funato et al. 2008; Hult et al. 2011; Masaki et al. 2003). Further studies are needed to determine the mechanism of action for the effects of htt in the regulation of body weight phenotype in BACHD mice and rats.

Weight loss is present in HD patients and it appears not to be associated with a decrease in food intake (consumed calories), an increase in energy expenditure or basal metabolic rate (Aziz et al. 2010; Morales et al. 1989; Pratley et al. 2000; Sanberg et al. 1981). The causes of weight loss in HD patients are currently unknown and further analyses are necessary to shed light on this important non-neurological symptom and to further assess the validity of our animal models.

### *2. Motor deficits*

*BACHD mice*

Gray and colleagues (2008) demonstrated in BACHD mice progressive motor deficits on the accelerating rotarod starting at 2 months of age. Other reports have confirmed a decline in motor coordination on a rotarod and in rearing-climbing tests in mice as young as 1 month of age. Hypoactivity in the dark phase of the light cycle in an open field test was reported at 7 months of age. Subtle gait abnormalities, such as shorter/longer stride length, splay, and wider base, were found in a paw-print test at 3 and 9 months of age, respectively (Kordasiewicz et al. 2012; Menalled et al. 2009; Pouladi et al. 2012; Southwell et al. 2009).

In our first study, we performed a comprehensive behavioral analysis and investigated motor deficits in adult male BACHD mice of 9 and 10 months of age. We found significant motor deficits in BACHD mice as they showed a shorter latency to fall off a Rotarod. Novel findings were the gait abnormalities in both static and dynamic parameters, such as the order in which the four paws are placed during free walking and the timing relationships between paw placements that were found in a catwalk test. Since a deficit in rotarod performance was as already observed at 1 month of age, it would be interesting to see if the catwalk phenotype observed at 3 months of age would also manifest itself in younger animals.

*BACHD rats*

Transgenic rats displayed a progressive deficit in their performance on an accelerating rod at 1 month of age (Yu-Taeger et al. 2012). In our study, transgenic rats showed motor deficits starting at 2 months of age in both, a fixed (constant) and accelerating speed rotarod test. At fixed speed, the rat's balance, muscle strength and fatigue is assessed. The accelerating speed test is more complex and demands to adapt gait, balance and coordination skills. Our results confirm that the rotarod is a robust test that is suitable for motor function assessment in animal models for HD.

In an open field test (Actimot), we found an increase in locomotor activity at 1 month of age, followed by a decrease that started at 4 months of age. Yu-Taeguer and colleagues (2012) found a decrease in locomotor activity and rearing during the dark phase at only 3 and 6 months of age (they did not test younger animals). Interestingly, Yu-Taeguer and colleagues used a different experimental set-up, more closely related to the home-cage and recorded motor activity over a prolonged period of time (12 h). The fact that, in spite of these differences, both studies show hypolocomotion emphasizes the robustness of the motor deficit in the BACHD model. Moreover, our study showed that short duration open field studies should be sensitive enough to detect the effects of potential treatments.

Finally, gait abnormalities like decreased step length, increased stride width, and decreased overlap between hind and front paw placement in footprints test were observed in 14 months old rats (Yu-Taeger et al. 2012). Longitudinal characterization of BACHD rats gait analysis with catwalk revealed subtle deficits at 12 months of age. We found a shorter stride length, shorter time for stand for both front and hind paws, and shorter time in front paw swing during walking. The catwalk system is a more sophisticated instrument for the assessment of gait than conventional footprint testing since it allows evaluation of both static and dynamic gait parameters. In light of the Catwalk phenotype in BACHD mice, we expected more robust deficits in the BACHD rats. The reason for the more subtle phenotype in rats is not clear. It is unlikely to involve differences in testing protocol since the same catwalk testing procedure was used in the same facility and by the same experimenter for the mouse and rat studies. Alternatively, differences in body weight and/or genetic background may have

contributed to the different findings. Body weight seems not a good candidate for explaining the differences between the mouse and rat findings since no difference in the print intensity of the paws (an indicator of weight load bearing), was observed between transgenic and WT mice and rats. Strain differences were reported in gait parameters on a catwalk test between Lewis, Wistar and Sprague Dawley rats (Koopmans et al. 2007). Although little is known about the relative paw placement when moving on a flat surface, differences in size and skeletal morphometry between rats and mice exist and could contribute to differences in standard gait between BACHD mice and rats (Herbin et al. 2007; Pereira et al.2006; Wooley et al. 2009). Further studies using more challenging conditions - like a ladder rung walking task that requires exact placement of all four limbs - could help to better understand the locomotor phenotype of BACHD rats and mice.

### *3. Emotional deficits*

*Anxiety*

Non-motor symptoms such as anxiety, depression, irritability and apathy are often associated with HD (Van Duijn et al. 2007). In adult BACHD mice, we found a *higher* level of anxiety-like behavior in an elevated zero-maze (EZM) test and a higher freezing response to a tone in a fear conditioning test. Similar findings have been reported in the literature. For example, 3 months old BACHD mice showed a higher preference for staying in a dark area in a light-dark choice test (Kordasiewicz et al. 2012; Menalled et al. 2009).

It remains to be determined to what extent the elevated zero maze (EZM) and the tone fear conditioning data can both be interpreted as supporting an anxious phenotype in the BACHD mice or whether these tasks measure largely independent emotional and/or cognitive processes. In BACHD rats, a *decrease* in anxiety level was reported in an elevated plus maze (EPM) test. At 4 months and at 12 months of age, the TG rats spend more time on the open arms of the maze compared with the WT rats. 3 Months old TG rats also expressed a lower level of freezing to a fearful stimulus in a fear conditioning test. However, this phenotype appears to be not confounded by differences in sensitivity to foot shocks (as shown in the flinch-jump test) or by motor deficits (same

base line activity).These results are consistent with a lower level of anxiety. But as mentioned before for the BACHD mice, it is again possible that the EPM and fear conditioning tasks measure different processes. The different phenotypes of BACHD rats and mice in EZM test, EPM test and fear conditioning models could involve differences in spatiotemporal nuclear accumulation of mhtt. In BACHD rats of 3 months of age, mhtt was found in the neocortex and in limbic area such as amygdala, nucleus accumbens, bed nucleus of the stria terminalis, hippocampus and later in the striatum. In BACHD mice, mhtt was detected later (at 12 and 18 months of age) and there were differences in the mhtt distribution patterns (in the cortex, striatum and occasionally in the globus pallidus and substantia nigra (Gray et al. 2008; Yu-Taeger et al. 2012). The role of the limbic circuitry in emotion and motivation is well documented (Cardinal et al. 2002; Davis and Whalen, 2001). We hypothesize that the molecular mechanisms that underlie these differences in emotional-like behavior between transgenic rats and mice involve differences in mhtt expression induced-dysfunctions in limbic areas.

*Depression*

Depression symptoms are common in HD with 60% of patients experiencing low mood (Thompson et al. 2012). A depression-like phenotype has also been found in transgenic HD models. Transgenic BACHD and YAC 128 mice of 12 months of age showed increased immobility in a Porsolt forced swim test (Pouladi et al. 2012). This phenotype was seen as early as at 2 months of age in BACHD mice (Hult et al. 2013). A potential concern is the ability of these mice to swim, since transgenic mice have age-dependent motor deficits that could confound results from behavioral tests that rely on motor behavior read-outs. It is somewhat reassuring that no correlation between body weight and immobility time or swim ability was found in YAC 128 and BACHD mice. To expand the findings in the forced swim test, a next step would be to test BACHD mice in a tail suspension test. Like the forced swim test, this test is frequently used to phenotype mutant mice, although its validity as a depression test is also limited. In contrast, the sucrose consumption test is a more valid model as it measures a major component of depression: anhedonic behavior, that is, the inability to experience pleasure. YAC 128 mice displayed anhedonic behavior in this test (Pouladi et al. 2009) and therefore the

sucrose test could yield valuable insights into a possible depression-like phenotype of BACHD mice and rats.

### *4. Cognitive deficits*

Cognitive dysfunction is one of the triad of symptoms of Huntington disease (HD) and it appears to be present 15 years before clinically relevant motor signs have emerged (Duff et al. 2010; Stout et al. 2011). We have investigated and reported different aspects of memory and information processing by BACHD mice and rats in different tasks.

*Sensorimotor gating*

Prepulse inhibition (PPI) of the acoustic startle reflex is a measure of sensorimotor gating abilities that correlates with information processing. PPI is defined as a reduction in the magnitude of a startle response when a relatively weak sensory event precedes a strong startle stimulus.

In BACHD mice, Menalled and colleagues (2009) reported a decrease in startle response at 6 and 9 months of age in transgenic females, and only at 9 months of age in males. PPI deficits were found at 7 and 9 months in transgenic males and females, respectively, and at 13 months in both genders. In BACHD rats, we have shown that 9 months old transgenic males have deficits in PPI, especially at pre-pulses 6 and 12 dB above background (study 2). Therefore, BACHD mice and rats displayed similar PPI phenotypes. PPI deficits have been shown in HD patients and abnormalities in its modulation have been associated with striatal dysfunction (Kodsi and Swerdlow, 1995; Swerdlow et al. 1995). It should be noted that the PPI deficit in transgenic rats was subtle. Testing rats at later ages (13-15 months) may reveal a more robust sensorimotor phenotype.

*Recognition memory*

Pre-manifest and manifest HD patients are impaired in recognition memory tasks (Aretouli and Brandt, 2010; Hodges et al. 1990; Labuschagne et al. 2013; Lawrence et al. 1996, 2000; Montoya et al. 2006). Object recognition and object location tasks are two paradigms widely used to assess episodic memory in rodents. Deficits have been

reported in BACHD and YAC128 mice of 7 months of age with no preference for a target object in both paradigms (Southwell et al. 2009; Doria et al. 2013). However, our investigation in BACHD rats (reported in study 3) has showed intact recognition memory for a novel object at 4 and 12 months of age. The reason for this lack of impairment on object memory is presently not clear. It is possible that the neural circuits that mediate object recognition in our task do not sufficiently recruit brain areas that are significantly impaired in HD like the striatum to show a deficit. Future studies that use recognition tasks that rely more heavily on the functioning of these impaired brain areas (as determined by, for example, fMRI studies) may help to reveal a deficit in object memory (Montoya et al. 2006).

*Associative memory, Reversal learning and Strategy shifting*

We found cognitive deficits in BACHD mouse and rat models for HD in several tasks. In a fear conditioning task, transgenic rats have deficits in associating / remembering stimuli that are predictive of a fearful event (foot shock). We were able to provide strong evidence of how a temporal distance between events influences associative learning in 3 months old transgenic rats in a Trace conditioning test; this paradigm is believed to involve the medial prefrontal cortex, the hippocampus and the basal ganglia (Gilmartin et al. 2010, 2012). Therefore, deficits in associative learning could be attributed to a dysfunction in the activation of these brain areas months before aggregates can be detected.

Reversal learning in a cross-maze test was impaired in 6 months old transgenic rats. It would be important to confirm this in older animals (12 to 13 months). Like transgenic rats, adult transgenic mice had difficulties in reversal learning and this translated into a high number of incorrect arm choices during the task. Although the strategy adopted during each stage of learning (i.e. acquisition & reversal/extended reversal) was not statistically significant in mice and rats, the majority of wild type rats and mice shifted from a body turn response to a spatial learning strategy. Memory inflexibility was observed in transgenics as they persevered in the same set of strategy irrespective of the learning tasks. In the light of the cross maze test results (study 1 and 3), it will be of interest to perform a longitudinal study assessing the progressive learning and memory

impairment in BACHD mice. This will be a quite large study as different animals are needed for each age-cohort to avoid that prior learning will affect subsequent learning/performance in the same task.

- ***Do cognitive deficits start prior to motor impairments?***

Cognitive impairments are clinical indicators of the disease process before motor dysfunction is diagnosed in HD, although this has not always been found in animal models (Stout et al. 2011). In BACHD mice and rats, the earliest motor deficit was found during rotarod testing at 2 months of age. No impairment in reversal learning was found during cross maze testing. These results, together with published data, indicate that motor impairments are the first symptoms to occur in BACHD mouse and rat models for HD (Abada et al. 2013a, 2013b, 2013c; Duff et al. 2010; Menalled 2009; Southwell 2009; Yu-Taeger et al. 2012) .

The relative *intact* memory functioning found at this early stage in BACHD mice and rats point to intact functioning of striatal and cortical networks, since it has been demonstrated that progressive striatal and cortical atrophy correlate with cognitive deficits in attention, working memory and executive functions (Peinemann et al. 2005). To our knowledge, no evidence for neurodegeneration has been reported in rodent HD models before 6 months of age. Additional investigations with, for example, electrophysiological assays that can help examine the activity of prefrontal-striatal networks may shed further light on potential presymptomatic changes that may not be detectable with cognitive behavioral assays in BACHD mice and rats (Höhn S et al. 2011). In addition, the use of further tests that more specifically probe the prefrontal-striatal network can be used, such as the attentional set shifting test or specific working memory tests such as a delayed (non)-matching to sample task.

| PHENOTYPE | | | ANIMAL MODELS | | HUMAN |
|---|---|---|---|---|---|
| | | | BACHD mice | BACHD rats | HD |
| **Weight** | | | Gain (from 2 months) | "normal" | loss |
| **Motor** | Rotarod | | decrease in latency (from 2months) | decrease in latency (from 2months) | Chorea, bradykinea, dystonia, loss of postural reflexes, deficits in UHDRS motor tests and many subtle gait abnormalities (stride length, cadence sway…) |
| | Open field | | hypoactivity (from 7 months) | hyperactivity (at 1 month) hypoactivity (from 4 months) | |
| | Ink paw print | static gait parameters | abnormalities (at 3 & 9 months) | abnormalities (at 14 months) | |
| | Catwalk | static and dynamic gait parameters | abnormalities (at 9 months) | subtle abnormalities (at 12 months) | |
| **Emotional** | Elevated zero maze (EZM) | | Anxiety-like phenotype (at 9 months) | N/A | impairments in UHDRS behavioral tests with anxiety, depression, irritability, apathy |
| | Elevated plus maze (EPM) | | Anxiety –like phenotype (at 2 months) | low level of anxiety (from 4 months) | |
| | Fear conditioning | | high level freezing (at 9 months) | low level freezing (at 3 months) | |
| **Cognitive** | PPI | | deficit (at 7 and 13 months) | deficit (at 9 months) | Reduced PPI |
| | ORT | | deficit (at 7 months) | "normal" | Impaired recognition memory |
| | Cross maze | reversal learning | Impairment (at 10 months) | Impairment (at 6 months) | impairments in reversal, WCST, EDS, MMSE, UHDRS cognitive tests |
| | | strategy shifting | difficulties at 10 months | difficulties (at 6 months) | |
| | Trace fear conditioning | Associative memory | N/A | Impairment (at 3 months) | |

**Table 1.** Behavioral phenotype of BACHD mice and rats model for HD. This comparative table is based on results of the 3 studies of this thesis as well as on reports from the literature (Hult Lundh et al. 2013; Menalled et al. 2009; Southwell et al. 2009; Yu-Taeger et al.2012).

- ***Is the disease progression similar in BACHD mouse and rat models for HD?***

We have summarized results from our studies and related published works from other groups in Table 1. In general, many behavioral phenotypes such as progressive deteriorations of motor and cognitive functions and the late onset of neuronal aggregates / dark cells in the cortex and striatum observed in BACHD mouse and rat models are consistent and comparable to human HD reports (Abada et al. 2013a, 2013b, 2013c; Gray et al 2008; Yu-Taeger et al. 2012). However, we found some differences in phenotype between BACHD mice and rats, in spite of the fact that they express the same genetic construct. Although the precise reasons for these differences are not known, these may involve differences in the regulatory sequences between mouse HTT, rat HTT and human mHTT (Ehrnhoefer et al. 2009) and the exact insertion of the mutant gene in the genome of the BACHD mice and rats.

- ***Which experimental assays are sensitive for therapeutic drug screening?***

HD is a progressive neurodegenerative disorder; the use of robust, sensitive and inexpensive assays which can identify behavioural markers at an early stage and evaluate numerous compounds is highly desirable. In this thesis, we have used assays that can evaluate motor, emotional and cognitive abnormalities in BACHD mice and rats.

For motor deficits assessment, the rotarod and open field seem to be reliable tasks since we were able to replicate data from previous reports (Menalled et al. 2009; Yu-Taeger et al. 2012). Both tests have been widely used for measuring efficacy of potential disease-modifying therapies. Compared with the rotarod test, the catwalk is a more sophisticated task with the advantage that it allows free movement of the animal during the test. It can detect more subtle information on gait impairments. However, it has a lower through-put than the rotarod and open field tests .Our data suggest that the deficits in catwalk test are less robust and smaller. This may be relevant when using this task for drug screening and testing. One reason for the lower throughput is the current

need for manual analysis of recorded foot prints which is a time consuming process. Finally, further tests across labs will help to demonstrate the reliability and efficacy of catwalk testing for phenotyping and drug testing.

To evaluate emotional symptoms, we have used the EZM test and a fear conditioning test. A low level of anxiety has been reported in an EPM test by Yu-Taeger and colleagues (2012). A concern with the EPM design (plus shape) is the problem of how to interpret occupation by the animal of the central compartment. The EZM design (annular runway) decreases this ambiguity and seems therefore a more suitable task. In view of the relatively small effects sized in both the EZM and the EPM, they may not consist of the tasks of choice for drug testing. Instead, the effect size in the mouse fear conditioning test was larger and therefore this test could potentially be used for drug screening (Inoue et al. 1996).

PPI, cross-maze and trace fear conditioning have been used to assess cognitive deficits in BACHD mice and rats models for HD. Our results indicate that BACHD mice and rats have subtle deficits in both PPI and cross maze tests. So far, only one report has demonstrated PPI deficits in HD patients and it would be good to see these findings replicated (Swerdlow et al. 1995). Testing BACHD mice and rats at older ages (more than 12 months of age), that is, when they display more profound neurohistopathological deficits, might reveal a more robust PPI and cross-maze phenotype. In contrast, assessment of associative memory with a trace fear conditioning test showed a robust deficit in BACHD rats at an early age (3 months). Therefore, from the current set of studies, the Trace fear conditioning task seems the best choice for drug testing.

## 5. Therapeutic approaches and plausible candidates

Initial studies in conditional transgenic mice models have shown that the phenotype can be rescued after onset of the disease, and thus, raising the possibility that HD may be reversible (Yamamoto et al. 2000). Therefore, in order to initiate clinical trials it is indispensable that 'successful' compounds *alleviate* the disease in animal models. Since HD is associated with neuropathology changes in the striatum and cortex, many therapies have focused on general neuronal protection strategies (Krobitsch and Kazantsev, 2011).

Combined treatment of coenzyme Q10 (***CoQ10***), a vitamin-like substance that generate energy in form of ATP, and ***Remacemide***, a prodrug and NMDA receptor antagonist, significantly improved motor performance, delayed body weight loss and the occurrence of neuronal intranuclear aggregates and increased survival in R6/2 and N171-82Q mice models for HD (Ferrante et al. 2002). Unfortunately, clinical trials of *CoQ10* and *Remacemide* as mono- or combination therapies where largely negative (HSG, 2001). One possible explanation for the false positive findings in the animal models is that the pathophysiology of neurodegeneration in these transgenic mice may not replicate HD illness. In addition, the disease stage at which the therapeutic trials were initiated in patients (symptomatic HD) differs from that of the transgenic mice (day 21, before onset of symptoms).

HD has also been linked to alteration of glutamate and dopamine pathways, especially the fronto-striatal circuitry. ***Riluzole***, a drug that prevents stimulation of glutamate receptors and that is used for amyotrophic lateral sclerosis (ALS) treatment is reported to significantly delay and reduce weight loss and htt aggregation in R6/2 mice (Schiefer et al. 2002). Clinical investigations with anti dopamine and glutamate agents such as *Tetrabenazine (**TBZ**)*, ***Amantadine***, ***olanzapine*** and *Riluzole* showed amelioration of chorea but these compounds induced many adverse effects, including depression and sedation (Novak and Tabrizi, 2010; Mittal and Eddy, 2013). Currently, there is no evidence of a universally effective treatment for HD; only Tetrabenazine, an approved drug from the US Food and Drug Administration (FDA), showed clear efficacy against

chorea (Fekete et al. 2012; Mestre et al. 2009; Pidgeon and Rickards, 2013; Scott, 2011).

An attractive therapeutic strategy would be to target mHTT itself. 'Gene silencing' or HTT lowering treatments is one of the promising experimental approach. Isis Pharmaceuticals and Roche started a collaboration using such an approach for the treatment of HD (Drug discovery news, 2013). Isis Pharmaceuticals is developing anti-sense oligonucleotides (***ASOs***) drugs for HD. Unlike other gene silencing strategies including short-hairpin RNA (shRNAs), small interfering and micro interfering RNAs (siRNAs and miRNAs), ASOs have the advantage of penetrating the blood brain barrier (BBB) when injected in the spinal fluid. Infusion of human ASO (HuASO) in R6/2, BACHD and YAC 128 mice or MkHuASO in Rhesus monkeys has been recently shown to produce sustained effects in rotarod and open field tests, and a prolonged survival (Kordasiewicz et al. 2012). These promising findings will hopefully translate into a positive proof of concept trial in patients. Another approach for novel therapeutics with an improved side effects profile consist of ASOs drugs that specifically silence mHTT – but not HTT.

Finally, **stem cell** research could provide an opportunity to design new therapeutic strategies. HD is mostly due to loss of MSNs in the brain. Obtaining new MSNs to replace injured cells would be straight forward approach (Dunnett and Rosser, 2013). As a consequence, embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) are capable of self-renewal and can differentiate into MSNs. These stem cells have the ability to make all the different cells of the body (Lescaudron et al. 2012; Perrier and Peschanski, 2012). However, this technology raises many challenges to meet: 1) direct these stem cells to make only MSNs, 2) demonstrate proper integration of stem cells in the central nervous system with long-term survival and no tumor formation. A sensitive and viable approach that can help overcome these challenges will be to use HD patients-derived stem cells because neurons with mutations that were corrected before transplantation should not be subjected to cell-intrinsic neurodegenerative signals (An et al. 2012; Jeon et al. 2012).

# Conclusion

In this thesis, we have characterized transgenic BACHD mice and BACHD rats that have incorporated the human full length mHTT in their genome on motor, cognitive and emotional tasks. We have demonstrated that, although a difference in the disease progression exists between transgenic mice and rats, both models present several behavioral-like symptoms similar to those reported for HD patients.

Cognitive symptoms appear years before clinically diagnose symptoms in HD. However, the studies we have presented in this thesis indicated that motor abnormalities are the first symptoms to be detected in transgenic BACHD mice and rats. The lack of significant cognitive deficits at the early ages has been highlighted as one of the most likely causes for discrepancy between experimental and clinical data. The development and use of techniques such as electrophysiology and neuroimaging can enhance our understanding of cognitive dysfunction associated with HD in animal models for HD. Finally, we have indicated relevant behavioral tasks for detecting and tracking different aspects of the disease progression.

HD pathology is not restricted to the brain; it is also observed in non neuronal tissues. For example, high level of blood pro-inflammatory immune system agents like cytokines and increased kynurenine/tryptophan ratio have been reported (Björkqvist et al. 2008; Stoy et al. 2005). This ‘new window’ from the blood may also help us to understand and monitor the disease progression earlier.

Currently, no effective treatment that can halt the disease progression or prevent its manifestation exists. The present thesis adds to the wide range knowledge of existing animal models because we have characterized 2 recently generated models for HD with the same genetical construct. The comparison of BACHD mouse, BACHD rat and other animal models for HD has given an outlook on differences and similarities in the disease progression. We come to the conclusion that, for an effective treatment, it is indispensable to use more than one genetical animal model and species for preclinical drugs testing.

# IV. Summary

# Summary

Huntington's disease (HD) is a devastating neurodegenerative disorder caused by a genetic mutation that produces an expansion of C-A-G repeats in the huntingtin (HTT) gene. This expansion results in a selective neuronal degeneration in striatal and cortical brain regions. The underlying mechanisms remain to be fully elucidated. Although HD is best known for chorea, the disease is actually a triad of progressive cognitive, psychiatric and motor symptoms and patients usually die within 15 to 20 years of onset. Cognitive impairments seem to occurs decades prior to the onset of motor symptoms. This potentially offers a long time window for treatments to delay or halt disease progression. Development of such treatments requires the generation of transgenic animal models for HD. Such models are now available and have already greatly enhanced our understanding of the pathogenesis of HD. But unsolved questions such as: "Do cognitive manifestations precede motor deficits in transgenic mouse and rat models for HD?" remain. We address this question in 3 studies that examine the development of motor, cognitive and psychiatric symptoms in BACHD mice and rats, two recently generated models for HD. Unlike other genetic models, BACHD mice and rats express the full length of the human mutant HTT (97 polyglutamine repeats) and present neuropathology similarities to that of patients.

We found progressive motor, cognitive and psychiatric deficits in transgenics BACHD mice and rats compared to their wild type littermate controls, on different tasks such as catwalk, rotarod, open field, zero-maze, cross-maze, prepulse inhibition and fear conditioning. The findings indicate that progressive cognitive deficits can be reliably detected with methodologically rigorous protocols. However some phenotypic variability (body weight and emotional-like behavior, for example) was found and might involve species differences and ectopic expression of the human mutant HTT gene. Finally, the time course for the emergence of the various symptoms indicates that motor abnormalities might be the first to occur in transgenic BACHD mice and rats.

In conclusion: we have identified robust cognitive and motor phenotypes for BACHD mice and rats that can be used to test novel compounds from HD drug discovery

programs. Certain tests like fear conditioning, lend themselves for translational approaches and characterization of HD patients in such tests would be a logical next step. Confirmation of a fear conditioning phenotype in HD patients would offer an additional functional read-out for drug testing and decrease program risk by bridging the gap between rodent and human testing.

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# VI. Acknowledgements

# Acknowledgements

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*Yah-se ABADA*

# VII. Publications and Posters

# Publications and Posters

- The following articles have been published or accepted for publication in international scientific journals and their manuscripts are included in this thesis:

- **Abada** Y-s K, Schreiber R, Ellenbroek B. Motor, Emotional and Cognitive deficits in adult BACHD mice: A model for Huntington's disease. Behav Brain Res. 2013 Feb 1;238:243-51. doi: 10.1016/j.bbr.2012.10.039. Epub 2012 Oct 30.

- **Abada** Y-s K, Nguyen HP, Schreiber R, Ellenbroek B. Assessment of motor function, sensory motor gating and recognition memory in a novel BACHD transgenic rat model for Huntington disease. PLoS One. 2013 doi: 10.1371/journal.pone.0068584

- **Abada** Y-s K, Nguyen HP, Ellenbroek B, Schreiber R. Reversal learning and associative memory impairments in a BACHD rat model for Huntington disease. PLOS ONE (2013) doi: 10.1371/journal.pone.0071633.

- The following posters were presented at international scientific meetings:

- 26-29 August 2011, European Behavioural Pharmacology society (EBPS)
  14th Biennial Meeting, Amsterdam – the Netherlands
  **Emotional and Cognitive deficits in adult BAC-HD mice: a model for Huntington's disease**
  Abada Y., Ellenbroek B.
  In: *Behavioral Pharmacology, volume 22 e-suppl A, page e 45, August 2011*

- 14-16 September 2012, European Huntington's disease network (EHDN)
  7th plenary meeting, Stockholm – Sweden

**Motor, Cognitive and Emotional investigation in a novel rat model for Huntington's disease**

Abada Y., Nguyen H. P., Schreiber R., Ellenbroek B.

In: *Journal of Neurology Neurosurgery & Psychiatry, September 2012 volume 83 suppl 1, page A17-A18; doi: 10.1136/jnnp-2012-303524.55*)