Pheno-Ratting
towards the Characterization of Motor and Psychiatric Phenotypes in the BACHD Rat Model of Huntington Disease

Giuseppe Manfré
The studies presented in this thesis were carried out at the Donders Institute for Brain, Cognition and Behaviour, Department of Cognitive Neuroscience, Radboud University Medical Center, Nijmegen, Netherlands, at the Institute of Medical Genetics and Applied Genomics, University of Tübingen, Tübingen, Germany, and at the Paris-Saclay Institute of Neuroscience, Orsay, France. Giuseppe Manfré was supported by a European Community’s Framework Programme FP7/2012 under grant agreement No. 317259.

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in the BACHD Rat Model of Huntington Disease

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General introduction and outline of the thesis
General introduction and outline of the thesis

Neuropsychiatric and neurological disorders constitute a major health problem in Europe, and their impact on public health and society is increasing with the aging of the population. According to the Strategic Research Agenda of the European Platform on Innovative Medicines (IMI) (http://www.imi.europa.eu/), one of the most important bottlenecks for finding more effective drugs for brain disorders is the development of model systems that translate to human pathology and are predictive of clinical efficacy.

The World Health Organization estimated in 2006 that neurological disorders, ranging from epilepsy to Alzheimer disease, from stroke to headache, affect up to one billion people worldwide (World Health Organization. The world health report 2006 – working together for health: World Health Organization, 2006). Neurologic disorders and cerebrovascular disease combined represent 7.1% of the total global burden of disease measured in DALY (disability-adjusted life-years) for all causes and ages (Chin & Vora, 2014).

One particularly relevant neurological disorder in the framework of the foregoing concerns is Huntington disease (HD). HD is an autosomal-dominantly inherited, fatal, neurodegenerative disorder. Its prevalence is estimated highest in Europe and populations of European descent (P. S. Harper, 1992). The average frequency of affected persons in Europe, North America and Australia amounts to a minimum of 6 per 100,000 (Pringsheim et al., 2012) and varies greatly geographically as a result of ethnicity, local migration and past immigration patterns. HD symptoms comprise adult-onset personality changes, generalized motor dysfunctions, and cognitive decline. The peak age of adult-onset HD is between 35 and 50 years. A small percentage of patients (10%) develop symptoms before age 20. Animal models of neurological disorders are essential for the assessment of new preventive interventions and therapeutic options (Cenci, Whishaw, & Schallert, 2002). Accordingly, part of that research effort involves identifying suitable animal models that provide a valid representation of the pathological and behavioral profile of the human disease and that can serve for the identification of therapeutic candidates and novel approaches to therapy (P. Kumar, Kalonia, & Kumar, 2010; Pouladi, Morton, & Hayden, 2013; Ross & Tabrizi, 2011).

Among mammalian species, rats were the first animals used for scientific purposes and have been considered for decades as a key model organism in biomedical science, including neurological disorders and behavioral neuroscience (Cenci et al., 2002; Homberg, Wöhr, & Alenina, 2017). Accordingly, some complex behaviors and physiological processes that can be readily studied in rats are difficult or impossible to investigate in mice (Ellenbroek & Youn, 2016; Homberg et al., 2017). Comparative analyses of movements in rats and primates show homology of many motor patterns across species. Advances have been made in identifying rat equivalents of akinesia, tremor, postural deficits and
dyskinesia, which are relevant to neurological disorders (Cenci et al., 2002). Other practical advantages of rats include their larger size, which facilitates direct invasive procedures, such as blood and cerebrospinal fluid collections, repetitive physiological measurements and surgical manipulations. Whereas understanding disorder symptoms is important, even more important is the understanding of phenotypes that precede the full-blown disorder symptoms and their stability during the course of the disorder, so that possible therapies can be more effective in preventing further development of these disorders. This insight is mostly lacking in clinical studies, because prospective longitudinal studies are very time consuming and costly. The relatively short life span of experimental rodents allows us to overcome this limitation of clinical (human) studies.

Recent progress in preclinical and clinical research on HD has been remarkable and is supported by a tight community of physicians, researchers, patients, and their families, creating a special dynamic that drives progress in the field (Reilmann, Leavitt, & Ross, 2014). HD has evolved into a model disease with the potential to treat CAG expansion carriers before manifestation of the disease, raising hopes for personalized preventive medicine. Hopefully, the collective efforts of the field will help pave the way to our ultimate goal—effective disease-modifying treatments for HD.

**Polyglutamine Diseases**

Polyglutamine (polyQ) diseases are inherited, fatal neurodegenerative disorders caused by an expansion of a coding trinucleotide (CAG) repeat, which is translated to an abnormally elongated glutamine (Q) tract in the respective mutant proteins. There are nine known polyQ diseases: dentatorubral-pallidoluysian atrophy (DRPLA), Huntington disease (HD), spinal-bulbar muscular atrophy (SBMA), and six spinocerebellar ataxias (SCA1, 2, 3, 6, 7, and 17). Except for SBMA, which is X-linked, members of this disease group are inherited in an autosomal dominant manner (Orr & Zoghbi, 2007). All the polyglutamine disorders share several common pathological features, including the nuclear accumulation and aggregation of the disease proteins (Weber, Sowa, Binder, & Hübener, 2014). Neuronal nuclear inclusions are considered to be a histopathological hallmark of the polyQ diseases and are even observed in disease brains in which normal polyQ proteins are predominantly expressed in the cytoplasm. Although the role of nuclear inclusions in pathology is not fully understood, it is clear that the inclusions result from the nuclear accumulation of polyQ-expanded proteins (Havel, Li, & Li, 2009).

**Natural history of Huntington Disease**

Huntington disease (HD) is a neurodegenerative genetic disorder formally described for the first time in 1872 by George Huntington (Huntington’s Disease Collaborative Research Group, 1993). It is caused by an unstable, expanded CAG base triplets in the coding region of the \( \text{IT15} \) gene (consequently later named \( \text{Huntingtin} \) gene, \( \text{HTT} \)) on chromosome 4p16 (Huntington’s Disease Collaborative Research Group, 1993), which results in an abnormal
polyglutamine sequence in the huntingtin protein (htt) (Gusella et al., 1983; Ross & Tabrizi, 2011). This disease is autosomal-dominantly inherited and has a broad impact on a person's functional abilities and usually in movement, such as progressive motor abnormality and chorea, intellectual deterioration and psychiatric disorders, including severe depression (Walker, 2007). Since the original description, the name of this condition has been changed from Huntington Chorea to Huntington Disease to acknowledge the multiple non-motor symptoms faced by patients with this disease (Novak & Tabrizi, 2010).

**Symptoms**
The symptoms of HD are the result of premature neuronal cell death, in particular in the striatum (Saudou & Humbert, 2016). After a variable ‘premanifest’ period, a prodromal phase characterized by subtle motor, cognitive and behavioural changes often precede a formal clinical diagnosis of motor onset by up to 15 years. Once signs and symptoms begin, they progress relentlessly over the course of the illness, which — with the exception of those patients with late-onset disease who may die of other causes — is fatal, with a median survival from motor onset of 18 years (Bates et al., 2015).

HD patients typically suffer from a triad of movement, psychiatric and cognitive symptoms. The onset of the symptoms, that can vary between individuals and affected members of the same family, usually occurs in mid-adulthood (30-40 years old), but the onset of disease may be earlier or later in life. The presence of 60 or more CAG repeats is associated with juvenile onset, typically manifesting with more severe symptoms including rigidity and a more rapid course of the disease (Huntington’s Disease Collaborative Research Group, 1993; Nance & Myers, 2001). For this reason, it is possible to divide HD into two phenotypes: juvenile (approximately 7% of individuals) and adult onset (Kirkwood, Su, Conneally, & Foroud, 2001). Declining cognitive functions, impairments of voluntary movements, awkward gait, slowed speech, bradykinesia, seizures and rigidity of limbs or trunk are the characteristic symptoms of juvenile HD (Kirkwood et al., 2001; Nance & Myers, 2001). Later in the progression of the juvenile HD the voluntary movement abnormalities and cognitive decline worsen (Kirkwood et al., 2001).

In contrast to juvenile-onset HD, the diagnosis of adult-onset HD is typically involuntary motor abnormalities and chorea that often decrease in a late stage, when parkinsonism, dystonia and rigidity dominate. In the early stages patients have deficits in executive system functioning, short term memory and visuospatial functioning. Especially affected are executive functions which include planning, cognitive flexibility, abstract thinking, rule acquisition, initiating appropriate actions and inhibiting inappropriate actions. The later stages are characterized by bradykinesia, spasticity, dysarthria, dysphagia and incontinence (Kirkwood et al., 2001). In addition to motor abnormalities, cognitive decline and personality changes are part of HD, and, like motor impairments, cognition deteriorates gradually. Additionally, cognitive deficits progress to more widespread global subcortical dementia with apathy, impulsiveness, depression and antisocial and suicidal
behaviour. Depression and apathy may be an integral part of the disease process rather than a response to the debilitation, because they can occur several years before the motor abnormalities begin (Kingma, van Duijn, Timman, van der Mast, & Roos, 2008; Kirkwood et al., 2001). Less common are delusional depression or schizophrenia-like-psychosis, which might require psychiatric treatment (Bates et al., 2015).

Beyond the well-established impairments of HD such as motor, neurocognitive and psychiatric symptoms, the presence of emotional disturbances have been documented. In particular it has been shown that HD patients have difficulty with the identification of the six classically basic facial emotional expression (Henley et al., 2012). Recent studies have shown that other than disgust also emotions like anger, fear or sadness (Johnson et al., 2007) are involved and this deficit might be already present at the pre-clinical stage. HD appears associated with generalized impairment in the detection and expression of emotions (Henley et al., 2012). An altered emotional processing can cause problems in the daily life and in the interpersonal relations, resulting in a negative impact in the social life (Vamos, Hambridge, Edwards, & Conaghan, 2017).

Present and future treatment of HD
To date, there is no cure for HD. Therapeutic strategies are designed in order to ameliorate the primary symptomatology and to improve the quality of life of patients (Handley, Naji, Dunnett, & Rosser, 2006). In order to improve the conditions of HD patients the current therapies use psychiatric agents for the control of behavioral symptoms, motor sedatives for the control of the chorea, cognitive enhancers and neuroprotective drugs (Kumar et al., 2015). Commonly prescribed drugs to reduce involuntary movements are dopamine antagonists (e.g. haloperidol) or presynaptic dopamine depleters (e.g. tetrabenazine). Psychiatric symptoms are treated with antidepressants (e.g. carbamazepine), antipsychotics (e.g. clozapine), anxiolytics (e.g. benzodiazepine) or tranquilizers (e.g. chlorpromazine) (Videnovic, 2013).

Physiotherapy can be also useful to strengthen muscles or loosen rigidities, and speech language therapy can ameliorate speech and swallowing problems. In addition, psycho-social support can help patients and their family members to cope with the burden of HD.

Thus, therapeutic strategies for HD have been developed for basically every aspect discovered to be involved in the disease pathogenesis, and at least minor benefits have been demonstrated for a multitude of compounds and approaches in preclinical studies (Zuccato, Valenza, & Cattaneo, 2010). Unfortunately, promising candidates have so far failed to exert disease-modifying effects in clinical trials in HD patients (Nguyen & Cenci, 2015). Among strategies such as enhancing mHTT degradation, improving neurotrophic support and reducing inflammation, the most promising therapeutic approach is the reduction of mHTT expression (Wild & Tabrizi, 2014). The single mutation and sole genetic cause responsible for HD enables the direct targeting of the inflicted protein on the tran-
scriptional or translational level. RNA interference (S. Q. Harper et al., 2005; Stanek et al., 2014), translational repression with antisense oligonucleotides (Carroll et al., 2011) and transcriptional repression using zinc finger proteins (Garriga-Canut et al., 2012) have proven to be successful and beneficial in animal models. Delivery and distribution issues as well as allele-selectivity and -specificity constitute the major areas of research in this area (Wild & Tabrizi, 2014).

Genetics and neuropathology
The mutant huntingtin (mhtt) protein is the result of the unstable CAG repeat in the HTT gene that causes a polyQ stretch near the N-terminus of the protein (Everett & Wood, 2004). The N-terminal fragment represents the “toxic” species and the proteases that generate HTT fragments are caspase-3, caspase-6, calpains and matrix metalloproteinase 10 (Sathasivam et al., 2013). The mutant htt protein undergoes proteolytic cleavage, misfolding and aggregation in all cells of the body (Cisbani & Cicchetti, 2012). This leads to impairments in transcription, axonal transport and mitochondrial function that cause atrophy due to massive neuronal loss (Hedreen & Folstein, 1995).

The length of the CAG/polyglutamine repeat sequence is inversely correlated with the age of disease onset, thus determining whether an individual will develop HD (Duyao et al., 1993). In the normal population the polymorphic CAG repeat is between 6 and 35 units, but the mutation becomes highly penetrant when the units are >40, provoking a disease process that inexorably leads to the onset of diagnostic motor signs (Huntington’s Disease Collaborative Research Group, 1993; Novak & Tabrizi, 2010). Repeats of 36-39 CAG units show reduced penetrance and are potentially unstable during inheritance, as some individuals have HD whereas others are not clinically diagnosed as having HD (Bates et al., 2015; Novak & Tabrizi, 2010). Due to the instability of the gene during gametogenesis, so-called anticipation in the offspring can occur. This is referred to the phenomenon in which the inheritance of the mutant HTT allele through the male germ line often leads to a more severe clinical course than inheritance through the female germ line (Walker, 2007). In general, children with HD show symptoms 8 years earlier than their fathers (Ranen et al., 1995). Importantly, the timing of onset of the motor signs is determined by the allele with the longer CAG repeat in a completely dominant manner. The second HTT allele (regardless of its length) does not alter the rate of the process that leads to clinical diagnosis. The pathogenetic mechanism of the CAG length dependence and allele dose independence is not known, but previous studies showed that the length of the CAG repeat, even in the normal range, correlates with measures in some cellular assays (for example, cellular energy charge or cellular adhesion assays) (Seong et al., 2005). This suggests that a gain of function that acts through augmentation or dysregulation of one or more normal functions of huntingtin might be involved (Bates et al., 2015).
HD: a basal ganglia disorder
Basal ganglia consist of four main interconnected nuclei: the striatum, the globus pallidus, the subthalamic nucleus and the substantia nigra (Pollack, 2001). The largest component is the striatum, and comprises two macroscopic nuclei, the caudate nucleus and the putamen (Yelnik, 2002). Both of them contain two different types of neurons: projection neurons and interneurons (respectively 90% and 10% of striatal neurons). The so called medium-sized spiny neurons (MSNs) are projections or striatofugal neurons, and they are all inhibitory neurons, using GABA as neurotransmitter (Lanciego, Luquin, & Obeso, 2012). The MSNs in the striatum receive information from incoming axons from the cerebral cortex. Striatofugal MSNs can also be divided according to their projections targets. The first group innervates the globus pallidus external nucleus and express dopamine subtype 2 receptors (D2R), which inhibit intracellular adenyl-cyclase and give rise to the indirect pathway. In the second group striatal MSNs directly project to globus pallidus internal nucleus and subthalamic nucleus and contain dopamine receptor subtype 1 receptors (D1R) which activates adenyl-cyclase and give rise to the direct striatopallidal pathway (Lanciego et al., 2012). The striatum receives also a dopaminergic input from the substantia nigra pars compacta (Yelnik, 2002). The indirect pathway serves to modulate the disinhibitory actions of the direct pathway, which reduces tonic inhibition when activated. In patients with HD, MSNs projecting to the external segment of the globus pallidus degenerate, causing impairments in the pathway. This leads to an inappropriate activation of the upper motor neurons, resulting in undesired ballistic and choreic movements (Graybiel, 2000).

HD causes neuronal loss particularly in the striatum with about 57% loss of cross-sectional area from the caudate nucleus and about 65% loss of the putamen, while in the advanced cases there is also a loss of neurons in the thalamus, substantia nigra pars reticulata and in the subthalamic nucleus (Rubinsztein, 2002). The MSNs in the striatum that contain the neuropeptide encephalin and express the D2-subtype of dopamine receptor appear to be the most vulnerable neuronal population (Augood, Faull, Love, & Emson, 1996; Sapp et al., 1995). Accordingly, HD is defined as a basal ganglia disorder. The basal ganglia dysfunction leads to an abnormal reduction in tonic inhibition, and thereby to excessive excitability of the upper motor neurons (Shoulson & Young, 2011).

Huntingtin protein
The huntingtin (htt) protein is a 348-kDa protein containing 3,144 amino acids. It is highly conserved from flies to mammals (Saudou & Humbert, 2016) and its normal function is not fully understood. The N-terminal region contains the expandable polyQ stretch and it is preceded by 17 amino acids that form an α-helical structure but can also adopt other conformations including α-helix, random coil and extended loop (Kim, Chelliah, Kim, Otwinowski, & Bezprozvanny, 2009) and it is followed by a proline-rich domain (PRD). Both the polyQ stretch and the PRD are polymorphic in the human population. The proline-rich
domain, which is important for protein-protein interactions with tryptophan or Src homology 3 (SH3) domains, can be variable in the non-HD population (Harjes & Wanker, 2003). Htt is also enriched in consensus sequences called huntingtin elongation factor 3, protein phosphatase 2A, and TOR 1 (HEAT) repeats that are organized into protein domains relevant for protein-protein interactions (Zuccato et al., 2010). It has been shown that the polyQ tract has an important role in binding the huntingtin protein to its large number of partners (Zuccato & Cattaneo, 2007). The protein might have a flexible or multifunctional structures that are able to assume specific conformations and activities depending on its binding partners, subcellular location, and time of maturation in a given cell type and tissue (Kim et al., 2009).

Htt contains three protease cleavage consensus sites that generate shorter fragments from the full-length HTT protein (Goldberg et al., 1996; Wellington et al., 1998). In addition to these three sites, there are an additional three caspase cleavage sites and two calpain cleavage sites N-terminal to the primary caspase cleavage sites (Kim et al., 2001). Both the wild-type and mutant forms of the HTT protein can be cleaved, producing fragments of varying length, function, and cellular localization (Lunkes et al., 2002; Ratovitski et al., 2007). Brain region-specific cleavage of HTT protein has also been described, suggesting that different fragment lengths may have cell-type-specific functions adding additional complexity to the function of this protein (Mende-Mueller, Toneff, Hwang, Chesselet, & Hook, 2001).

Despite the association of mutant huntingtin with selective neurodegeneration in specific brain regions, HTT is ubiquitously expressed, at low levels, throughout the body (Van Raamsdonk et al., 2005). The normal functions of huntingtin are not fully understood, but some broad biological functions have been discovered (Saudou & Humbert, 2016). For instance, in vitro and in vivo studies have shown that the protein has a prosurvival role by blocking the formation of a functional apoptosome complex and the consequent activation of caspase-3 and caspase-9 (Rigamonti et al., 2001). Furthermore, huntingtin has a critical role in the development of the nervous system and influence the brain-derived neurotropic factor (BDNF) expression that is important for the survival of striatal neurons and for the activity of cortico-striatal synapses (Zuccato & Cattaneo, 2007). Huntingtin is associated with vesicle membranes and microtubules and also appears to be associated with clathrin through huntingtin-interacting protein. For these reasons, huntingtin might have a role in vesicle transport and synaptic function (Rubinsztein, 2002). Although a number of molecular dysfunctions have been correlated to the pathogenesis of HD, the specific biochemical functions of huntingtin protein remain largely unknown.

**Cell and animal models of HD**

Molecular aspects of HD have been studied intensely in mammalian cells including lymphoblasts (Trottier et al., 1995), fibroblasts (Persichetti et al., 1996), embryonic stem cells (Lu & Palacino, 2013; Metzler et al., 1999) and inducible pluripotent stem cells.
(The HD iPSC Consortium, 2012) from HD patients and control subjects. In addition, cell lines (Ehrlich et al., 2001; Goldberg et al., 1996; Li, Cheng, Li, & Li, 1999; Li & Li, 1998; Trettel et al., 2000) and primary cell cultures (Saudou, Finkbeiner, Devys, & Greenberg, 1998) expressing mutant HTT (mHTT) with various lengths of the protein and polyQ tract have been used. In order to further understand the disease on organismic level, animal models of HD have been established. Small invertebrates such as worms and flies can be used to study the order and progression of molecular HD-related phenotypes, while larger, mammalian species such as mouse, rat, sheep, pig and monkey enable the analysis of systemic abnormalities and the interplay between cell populations in the body. Of these, rodent models certainly had the most impact on the evaluation of disease mechanisms as well as preclinical testing.

**Invertebrate models of HD**

The transgenic *Caenorhabditis elegans* model presents truncated amino-terminal fragments of mHTT between 57 to 171 amino acids in targeted neurons. Transgenic expression of mHTT in worms results in neuronal dysfunction and neurodegeneration (Faber, Alter, MacDonald, & Hart, 1999). The *Drosophila melanogaster* models use the USA-GAL4 system to drive the expression of full-length or truncated N-terminal fragments (65-548 amino acids) of mHTT in cellular populations. HD fly models show a progressive degenerative phenotype and motor abnormalities (Lee, Yoshihara, & Littleton, 2004).

**Rodent models of HD**

Up to now, rodents are the most commonly animal models used for HD and over 20 different models have been created. The R6/1 and R6/2 mouse models expresses exon 1 of human HTT, respectively with 116 and 144 CAG repeats under the control of the human HTT promoter (Mangiarini et al., 1996). In particular, in the R6/2 mice the CAG repeat tract appeared to be unstable in the germ cells, and the transmissible CAG repeat expansions were greater than 400 CAGs. This means that in many colonies of R6/2 the size of expansion is greater than originally (Dragatsis et al., 2009). The N71-Q82 mouse model expresses a truncated HTT cDNA with 82 CAG repeats under the control of the mouse prion protein promoter (Schilling et al., 1999). All of the truncated N-terminal fragment models typically exhibit a rapid onset of symptoms, including motor and cognitive abnormalities, weight loss and a reduced lifespan. These symptoms are accompanied by a widespread and generalized degenerative phenotype (for a detailed review see Pouladi et al., 2013).

By knocking in a human HTT exon 1 with an expanded CAG repeats expansion in the endogenous mouse Htt gene locus, full length mHTT rodent model have been created. *HdhQ111, CAG140* and *HdhQ150* mouse models are genetically precise, with one or two copies of the mutant HD gene and temporally and spatially appropriate levels of mHTT expression (Heng, Tallaksen-Greene, Detloff, & Albin, 2007; Lin et al., 2001; Menalled, Sison, Dragatsis, Zeitlin, & Chesselet, 2003; Tallaksen-Greene, Crouse, Hunter, Detloff, & Albin,
2005; Wheeler et al., 2002). HdhQ111 and HdhQ150 show both a somatic instability of CAG repeats, HdhQ150 has the mouse proline-rich region, while the HdhQ111 and CAG140 mice have the human proline-rich region. Knock-in mice have been shown to develop neurological and neurodegenerative phenotypes (Hickey et al., 2008; Lerner, Trejo Martinez, Zhu, Chesselet, & Hickey, 2012; Menalled et al., 2003; Wheeler et al., 2002).

Another group of full-length mHTT rodent models was generated using genomic-type constructs such as yeast artificial chromosomes (YACs) technology and bacterial artificial chromosomes (BACs) (Gray et al., 2009; Hodgson et al., 1999; Slow et al., 2003; Yu-Taeger et al., 2012). These models have human genomic mutant HTT transgenes, including all the introns and exons and the regulatory sequences up to 24 kb upstream and 117 kb downstream of the gene, ensuring appropriate temporal and tissue-specific expression of mHTT (Gray et al., 2009; Hodgson et al., 1999; Slow et al., 2003; Yu-Taeger et al., 2012). The YAC128 mice model expresses the transgene with 128 CAG repeats, while BACHD mice and rats express mutant HTT with 97 CAG repeats. Both YAC128 and BACHD rodent models develop progressive motor, cognitive and psychiatric symptoms, as well as selective striatal and cortical atrophy (Ehrnhoefer, Butland, Pouladi, & Hayden, 2009). Although these models share many phenotypic features, it is possible to find differences between neurochemical and aggregation phenotypes (Pouladi et al., 2012).

Large animal models of HD

Different large animal models of HD have been developed such as a non-human primate model (rhesus monkey, Macaca mulatta), a Tibetan miniature pig model, and a sheep model (Ovis aries). The first two models were generated using fragments of human HTT that included a CAG repeat expansion, while the sheep model uses the full-length human coding sequence of HTT that is expressed from a transgene.

The transgenic monkey was created by the injection of oocytes with lentiviruses expressing exon 1 of HTT carrying 84 CAG repeats, and the expression was controlled by the ubiquitin promoter (S.-H. Yang et al., 2008). The hallmark features of HD, including the presence of nuclear inclusions and neuropil aggregates, were observed in the brains of the symptomatic HD monkeys. The pathology was more similar to that observed in the R6/2 mouse rather than in human brain, because both models contained a transgene with an N-terminal HTT fragment controlled by the ubiquitin promoter, that is upregulated in HD.

The transgenic HD sheep was created by micro-injection of a full-length human HTT cDNA containing 73 CAG repeats under the control of the human HTT promoter (Jacobsen et al., 2010). It has been reported that the brains of this showed characteristic inclusions at 18 months of age, without showing the full HD phenotype. The Tibetan miniature pig model expressed the N-terminal of mHTT, containing a repeat of 105 glutamine residues and the expression of the construct was under the control of the cyclomegalovirus enhancer and chicken β-actin promoter (Yang et al., 2010). A large animal model recently
developed is the transgenic (tg) HD minipig (Baxa et al., 2013), which presents a stable expression of the N-terminal truncated form of human huntingtin transgene and is currently being tested in different motor, cognitive and behavioral tests (Schramke et al., 2016; Schuldenzucker et al., 2017).

The urgency of a good animal model for the study of Huntington Disease
Since the discovery of the HTT mutation 25 years ago, more than 13,000 papers have been published on HD, approximately half of which relate to attempts to model various aspects of the disease. As discussed in the previous section, different animal models have been established, elucidating important pathways that are disrupted in HD and providing important insights into the pathogenesis of this disease. These developments have been accompanied by the identification of several therapeutic candidates and novel approaches to therapy (Ross & Tabrizi, 2011). Over the past two decades 99 clinical trials have been performed in HD investigating 41 different compounds (McColgan & Tabrizi, 2017). However, the success rate is low, with only 3.5% of trials making from phase I to approval (Travessa, Rodrigues, Mestre, & Ferreira, 2017). Thus, despite the considerable progress in our understanding of the disease, an effective treatment that either prevents or slows the pace of HD remains out of reach. The slow rate of progression of HD often necessitates lengthy and therefore costly clinical trials. In addition, the number of patients with HD who are available to participate in such trials is limited, which means that a relatively small number of compounds can be tested every time. Thus, animal models will continue to have a role not only as a filter for test compounds before the initiation of human clinical trials but also as a means for identifying candidate compounds with therapeutic promise. The translational potential of an animal model of disease may be gauged on the basis of its construct, face and predictive validity (Belzung & Lemoine, 2011). Rodent models of HD are versatile, especially when considering the high number of established batteries of tests available that allow assessment of motor, cognitive and psychiatric-like features of the disease. Studies in rodents that emphasize careful behavioral analysis are being developed as effective and inexpensive models that complement clinical studies.

The BACHD rat model
The BACHD rat is the latest rat model of HD, and expresses the full length human huntingtin (fl-mhtt) with 97 CAG-CAA repeats under the control of the human HD promoter gene and all its regulatory elements to determine its similarity to the human condition (Gray et al., 2009; Yu-Taeger et al., 2012). The BACHD rat model is particularly relevant since it presents neuropathological and behavioral phenotypes reminiscent of symptoms seen among HD patients.
Neuropathology
Regional distribution pattern of mhtt-positive aggregates was investigated in BACHD rats, which exhibited widely distributed aggregates in all regions, with prominent expression in the neocortex and in limbic areas including nucleus accumbens, hippocampus, bed nucleus of the stria terminalis, and the amygdala. Very few aggregates were found in the dorsolateral caudate-putamen, the lateral globus pallidus, and the substantia nigra (Yu-Taeger et al., 2012).

Both the size and number of aggregates increased with age, showing the largest and most abundant aggregates in amygdala, in the CA3 region of the hippocampus, and in the cerebral cortex, indicating that aggregate formation continued to progress in these regions (Yu-Taeger et al., 2012). Moreover, dynamic PET scans in the striatum of BACHD rats presented a decrease in the striatal uptake of the D2-receptor antagonist [11C] raclopride, showing a reduced dopamine receptor binding (Yu-Taeger et al., 2012).

Obesity
BACHD rats are obese and suffer from discrete developmental deficits. Although BACHD rats do not differ from WT rats in terms of body weight, they display several changes in body composition, carrying an excess amount of adipose tissue (Jansson, Clemens, Riess, & Nguyen, 2014). When assessing the motivation to lever push for a food reward, BACHD rats were found to be less motivated than WT rats, although this phenotype was dependent on the food deprivation strategy (Jansson et al., 2014).

Gait and motor dysfunction
In order to measure forelimb and hindlimb motor coordination, BACHD rats have repeatedly been tested in the Rotarod test (Abada, Nguyen, Schreiber, & Ellenbroek, 2013; Clemens et al., 2015; Yu-Taeger et al., 2012), showing early impairments which progressively worsen over time. Altered activity in the PhenoMaster (TSE Systems, Germany) and in an open field test-like setup as well as abnormalities in unhindered walking gait have also been reported (Abada, Nguyen, Schreiber, et al., 2013; Yu-Taeger et al., 2012). Moreover, gait deficits have been investigated using the Catwalk system, showing that 12 months-aged BACHD rats exhibited shorter stride length, shorter stand and shorter front limb swing, with no differences in general walking speed (Abada, Nguyen, Schreiber, et al., 2013). Lower rearing and ambulatory activity were observed with the PhenoMaster system at 3 and 6 months of age (Yu-Taeger et al., 2012). In an open field test-like setup BACHD rats exhibited an initial hyperactivity followed by hypoactivity starting at 4 months of age (Abada, Nguyen, Schreiber, et al., 2013).

Cognition
In a fear conditioning set-up, BACHD rats exhibited associative memory deficits by 4 months of age while an impairment of their reversal learning performance emerged at
6 months when rats were tested in a cross maze task (Abada, Nguyen, Ellenbroek, & Schreiber, 2013). In an object recognition task, BACHD animals presented intact object memory at 4 and 12 months of age. BACHD also showed a subtle deficit in prepulse inhibition of acoustic startle (Abada, Nguyen, Schreiber, et al., 2013). A recent study revealed an impaired performance in two Skinner box tests (delayed alternation and the delayed non-matching to position tests), indicating general problems with handling basic aspects of the tests, while short-term memory appeared to be intact (Clemensson, Clemensson, Riess, & Nguyen, 2017).

**Psychiatric endophenotypes**

In the tail suspension test (TST), broadly used to measure depressive-like behavior, characteristic hindlimb clasping behavior has been observed in BACHD rats starting at three weeks of age (Yu-Taeger et al., 2012). Starting at 4 months of age, BACHD rats display also changes in emotionality as suggested by the decreased anxious-like behavior in the elevated plus maze (Yu-Taeger et al., 2012). At 4 months, BACHD rats exhibited behavioral hyper-reactivity to threats in social (social behavior test) and avoidance learning (signaled active avoidance, SAA) tasks. At 9 months of age, deficits in prepulse inhibition became evident as well (Abada, Nguyen, Schreiber, et al., 2013). 18 month-old BACHD rats made fewer choices during the Rat Gambling Task (RGT) and were less efficient in choosing advantageous options than younger animals (Adjeroud et al., 2015).

**Validity of the BACHD rat model for HD**

In principle, the relevance of a given animal model of a human disease is often judged on the basis of three broad measures of validity: the animal model’s construct validity, face validity and predictive validity.

*Construct validity* pertains to how closely the animal model reproduces the pathogenic lesion that underlies the disease in humans. For genetic diseases such Huntington disease, a model with high construct validity would reproduce the human mutation in the context of the full length human gene under the control of the gene’s endogenous promoter. Therefore, the construct validity of models expressing full-length human mutant huntingtin (HTT) is greater than those expressing full-length mouse mutant HTT (Pouladi et al., 2013). The BACHD rat model contains human genomic DNA spanning the full-length HTT gene with 97 CAG/CAA repeats, under the control of the HTT human promoter and including all regulatory elements (Yu-Taeger et al., 2012).

*Face validity* refers to whether the overall phenotypes of a disease model, both behavioral deficits and neuropathology, faithfully recapitulate the symptoms and phenotypes associated with the human disease. For HD, animals with high face validity would reproduce progressive motor and cognitive deficits as well as psychiatric-like disturbances (Pouladi et al., 2013). As mentioned before, BACHD rats present motor, emotional and cognitive impairments resembling symptoms seen among HD patients.
Overview of the behavioral phenotypes investigated within this thesis.

- Fine motor control
- Muscle strength and endurance
- Locomotor activity
- Social behavior
- Anxiety-like behavior
- Choice impulsivity
- Motor/Action impulsivity
- Social behavior
- Anxiety-like behavior
(Abada, Nguyen, Ellenbroek, et al., 2013; Abada, Nguyen, Schreiber, et al., 2013; Clemensson, Clemensson, Fabry, Riess, & Nguyen, 2017; Clemensson, Clemensson, Riess, et al., 2017; Lamirault et al., 2017; Manfré et al., 2016; Yu-Taeger et al., 2012). Furthermore, neuroanatomically and histologically, the models would display selective, age-dependent striatal and cortical neuronal loss and atrophy, nuclear inclusions and neuropil aggregates (Pouladi et al., 2013).

Predictive validity is judged based on how closely improvements in response to treatment in the animal model parallel, or predict, improvements in patients. For treatable conditions, the predictive validity of an animal model may be tested, but for diseases like HD that are without an effective treatment, assessment of the predictive validity of an animal model is currently impossible (Pouladi et al., 2013).

Aim and outline of this thesis

Previous experiments have been already performed in this transgenic rat model, but they are lacking of information about important phenotypes preceding the onset of symptoms in BACHD rats. Despite the extensive use of the BACHD rat line in the last years (Abada, Nguyen, Ellenbroek, et al., 2013; Abada, Nguyen, Schreiber, et al., 2013; Clemensson, Clemensson, Fabry, et al., 2017; Clemensson, Clemensson, Riess, et al., 2017; Yu-Taeger et al., 2012) the studies performed so far did not cover fine and gross motor function as well as psychiatric endophenotypes such as social behavior and impulsivity.

The investigation of the previously mentioned domains is necessary for the early detection of the first core symptoms of the disease and the monitoring of its development so that possible therapies can be more effective in preventing further development of neurodegeneration.

Although patients and several HD mouse models have been found to present cognitive symptoms already before the start of any motor symptoms (Carter et al., 1999; Lichter & Hershey, 2010; Paulsen et al., 2001; Van Raamsdonk et al., 2005), this could not be demonstrated for the BACHD rat. Conversely, transgenic rats have repeatedly been found to show early impairments in the Rotarod test (Abada, Nguyen, Schreiber, et al., 2013; Clemens et al., 2015; Yu-Taeger et al., 2012) which progressively worsen over time (Abada, Nguyen, Schreiber, et al., 2013; Clemens et al., 2015; Yu-Taeger et al., 2012). Altered activity in the PhenoMaster (TSE Systems, Germany) and in an open field test-like setup as well as abnormalities in unhindered walking gait have also been reported (Abada, Nguyen, Schreiber, et al., 2013; Yu-Taeger et al., 2012). These findings suggest that a more in-depth investigation of various aspects of motor function in BACHD rats is needed. In chapter 2 we further dissect the motor phenotype of this transgenic rat model of HD by combining traditional and modern paradigms, hypothesizing that this impairment might be due
to deficits in: 1) fine motor control; 2) muscle strength and/or endurance; 3) locomotor activity.

Cognitive and psychiatric symptoms, including perseveration, lack of insight, distractibility, impaired social life, anxiety and impulsivity, affect HD patients before motor symptoms (Dale & van Duijn, 2015; Novak & Tabrizi, 2010; Rosenblatt, 2007; Vamos et al., 2017) and have thus an early deleterious impact on the quality of HD patient’s life. BACHD rats’ psychiatric phenotype has been only partly investigated indicating that the BACHD rat line requires further phenotyping to parallel their impairments to patients’ symptoms. In **chapter 3**, we focus on social behavior of BACHD rats as its changes are an important component of neuropsychiatric symptoms. Some aspects of social behavior have been partly examined in different rodent models of HD, showing altered social interaction (Nguyen et al., 2006; Pietropaolo, Delage, Cayzac, Crusio, & Cho, 2011; Wood & Morton, 2015) and social preference (Pietropaolo et al., 2011), reduced social memory and recognition (Ciamei & Morton, 2008; Rudenko, Tkach, Berezin, & Bock, 2010) and even increased levels of aggression (Shelbourne et al., 1999), supporting the view of impaired social behavior also in various rodent models of HD. We therefore investigated different parameters of the social behavior repertoire. Additionally, gene expression analyses were performed to evaluate a potential involvement of D1 and D2 dopaminergic receptors and the contribution of brain-derived neurotrophic factor (BDNF) to the observed behavioral alterations.

In **chapter 4** we examined impulsivity traits in BACHD rats by using two different paradigms, the delay discounting task (DD), and the Differential Reinforcement of Low Rate of Responding task (DRL), to assess choice and action impulsivity, respectively.

In **chapter 5** risk assessment and anxiety-like traits were characterized in a separate cohort of animals tested longitudinally, using the elevated plus maze and light-dark box. Additionally, an automated tool for assessing anxiety within a home cage-like environment allowed us to further investigate anxiety through an anxiety test “light spot” (LS). This home cage test provides the opportunity for in-depth analysis, making it a potentially useful tool for detecting subtle or complex anxiety-related traits.

Finally, **chapter 6** provides a general discussion and future perspectives regarding the results reported in this thesis.
References


General introduction and outline of the thesis


The BACHD rat model of Huntington Disease shows specific deficits in a test battery of motor function

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Abstract

Rationale: Huntington disease (HD) is a progressive neurodegenerative disorder characterized by motor, cognitive and neuropsychiatric symptoms. HD is usually diagnosed by the appearance of motor deficits, resulting in skilled hand use disruption, gait abnormality, muscle wasting and choreatic movements. The BACHD transgenic rat model for HD represents a well-established transgenic rodent model of HD, offering the prospect of an in-depth characterization of the motor phenotype.

Objective: The present study aims to characterize different aspects of motor function in BACHD rats, combining classical paradigms with novel high-throughput behavioral phenotyping.

Methods: Wild-type and transgenic animals were tested longitudinally from 2 to 12 months of age. To measure fine motor control, rats were challenged with the pasta handling test and the pellet reaching test. To evaluate gross motor function, animals were assessed by using the holding bar and the grip strength tests. Spontaneous locomotor activity and circadian rhythmicity were assessed in an automated home-cage environment, namely the PhenoTyper. We then integrated existing classical methodologies to test motor function with automated home-cage assessment of motor performance.

Results: BACHD rats showed strong impairment in muscle endurance at 2 months of age. Altered circadian rhythmicity and locomotor activity were observed in transgenic animals. On the other hand, reaching behavior, forepaw dexterity and muscle strength were unaffected.

Conclusions: The BACHD rat model exhibits certain features of HD patients, like muscle weakness and changes in circadian behavior. We have observed modest but clear-cut deficits in distinct motor phenotypes, thus confirming the validity of this transgenic rat model for treatment and drug discovery purposes.

Keywords: Huntington disease, polyglutamine disease, model characterization, motor function, transgenic rat, neurodegenerative disorders, fine motor control, automated home-cage monitoring.
Introduction

Huntington disease (HD) is a severe autosomal dominant neurological disorder. Typically, onset of symptoms is during middle age and it consists of a triad of motor, cognitive and psychiatric symptoms (Walker, 2007). The motor symptoms of HD are varied and encompass involuntary movements such as chorea as well as impaired voluntary movements, which cause limb incoordination and impaired hand function (Novak and Tabrizi, 2010). These symptoms are worsened by loss of postural reflexes and their pattern tends to change over time, with chorea declining and dystonia, rigidity, and bradykinesia becoming more marked (Nguyen and Cenci, 2015; Novak and Tabrizi, 2010). Motoric symptoms are frequently involved in the cause of death, along with complications such as dysphagia and aspiration (Nguyen and Cenci, 2015; Walker, 2007). Typical latency from diagnosis to death is 20 years (Walker, 2007; Watt, 1990). The disease is caused by an expanded CAG mutation in the huntingtin (HTT) gene (Huntington’s Disease Collaborative Research Group, 1993), leading to initial atrophy and cell loss in the neostriatum (caudate nucleus and putamen in humans), then spreading to cortical areas and eventually affecting the whole brain (Vonsattel et al., 1985). Although disease progression is currently untreatable, efforts are aimed at identifying novel symptomatic, neuroprotective and reparative treatments (Frank, 2014). The latest drug approved for use in the United States, deutetra-benazine, adds to the other drugs that can alleviate some of the choreic and psychiatric symptoms of HD (Frank, 2014; Frank et al., 2016). Part of that research effort involves identifying suitable animal models that provide a valid representation of the pathological and behavioral profile of the human disease and that can serve for the identification of therapeutic candidates and novel approaches to therapy (Kumar et al., 2010; Pouladi et al., 2013; Ross and Tabrizi, 2011). Between mammalian species, rats were the first animals used for scientific purposes and have been considered for decades as a key model organism in biomedical science, including neurological disorders and behavioral neuroscience (Cenci et al., 2002; Homberg et al., 2017). Accordingly, some complex behaviors and physiological processes that can be readily studied in rats are difficult or impossible to investigate in mice (Ellenbroek and Youn, 2016; Homberg et al., 2017). Comparative analyses of movements in rats and primates show homology of many motor patterns across species. Advances have been made in identifying rat equivalents of akinesia, tremor, postural deficits and dyskinesia, which are relevant to neurological disorders (Cenci et al., 2002).

The BACHD rat is a recently generated transgenic rat model expressing full-length human mutant huntingtin (mHTT) and is currently being characterized in order to understand its advantages and limitations concerning modeling of HD. BACHD rats present a wide range of behavioral abnormalities reminiscent of the cognitive, emotional and motor alterations observed in HD patients (Abada et al., 2013a, 2013b; Clemens et al., 2015; Clemensson et al., 2017a, 2017b; Jansson et al., 2014; Manfré et al., 2016; Yu-Taeger et al., 2012). Previous characterization studies in these rats showed the presence of emotional
alterations, as suggested by a decreased anxious-like behavior in the elevated plus maze (Yu-Taeger et al., 2012). BACHD rats also exhibited associative memory deficits in a fear conditioning setup, impairments of their reversal learning performance in a cross maze task (Abada et al., 2013a) as well as deficits in prepulse inhibition (Abada et al., 2013b). Furthermore, BACHD animals exhibited signs of fronto-striatal impairment in different Skinner box tasks for short term memory (Clemensson et al., 2017b) and an impulsive-like phenotype was shown in a delayed discounting paradigm and in the Differential Reinforcement of Low Rate of Responding task (Manfré et al., 2016).

Although patients and several HD mouse models have been found to present cognitive symptoms already before the start of any motor symptoms (Carter et al., 1999; Lichter and Hershey, 2010; Paulsen et al., 2001; Van Raamsdonk et al., 2005), this could not be demonstrated for the BACHD rat. Instead, BACHD rats have repeatedly been found to show early impairments in the Rotarod test (Abada et al., 2013b; Clemens et al., 2015; Yu-Taeger et al., 2012) which progressively worsens over time (Abada et al., 2013b; Clemens et al., 2015; Yu-Taeger et al., 2012). Altered activity in the PhenoMaster (TSE Systems, Germany) and in an open field test-like setup as well as abnormalities in unhindered walking gait have also been reported (Abada et al., 2013b; Yu-Taeger et al., 2012). These findings suggest that a more in-depth investigation of various aspects of motor function in BACHD rats is needed. Therefore, the aim of this study was to further dissect the motor phenotype of this transgenic rat model of HD by combining traditional and modern paradigms. We hypothesize that this impairment might be due to deficits in: 1) fine motor control; 2) muscle strength and/or endurance; 3) locomotor activity.

One of the fine motor functions that is possible to investigate is skilled reaching (the conventional term for the reach-to-eat act), which is a form of prehension in which a hand is used to grasp a food item and place it into the mouth for eating (Alaverdashvili and Whishaw, 2013). Skilled reaching is an everyday activity for humans (Sacrey and Whishaw, 2010). As mentioned earlier, rats serve as excellent models to reproduce deficits in motor ability, such as the ability to manipulate or reach various objects (Klein and Dunnett, 2012). Furthermore, manual dexterity is also a central daily activity and it is commonly disrupted by nervous system damage, often with permanent effects (Iwaniuk and Whishaw, 2000). Rodents use their forepaws in dexterous ways that are in some capacities homologous to humans (Cenci et al., 2002; Iwaniuk and Whishaw, 2000). Since HD patients have difficulties in manipulating and reaching objects, translating such behaviors to an animal model is of great interest, providing more information on a potential read-out for future treatments (Klein et al., 2012).

Another cause that might underlie the motor deficits of BACHD animals is an impairment in gross motor function. HD patients and animal models of HD present signs of peripheral motor pathology, including gait abnormality and muscle wasting (De Aragão et al., 2016). Hence, characterizing this aspect in rodent models of progressive neurodegenerative and muscle wasting diseases requires a battery of tests. Classical
Motor characterization of BACHD rats

behavioral assays such as the holding bar and the grip strength test are still of high value to assess muscle function and coordination (Brooks and Dunnett, 2009; Klein et al., 2012; Nguyen and Cenci, 2015).

A further aspect that can be evaluated concerns altered locomotor activity, since HD patients exhibit imbalance, trouble in walking, clumsiness and unsteadiness (Di Maio et al., 1993). In previous studies, motor phenotypes in rodent models of HD and Duchenne muscular dystrophy have been assessed using different setups, showing consistent results (Hara et al., 2002; Hickey et al., 2008). Accordingly, in the last decade there has been a concerted effort towards automating methods for continuous automated home-cage assessment and for measuring motor function (Bains et al., 2017; Chort et al., 2013; Vandeputte et al., 2010). Such technologies are aimed at capturing a wider range of behaviors and are free from experimenter bias through the possibility to house rodents in automated home-cage environments for extended periods of time and to measure voluntary activity without interference from the investigator (Schaefer and Claridge-Chang, 2012). Here, BACHD and control rats were tested in an instrumented home-cage (PhenoTyper®, Noldus Information Technology) to monitor locomotor and circadian activity over time.

In the present study, we focused on tasks that may reveal subtle motor disturbances characteristic of human HD. To expand the repertoire of meaningful motor function tests, we combined classical behavioral paradigms with automated home-cage observations which allow high-throughput testing. The tests were performed at three different ages (2, 7 and 12 months) to assess the onset and progression of specific motor symptoms and provide read-out parameters for future pre-clinical studies applying novel drugs for the treatment of HD.

Material and methods

Animals
Wild-type (WT) and transgenic (hemizygous BACHD; TG5 line) male rats carrying the mutant human huntingtin gene, under the control of the human huntingtin promoter and its regulatory elements were used. The transgene contained 97 CAG-CAA mix repeats, and additional 20 kb upstream and 50 kb downstream sequences ensured stability of the repeat length (Yu-Taeger et al., 2012). The construct has previously been used to generate the BACHD mouse (Gray et al., 2008). All animals were maintained on Sprague Dawley background and genotyped according to previously published protocols (Yu-Taeger et al., 2012). As the study was a collaborative effort, two different cohorts have been used at University of Tuebingen (Tuebingen, Germany) for Experiment 1, and at Radboudumc (Nijmegen, The Netherlands) for Experiment 2. Each group of animals was subjected to a different battery of tests, as described in the study design section.
For Experiment 1, 12 BACHD rats and 12 WT rats were obtained from in-house breeding with hemizygous BACHD males from the TG5 line (Yu-Taeger et al., 2012) paired with WT females (Charles River, Germany). Rats were weaned at 21 days of age and housed in genotype-matched groups of three rats per cage in type IV cages (38 x 55 cm) with high lids (24.5 cm from cage floor), containing wooden houses, nesting paper and wooden bedding material. All rats used for testing were handled on a daily basis. The animal facility kept 21–23°C, 55–10% humidity, and was set to a partially reversed light/dark cycle with lights on/off at 02:00/14:00 during summer, and 01:00/13:00 during winter. Food and water regimen is described in the study design section. All experiments were approved by the local ethics committee (Regierungspräsidium Tübingen) and carried out in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU).

For Experiment 2, 15 transgenic males were supplied from the original BACHD colony of Charles River (Wilmington, MA, USA) and an in-house breeding colony was preserved and maintained at Radboudumc (Nijmegen, The Netherlands) by cross-breeding these males with wild-type female rats (Charles River, Germany). WT and BACHD animals (n=12/group) were weaned at 21 days of age and group-housed two per cage with littermates of the same genotype in type IV cages (38 x 55 cm) containing plastic houses, nesting paper and wooden bedding material. Cages were in a constant temperature-humidity room (19.5 °C - 54% humidity) with a regular 12 h light/dark cycle with lights on/off at 8:00/20:00. Food and water were provided ad libitum.

Body weights were measured regularly throughout the study in both groups, and as seen with other cohorts of male BACHD rats, there was no difference in body weight between the genotypes (data not shown). It should be noted though that despite the unchanged body weight, BACHD rats have been reported to have a reduced bone and muscle mass and an increased fat mass from 3 months of age onwards (Jansson et al., 2014) but these parameters could not be assessed in the present study.

All experiments were positively evaluated by the Animal Ethics Committee (RU-DEC, Nijmegen) and performed under a project license from the Central Committee on Animal Experiments (CCD, The Hague), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals.

Study Design
There were two experiments conducted for this study.

Experiment 1: Fine Motor Control
Prior to behavioral testing described below, all animals (2-month aged) were subjected to the Rotarod test (for detailed protocol see Clemens et al., 2015). This was done to ensure that BACHD rats exhibited the strong phenotype observed in previous studies (Abada et
al., 2013b; Clemens et al., 2015; Yu-Taeger et al., 2012) and to avoid having a group of rats not being representative for the model. After confirming the presence of the Rotarod impairment (data not shown), animals were divided in two groups (n=6/group) and longitudinally tested at 2, 7 and 12 months of age according to the following scheme. On any given day, one group was challenged with the pasta handling and the pellet reaching test, while the rats of the other group rested. Therefore, groups were assessed on alternating days until stable performance (10 sessions/age point for the pasta handling and 8 sessions/age point for the pellet reaching test). Unless otherwise noted, behavioral testing took place during the light phase to allow optimal visualization of fine movements.

During non-testing periods, all animals were given access to food and water ad libitum. Two weeks before each testing phase, daily food amount was progressively reduced until rats reached 85% of their respective free-feeding body weights. Afterwards, they were fed a daily ration in order to maintain this restriction level, taking normal growth into consideration. The animals were weighed every morning to assess food restriction levels and fed in their home-cages at 17:00 h. Following the testing periods, animals were again fed ad libitum.

During scoring of both behavioral tests, the experimenters were blind to the rats’ genotypes, while this was not the case when the videos were gathered. After being tested at 12 months of age, all rats were used for additional food consumption tests published elsewhere (Clemensson et al., 2017a).

**Experiment 2: Gross Motor Function and Locomotor Activity**
Animals were divided in two groups of WT and BACHD (n=6/group) and longitudinally tested at 2, 7 and 12 months of age according to the following scheme. On day 1, rats were challenged with the holding bar test and subsequently housed in the PhenoTyper® cages for six consecutive days (day 1-6) to assess locomotor activity (de Visser et al., 2006). Animals were then taken out of the PhenoTyper® and socially housed for 48 hours (day 7-8) as resting period. On days 9-10, animals were, respectively, trained and tested for grip strength. All behavioral tests were performed during the light phase and carried out by a single experimenter, while another experimenter (blind to the rats’ genotype) was videotaping or scoring the behavior.

**Behavioral Procedures**

**Experiment 1: Tests for Fine Motor Control**

**Pasta handling test**
The pasta-handling test was used to evaluate forepaw dexterity in the BACHD rats. This test has been found to be sensitive to a wide range of injuries and impairments (Allred et al., 2008, Tennant et al., 2010). Although the original protocols suggest using 7 cm-long spaghetti pieces, we have found that our rats eat in a hunched-over position, causing them to frequently break pasta pieces of such lengths. For the current study, the rats were therefore given strands of uncooked spaghetti (1.5 mm in width and 0.15 g/piece, Barilla,
Italy) that were cut to lengths of 5 cm. Prior to the first test occasion, rats were habituated to the pasta by placing 8 pieces into their social home-cages during 5 consecutive days. Afterwards, rats were given three habituation sessions in the test setup that was used. The test used a glass cage (28.5 x 29 x 29.5 cm) with mirrors on the floor and along two walls, which ensured a good view of the test animals. The sessions followed a similar structure during both, habituation and testing. At the start of each session, a rat was placed inside the setup. Afterwards, a single piece of spaghetti was dropped into the cage, and the rat was allowed to consume it. When the rat consumed the first piece, a new spaghetti piece was dropped into the cage. During habituation sessions, no video recordings were made, and the sessions ended either after consumption of two spaghetti pieces, or when 5 minutes had elapsed. Rats that did not consume the first pasta piece within the set time limit were given an additional session at the end of the day, during dark phase, to promote habituation. After the habituation sessions, all rats were given ten test sessions, organized as described in the study design section. For these sessions, pasta pieces were filed down to achieve blunt edges and marked with an ultrafine tip marker at specific intervals (1 cm increments) in order to facilitate visualization of the movement of the pasta strand during eating. During each session, rats were given between 2 and 5 pasta pieces, depending on their behavior, with the aim to obtain two consumption videos of good quality from each rat. Sessions during which a rat broke the pasta piece were excluded and not counted as consumption video. A minimum of 20 consumption videos was gathered for each rat and test age. For occasions where the initial ten testing sessions were insufficient to achieve this, additional test sessions were given. Rats were videotaped with a handheld camera (Sony HD Handycam, Japan) positioned to optimize the view of paw movements. Several behavioral parameters were scored from the videos. Some of these concerned detailed scoring of biting and chewing behavior, and are further described in the Supplementary material. The primary readouts, however, concerned the rats’ forepaw use. For this, the total time spent actively handling the pasta piece was measured using a stopwatch during normal speed playback of the videos (MacLaren et al., 2014). This parameter specifically excluded occasions where the rats chewed, flipped over or dropped the pasta piece to obtain the time they actively manipulated it with their forepaws. Slow motion video playback (~50% of real-time) was then used to quantify the number of forepaw adjustments. The total number of adjustments of each paw was counted per trial. A normal adjustment was defined as any distinct removal and replacement of the paw, or of any number of digits, on the pasta piece after eating commences.

**Pellet reaching test**

To assess skilled reaching, the rats were assessed in a pellet reaching test. Other protocols of such behaviors are sensitive to discreet neuropathologies (Farr and Whishaw, 2002; Klein and Dunnett, 2012). All sessions were conducted in a transparent Plexiglas box (35 cm long x 35 cm wide x 35 cm tall) placed on a table surface. Each side presented two 1
Motor characterization of BACHD rats

**Text Content**

About 40 cm wide, slit vertical openings that allowed the animals to reach for 45 mg grain-based precision pellets (Bio-Serv, Dustless Precision Pellets F0021, purchased through Bilaney Consultants, Duesseldorf, Germany) placed on a wooden frame attached to each side of the box, and allocated 3 cm above the floor. Before the first test occasion, the rats were habituated to the pellets by placing a spoonful into their home-cages for 5 consecutive days. Animals were then positioned in the apparatus individually and first acclimatized to the chamber with two shaping sessions in the dark phase, during which 20 pellets were placed, one at a time, on the wooden frame, at a distance of 1.5 cm from the openings. After shaping, limb preference of individual animals was determined by challenging each rat with a single session during which 100 reaching attempts were scored. During this, the pellets were placed centrally in front of one opening to allow the rat to use either paw. When 80% out of 100 reach attempts were made with one limb during this single session, that limb was identified as the preferred one. In the subsequent training sessions, food pellets were located contralateral to this limb at a distance of 1.5 cm from the slit openings. This enabled the animals to reach for pellets with their preferred limb. Animals that were not attempting to reach the pellets were further trained during the dark phase, placing the pellets at 1 cm distance from the slit openings to encourage reaching attempts. Some animals used their tongue instead of their paws and were further trained, during the dark phase, to reach the pellets at a distance of 2 cm from the openings. When all animals had made 20 reaching attempts within a single session, with the pellet placed 1.5 cm away from the slit, they progressed to the testing sessions. It took a total of five days for all rats to reach this criterion. During test sessions, rats were allowed to reach for a total of 30 pellets (considered as 30 separate trials). The first ten pellets were considered as warm up trials, and the following 20 were considered testing trials. The pellets given on subsequent trials were placed so that the pellet position alternated between two sides of the box, but randomized between the two available slits on each wall. Through this, a predictable pattern of side alternation, but unpredictable pattern of specific pellet position was obtained. This was done to induce a partial searching behavior among the rats, which ensured goal-directed reaches. It should be noted that the exact use of the different slits was still balanced so that no opening was overrepresented during testing. Rats were given one test session per day until they reached a stable performance. This resulted in a total of 8 test sessions per age point. The success rate of the rats’ reaching attempts was used as outcome measure. Reaches were considered successful if the rat had grabbed the pellet on the first reach attempt and managed to retrieve and eat it without dropping it into the cage bedding. All other behaviors were considered failure. Success rates were calculated as the number of successful reaches out of the final 20 pellets offered at each session. Recordings were made with a small action camera (Mini WiFi Camcorder 1, Rollei, Germany) connected to a computer using AVS videorecorder (http://www.avs4you.com/AVS-Video-Editor.aspx). Slow motion video playback (~50% of real-time) was used to calculate the number of successful reaches.
Experiment 2: Tests for Gross Motor Function and Locomotor Activity

**Holding bar test**

The holding bar test was used to assess forelimb hanging strength and balance over time (Li et al., 2004; Putten et al., 2012). The test apparatus consisted of a 37 cm-wide and 3 mm-thick wooden bar tightly secured between two vertical stands placed around 75 cm above a pillow. The height was sufficient to encourage the animals to hold the bar, but also low enough to prevent them from injuries when falling down. Each animal was handled via the body and brought near the bar, allowing the grasping of the bar with the forepaws only. Each rat was videotaped with an iPhone® 6S (Apple, Cupertino, CA, United States) and given 3 consecutive trials (ITI = 1 min). The average of the three trials was used as main outcome measure. Moreover, the Holding Impulse \((s^*g) = \text{hang time (s)} \times \text{body mass (g)}\) was used to correct for the negative effects of body mass on the hanging time, and it reflected the minimal amount of sustained tension (impulse) that the animal developed for supporting itself on the bar against gravity for the longest period of time (Putten et al., 2012). When improper behavior occurred (e.g. balancing on top of the bar or deliberately jumping off the bar) the trial was omitted and repeated. Suspension latencies were scored with slow motion video playback (~50% of real-time) using The Observer XT 12 (Noldus Information Technology, Wageningen, The Netherlands).

**Grip strength**

This test is based on the tendency of a rat to instinctively grasp a bar or a grid when suspended by the body, and permits assessment of the strength of the forelimbs. The test apparatus (Grip Strength Meter, Ugo Basile, Italy) consisted of a grasping bar attached to a force transducer in order to measure the maximum force applied by the rat during the pull. The unit of force used was grams-of-force. Each animal was handled via the body and brought near the bar, allowing the grasping of the grid with both forepaws and then gently pulled back until they released it. Animals were trained and tested on two consecutive days, using the same protocol. Five such measurements were obtained for each animal, and the resting period between each pull was one minute (Aartsma-Rus and van Putten, 2014; Jeyasingham et al., 2001).

**Locomotor activity in the PhenoTyper**

Locomotor activity was recorded by videotracking in the PhenoTyper (Noldus Information Technology, Wageningen, The Netherlands), an instrumented home-cage in which rodent behavior was automatically monitored through a video-based observation system, as described in detail by de Visser et al. (de Visser et al., 2006). The cages (45 cm x 45 cm x 45 cm) were made of transparent Perspex walls with an opaque Perspex floor covered with cellulose-based bedding (Cellu-Dri, LBS Biotechnology, United Kingdom), and equipped with a water bottle, a feeding station and a shelter in one corner (14.3 cm x 14.3 cm x 11.5 cm). Food and water were provided \textit{ad libitum}. Video tracking was performed by an infrared-
sensitive video camera installed in the top unit of each cage, infrared lighting sources and hardware and software needed for videotracking. EthoVision 9 was used for data acquisition and Ethovision XT 11.5 for analysis (Noldus Information Technology, Wageningen, The Netherlands). Rats were introduced into the PhenoTyper during the light phase (between 12:00 and 16:00 h) and monitored for six consecutive days. Spontaneous locomotor activity was assessed, scoring the following parameters: distance moved, velocity, number of jumps and time spent on top of the shelter. We additionally investigated potential sleep disruption and/or altered circadian rhythmicity, taking into account the time spent inside the shelter. All parameters were calculated using the means of day 4-6 (de Visser et al., 2006) and results were then split into dark and light phases, according to the day-night cycle of the animals. No human interference took place between the start and the end of the observations (hence, no intermediate cleaning of the cages).

**Statistical Analyses**

All statistical analyses were conducted using GraphPad Prism v.6.0 (GraphPad Software, San Diego California USA, http://www.graphpad.com). Two-way repeated measures ANOVAs were used to analyze all parameters. Age was used as within-subject factor, and genotype as between-subject factor. Bonferroni post-hoc test was used to follow up any significant effect of genotype found in the two-way ANOVAs. A \( p \)-value < 0.05 was considered statistically significant.

During **Experiment 1**, two BACHD rats did not consume the pasta strands during every testing day, and were therefore excluded from the analysis. Between two testing periods of **Experiment 2**, two BACHD rats fell ill and had to be sacrificed and removed from the experiment at 9 and 11 months of age, respectively. In both cases, the illnesses concerned tumors. Due to technical problems, the locomotor activity/circadian rhythmicity data from two WT and four BACHD rats could not be analyzed.

Thus, the n of the analyses changed as follows: for the pasta handling test (WT: 12, BACHD: 10), for the pellet reaching task (WT: 12, BACHD: 12), for the holding bar test (WT:12, BACHD: 10), for the locomotor activity/circadian rhythmicity (WT: 8, BACHD: 8), for the grip strength (WT:12, BACHD: 10). Age development analyses excluded data from animals that were not assessed at all ages. No other exclusion criteria were used.
Results

Experiment 1: Fine Motor Control

Pasta Handling Test

Figure 1A shows a photo of a rat during the pasta-handling test, displaying its typical paw placement during consumption. The number of adjustments required to eat the pasta piece was calculated over different sessions at the three different test ages (Figure 1B). All groups were found to use less adjustments by the final age of testing (age effect: F(2, 40) = 17.51, p<0.0001) without any significant genotype or interaction effects. The handling time also changed with age (F(2, 42) = 16.08, p<0.0001), with both WT and BACHD rats showing a longer handling time during the first test age compared to the following two (Figure 1C). There was no difference between WT and BACHD rats' handling time at any age (genotype effect: F(1, 21) = 0.3779, p=0.5454). The rate of adjustment, shown in Figure 1D, served as an indication of the number of adjustments over the total time of handling, which did not unravel significant age, genotype or interaction effects (age effect: F(2, 40) = 2.195, p=0.1246; genotype effect: F(1, 20) = 0.02155, p=0.8848; interaction effect: F(2, 40) = 0.1206, p=0.8867).

We further performed a detailed analysis of parameters related to biting and chewing, which did not reveal any prominent phenotypes, and are described and discussed in the Supplementary material.

Pellet Reaching Test

Figure 2A,B show the pellet reaching setup with an animal engaging in the test. Figure 2C illustrates the mean reaching scores in the pellet reaching test. Performance did not differ between the two groups at any age, although success rate of both genotypes dropped over time (age effect: F(2, 22) = 10.26, p=0.0007; genotype effect: F(1, 11) = 0.2214, p=0.6472). The major reason for failure to retrieve a pellet in either group was that pellets were either dropped during the reaching attempt(s) or displaced from the frame. It should be noted that the protocol and setup for the pellet reaching resulted in rats frequently standing at an angle to the openings rather than straight in front of them.

However, more specific scoring suggested that differences in body position did not affect the rats’ success rate, and did not differ between genotypes (data not shown).

Experiment 2: Gross Motor Function and Locomotor Activity

Holding Bar

Figure 3A presents the average holding time of three trials, which reduced in both genotypes between 2 and 7 months of age, showing significant age (F(2,40) = 10.06, p=0.0003) and age x genotype effect (F(2,40) = 6.226, p=0.004). BACHD rats exhibited significant impairment compared to WT rats at 2 months of age only, as shown by the reduced average hanging time (genotype difference in post-hoc analysis 2 months: 

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Figure 1 Basic parameters of the pasta handling test.

(A) A photo displaying a rat during consumption of a spaghetti piece. Rats grasp the pasta pieces with both front paws and guide them using a coordinated asymmetrical pattern. The "grasp" paw holds the pasta in a whole-paw grasp, typically positioned lower (away from the mouth) on the piece at the start of eating. The other paw is named the "guiding" paw due to the fact that it is closer to the mouth than the grasping paw and is used to guide the pasta accurately between the teeth (Allred et al., 2008). (B) Age development of the number of adjustments needed for consuming a single spaghetti piece. (C) Age development of the total time spent handling a single spaghetti piece. (D) Age development of the rate of adjustment (number of adjustments over the total handling time). Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. (***) $p < 0.001$, (****) $p < 0.0001$, ns (not significant)
Chapter 2

At 7 and 12 months of age, a genotype effect was not evident ($F_{(1, 20)} = 3.452, p=0.078$).

The holding impulse decreased in WT rats with increasing age, while it non-significantly increased in BACHD rats (age effect: $F_{(2, 40)} = 0.2972, p=0.1106$; genotype effect: $F_{(1, 20)} = 2.788, p=0.1106$; Figure 3B), resulting in a significant age x genotype effect ($F_{(2, 40)} = 7.318, p=0.002$). Although ANOVA revealed no overall genotype effect, post-hoc analyses indicated the presence of deficits in the holding impulse in BACHD rats at 2 months of age ($p<0.01$).

Figure 2 Pellet Reaching test.

(A) Olfactory engagement with a food pellet and body posture of a rat before initiating a reach. (B) A rat reaching for a pellet through one of the slit openings. (C) Timeline of mean reaching success (per 20 reaches) in BACHD and WT control rats tested at 2, 7 and 12 months of age. Data are expressed as mean success rate over eight different sessions ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. (*** $p < 0.001$, ns (not significant))
Grip Strength

Figure 3C represents the average of the three highest values of grip strength, which did not show any significant genotype ($F_{(1, 20)} = 3.861, p=0.0635$), age ($F_{(2, 40)} = 2.72, p=0.0781$) or interaction effects ($F_{(2, 40)} = 0.07663, p=0.9264$). Figure 3D shows the rats’ performance normalized to the animals’ individual body weight. The performance of BACHD and WT rats worsened between the age of 2 and 12 months, as reflected by a significant age effect ($F_{(2, 40)} = 123.1, p<0.0001$). The ANOVA revealed a general genotype effect ($F_{(1, 20)} = 6.186, p=0.0218$), but no significant genotype x age interaction. Post-hoc analyses indicated that BACHD rats had significantly increased grip strength at 2 months of age ($p<0.05$).

Figure 3 Holding Bar and Grip Strength tasks.

(A) Mean latency in the Holding Bar maneuver. (B) The Holding Impulse, outcome measure (s*g) reflecting the minimal amount of sustained tension (impulse) that is needed to oppose gravity. (C) Performance in the Forelimb Grip Strength test. (D) Performance in the Forelimb Grip Strength test normalized for body weight. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$, ns (not significant)
Locomotor Activity in the PhenoTyper

Dark phase

Distance travelled (age effect: \( F_{(2, 32)} = 134.5, p<0.0001; \) Figure 4A) and velocity (age effect: \( F_{(2, 32)} = 112.8, p<0.0001; \) Figure 4B) during the dark phase was gradually reduced with age in animals of both genotypes. At each age, these parameters indicated similar behavior of WT and BACHD rats (distance travelled genotype effect: \( F_{(1, 16)} = 0.35, p=0.5624 \)) (velocity genotype effect: \( F_{(1, 16)} = 1.101, p=0.3095 \)). However, BACHD rats showed a significant decrease of the number of jumps on top of the shelter compared to age-matched WT rats, exhibiting significant age (\( F_{(2, 32)} = 3.886, p=0.0308 \)) and genotype differences (Figure 4C; post-hoc analyses 2 months: \( p<0.05 \)). On the other hand, animals of different genotypes did not present any significant differences in the time spent on the shelter (genotype effect: \( F_{(1, 16)} = 2.245, p=0.1535; \) Figure 4D). Moreover, BACHD and WT rats did not spend different amounts of time inside the shelter (genotype effect: \( F_{(1, 16)} = 0.7144, p=0.4105 \)); there was only a significant effect of age (\( F_{(2, 32)} = 16.77, p<0.0001; \) Figure 4E).

Light phase

The overall locomotor activity changed with age in both WT and BACHD, showing a significant age effect in the distance moved (\( F_{(2, 32)} = 45.45, p<0.0001; \) Figure 5A), in the velocity (\( F_{(2, 32)} = 11.47, p=0.0002; \) Figure 5B) and in the time spent on the shelter (\( F_{(2, 32)} = 7.809, p=0.0017; \) Figure 5D). BACHD rats showed a significant reduction of the distance travelled and the velocity, exhibiting significant genotype differences in post-hoc analyses for distance moved (2 months: \( p<0.01, 7 \) months: \( p<0.001, 12 \) months: \( p<0.0001; \) Figure 5A), for velocity (2 months: \( p<0.001, 7 \) months: \( p<0.05, 12 \) months: \( p<0.001; \) Figure 5B) and for number of jumps on top of the shelter (2 months: \( p<0.01; \) Figure 5C). Moreover, BACHD rats showed decreased time spent on the shelter (Figure 5D), exhibiting a significant genotype effect in post-hoc analyses (12 months, \( p<0.0001 \)) and significant interaction between genotype and age (\( F_{(2, 32)} = 6.750, p=0.0036 \)). On the contrary, BACHD rats spent significantly more time in the shelter, showing a genotype effect (\( F_{(1, 16)} = 6.132, p=0.0248 \)) in the time spent inside the shelter (Figure 5E), without showing any age (\( F_{(2, 32)} = 1.408, p=0.2594 \)) or interaction (\( F_{(2, 32)} = 0.7329, p=0.4884 \)) effect.
Figure 4 Home-cage assessment in the PhenoTyper – Active/dark phase activity.

(A) Distance Moved (B) Mean velocity (C) Number of jumps onto the shelter (D) Time spent on the shelter (E) Time spent inside shelter. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. (*) p < 0.05, (****) p < 0.0001, ns (not significant)
Figure 5  Home-cage assessment in the PhenoTyper – Inactive/light phase activity.

(A) Distance Moved  (B) Mean velocity  (C) Number of jumps onto the shelter  (D) Time spent on shelter  (E) Time spent inside shelter. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001, (****) p < 0.0001, ns (not significant)
Discussion

In previous studies BACHD transgenic rats presented a strong motor coordination deficit in the Rotarod test (Abada et al., 2013b; Clemens et al., 2015; Yu-Taeger et al., 2012), mild gait impairments and altered open field activity (Abada et al., 2013b). The use of multiple motor paradigms in the present study allowed us to further investigate fine motor skills, muscle strength and endurance as well as several parameters of activity-related motor function in a home-cage-like environment. Taken together, these findings expand the existing knowledge of the behavioral phenotypes of the BACHD rat, showing the presence of selected behavioral deficits.

Regarding fine motor control, BACHD rats did not present any significant impairment. Although HD patients are significantly impaired in fine motor skills such as skilled reaching or manual dexterity (Klein et al., 2011), both parameters appeared to be intact in BACHD rats when challenged with the pasta handling and the pellet reaching tests. The results are, however, in line with previous observations in another rat model of HD (Fielding et al., 2012). Overall, these results suggest that fine motor aspects most likely did not contribute to the gross motor deficits seen in Rotarod performance or in the gait pattern.

Concerning muscle function, BACHD rats appeared significantly impaired in the holding bar task at 2 months of age, suggesting reduced muscle endurance. However, no differences were found at 7 and 12 months of age, as the WT rats’ performance dropped down to BACHD rat level at these ages. The difference at 2 months of age is possibly related to a reduced amount of muscle mass and increased amount of fat mass carried by BACHD rats, as found in a previous study (Jansson et al., 2014). A reduced performance in the holding bar test could therefore be expected, as the animals have to hold the same body weight with a significantly lower amount of muscle mass. On the other hand, the drop that WT animals exhibited might be either due to repeated testing or due to the large increase in body weight from 2 to 7 months of age, which might have made it impossible for the rats to hang longer than a certain minimum amount of time. Thus, the test might not allow us to draw final conclusions on muscle endurance in older rats.

Conversely, the grip strength test did not show an indication that BACHD rats’ “passive” forelimb grip strength was reduced, even at the youngest age. A possible explanation could be that in the holding bar test the rats have to work against gravity and their own weight, while this is not the case in the grip strength test. Due to the abnormal body composition of BACHD rats with reduced muscle and increased fat mass, workload during the holding bar test is therefore higher than that of WT rats. In contrast, WT and BACHD rats are likely exposed to a more comparable workload during the grip strength test.

Additionally, previous studies provided evidence of peripheral phenotypes such as weight loss and muscle wasting in HD patients (Cepeda et al., 2007; Imarisio et al., 2008; Miller and Bezprozvanny, 2010), progressive skeletal muscle atrophy in HD patients and in
the R6/2 mouse model for HD (She et al., 2011; Zielonka et al., 2014). The impairment seen in the holding bar test might be also related to deficits in the joints, ligaments and tendons, all structures which are compromised in HD patients and in the BACHD mouse model (De Aragão et al., 2016; Nguyen and Cenci, 2015). Further studies would be required to investigate if the deficit we have reported is due to the reduced muscle mass or to a peripheral pathology or a functional impairment.

In addition to the assessment of motor phenotypes with classical tests, we implemented the observation in an automated home-cage system to investigate general aspects of motor function related to rats’ baseline behavior. In this setup, we found BACHD rats to show a generally reduced number of jumps on the shelter, while the time spent on the shelter was similar to WT rats. This suggests a specific motoric difficulty to reach the top of the shelter rather than a reduced motivation to use it. Interestingly, this phenotype was present at all investigated ages.

Furthermore, we found BACHD rats to have a generally reduced locomotor activity, specifically restricted to the light phase. These data suggest that, in our setting, the overall walking ability and activity of the rats was relatively preserved, as it was unaffected during the major activity phase. This is in line with previous observations in BACHD rats of the same age, which showed only mild gait abnormalities in both static and dynamic parameters during Catwalk testing (Abada et al., 2013b). Conversely, the results are in contrast with a lower rearing and ambulatory activity observed with the PhenoMaster system at 3 and 6 months of age (Yu-Taeger et al., 2012) and with an initial hyperactivity followed by hypoactivity starting at 4 months of age exhibited by BACHD rats in an open field test-like setup (Abada et al., 2013b). The different outcome could be due to the different test protocols and test setups used. Most prominently, we screened the animals for 6 consecutive days, while the other two studies screened for 22 hours and 1 hour, respectively. Thus, those results will probably rather reflect the rats’ response to novelty, while we have investigated their baseline behavior.

The reduction in activity during the light phase might suggest the existence of an altered circadian rhythm, which would be in line with previous studies in HD patients and in the BACHD mouse model (Kuljis et al., 2012; Morton et al., 2005). However, further and more detailed studies are necessary to investigate the mechanism underlying this behavioral alteration.

The robustness of our motor assessment study is that we performed a battery of behavior experiments, combining novel and classical test setups under well controlled environments (temperature, humidity, food restriction) making the monitoring of behavior across different ages possible. However, it has to be noted that we performed multiple behavioral tests with the same group of rats. Repeated testing might influence the outcome of the tests (carry-over effect), although such an approach offers a better possibility to investigate phenotype onset and development than in separate cohorts of rats. Another limitation is that we performed the classical tests during the light phase in order to enable
better visualization of the rats’ behavior. However, it is known that behavioral readouts differ when assessed during the light or dark phase, and the dark phase still represents the natural activity phase of rats.

Taken together, our study revealed specific motoric impairments in the BACHD rats, which might be related to a reduced muscle mass and increased fat mass, as reported earlier (Jansson et al., 2014). We suspect that different factors might have influenced BACHD rats’ strong impairment in the Rotarod test reported in previous studies. Although there is definitely a motor component, the deficit may also be related to other, non-motor phenotypes such as motivation or anxiety, as such differences have been found early on (Clemensson et al., 2017b; Jansson et al., 2014; Yu-Taeger et al., 2012). Most importantly, the study revealed novel readouts that can be used for addressing the efficacy of novel therapies on different parameters of motor function in pre-clinical research.

Conflict of interest
At the time of the studies, the authors G. Manfré, E.I. Kyriakou and Dr. J.E. van der Harst were working for the EU funded “PhenoRat” project of which Noldus Information Technology was an industrial partner. Dr. J.E. van der Harst was part-time scientific project advisor for “PhenoRat” employed by Noldus Information Technology.

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References


Supplementary material

Material and methods

In addition to the parameters discussed in the main article, parameters relating to orofacial aspects of food consumption and consumption speed were scored from subsets of the videos obtained from the pasta handling test.

The full consumption time was measured from the point that a rat positioned the spaghetti piece into its mouths and started biting, to the point where the end of the pasta piece disappeared into its mouth. This parameter was scored at all ages. For videos gathered at two months of age, separate measurements of the time the rats spent actively biting on the pasta piece and the time they spent chewing and swallowing were also taken. The periods of active biting were clearly identifiable by the audible bite sounds that the rats produced. The periods of chewing were also clearly identifiable by the rat removing the spaghetti piece from its mouth and making characteristic chewing motions with their jaws. An additional and more discreet behavior was, however, included in these chewing periods. The rats would on occasion make breaks from biting on the spaghetti piece, and sit motionless with closed mouths, while still seemingly maintaining focus on the task at hand. The behavior was coupled with distinct sounds, and was deemed to be part of the feeding behavior, as we have not encountered it in other circumstances. Periods where the rats adjusted the position of the spaghetti piece, or stopped focusing on the spaghetti piece entirely, were not included in the measurement of consumption time, biting time or chewing time. Notably, these behaviors were all excluded despite the fact that rats would often couple them with some chewing motions. This was done to obtain a similar scoring protocol to what has been used in other studies of the BACHD rats’ food consumption behavior (Clemensson et al., 2017). The parameters above were scored using the open-source BORIS software for video annotation (Friard and Gamba, 2016). Videos gathered at 12 months of age were subjected to additional analyses to obtain the number of bites needed to consume a spaghetti piece and measure the temporal spacing of individual bites. This analysis was performed with The Observer XT 12.5 (Noldus Information Technology, Wageningen, The Netherlands), which allowed simultaneous viewing of a pasta handling video and its audio track. The timestamp of each audible bite sound was noted, and further processed to obtain the parameters of interest.
Statistical analysis

Videos where the full feeding was not recorded, where the rats broke the pasta piece, or handled it with only one paw were excluded from the analysis of all parameters. For the analysis of consumption time, biting time and chewing time, average values were obtained for each test age and rat, during periods of stable performance. Ultimately, the data was for a given rat was based on an average of 15 videos (i.e. individual pasta piece consumptions) during the first test age, 11 videos during the second test age, and 5 videos during the final test age. Sound analysis was based on four trials per rat. The videos were selected to obtain a sample of videos with optimal audio quality, during a period of stable performance.

Age progression of consumption time was subsequently analyzed with a two-way ANOVA, using age as within-subject factor, genotype as between-subject factor and Sidak’s post-hoc test. One WT and three BACHD rats were excluded from the analysis, as data was not available for all ages. Thus the final n for this analysis was 11 WT rats and 9 BACHD rats. The analyses of consumption time, time spent biting and time spent chewing measured at the first test age used simple comparisons between WT and BACHD rats, and thus used individual t-tests. The n for these analyses were 12 for both genotypes. The total number of bites made during consumption of a spaghetti piece also used a simple comparison between WT and BACHD rats, but used a Mann-Whitney U test as the data from the BACHD rats did not appear to be normally distributed. Finally, data for frequency distribution of bite intervals were analyzed with two-way ANOVAs, using genotype as between-subject factor, bite interval as within-subject factor, and Sidak’s post-hoc test.

Results

The time needed for consuming the spaghetti piece changed with age (Age effect: \(p<0.001\)), with both WT and BACHD rats showing a seemingly longer consumption time during the first test age compared to the following two (Supplementary Figure 1A). There was no difference between WT and BACHD rats’ consumption time at any age. Interestingly, most of the consumption time was made up of time spent actively biting on the spaghetti piece, while very little time was spent on chewing and swallowing behaviors (Supplementary Figure 1B, 1C, 1D).

Supplementary Figure 2A shows an example of the audio readout that was used to score individual bites. There was no significant difference between WT and BACHD rats in terms of the total number of bites that were needed in order to consume a full spaghetti piece (Supplementary Figure 2B) or in terms of the absolute or relative frequency distribution of bite intervals (Supplementary Figure 2C, 2D). Most bites were evenly spaced, with intervals around 0.11-0.20 seconds, although some bites appeared in much
closer succession, showing intervals of 0.00-0.05 seconds. This is also evident from the audio printout shown in Supplementary Figure 2A. It should, however, be noted that this might not constitute individual biting motions, but rather a single bite causing the spaghetti piece to break in multiple places (e.g. inside the rats’ mouth). Although the analysis presented in Supplementary Figure 2 did not reveal any significant difference between WT and BACHD rats, it should be noted that one BACHD rat performed a remarkably high number of bites compared to the rest of the group. Excluding this rat from analysis did result in significant differences being detected between the genotypes ($p$<0.05 for Supplementary Figure 2B, genotype effect: $p$<0.05, interaction effect:

Supplementary Figure 1 Spaghetti consumption behaviors.

(A) Age development of the time needed to consume a spaghetti piece. Group mean plus standard error is indicated. Results from two-way ANOVA are displayed in bottom left corner of graph. Results from post-hoc test are displayed for data points where performance of WT and BACHD rats differed significantly. Detailed analysis of spaghetti consumption at two months of age is displayed in separate graphs for the full consumption time (B), the total time spent actively biting on the spaghetti piece (C), and the total time spent on chewing and swallowing (D). Graphs indicate performance of individual rats and group mean. (***$p$ < 0.001, ns (not significant)
p<0.01, post-hoc results for 0.00-0.05 bin; p<0.01, post-hoc results for 0.11-0.15 bin; p<0.05 for Supplementary Figure 2C; unchanged results for Supplementary Figure 2D). This analysis approach was, however, not considered to be appropriate, as there was no reason to assume that the outlier was caused by sub-optimal experimental conditions, but was likely just a representative of biologically based variation between rats.

**Supplementary Figure 2** Bite sound analysis of spaghetti consumption.

(A) An example of the audio printout that was analyzed to obtain the data of interest. Note that the second and seventh bite sounds appear in very close succession of the previous bite sounds. (B) Basic comparison of the total number of bites needed to consume a single spaghetti piece. Performance of individual rats and group mean is indicated. (C) Frequency distribution of bite intervals, presented in terms of the absolute number of bites. Group mean plus standard error is indicated. Results from two-way ANOVA are displayed in top left corner of graph. Results from post-hoc test are displayed for data points where performance of WT and BACHD rats differed significantly. (D) Frequency distribution of bite intervals, presented in terms of the relative numbers of bites. Group mean plus standard error is indicated. Results from two-way ANOVA are displayed in top left corner of graph. Results from post-hoc test are displayed for data points where performance of WT and BACHD rats differed significantly. (***) p < 0.001, ns (not significant)
Supplementary Figure 2  Continued.
Discussion

There was no difference between WT and BACHD rats in terms of how much time they needed to consume the spaghetti pieces. This is in line with results from measuring consumption rate of small (45mg) reward pellets (Jansson et al., 2014), but in contrast with results from measuring consumption rate of single pellets of standard rodent chow (Clemensson et al., 2017). In the latter case, BACHD rats have been found to reliably require longer time than WT rats when consuming chow pieces of comparable size and shape. As discussed in (Clemensson et al., 2017), the reason for this discrepancy might be due to the different involvement of chewing behaviors in the three food consumption tests. Consuming the small reward pellets appears to involve very little chewing (Jansson et al., 2014), which is also true for the consumption of spaghetti pieces (Supplementary Figure 1D). In contrast, consumption of chow pieces, involves frequent, and extensive chewing (Supplementary Figure 8 in (Clemensson et al., 2017)). Thus, as a clear impairment in consumption rate has only been seen when assessing consumption of chow pieces, it is likely that BACHD rats show generally unimpaired biting and swallowing, while chewing motions are more strongly impaired. Still, discreet impairments have been noted also during biting behaviors (Clemensson et al., 2017). In line with this, the results from the current study show some indications that BACHD rats might suffer from discreet biting impairments, although the results are unclear (i.e. only significant if outliers are excluded). Essentially, although there does not appear to be any overt impairment present, BACHD rats might suffer from discreet deficits, resulting in them taking slightly fewer bites compared to WT rats. Unfortunately, the audio quality of the videos recorded in the current study was of generally poor quality, resulting in a quite limited data set (i.e. four spaghetti pieces per rat). A more extensive study is thus needed in order to determine if the slight trends noted in the current results are indicative of an actual phenotype, or due to chance.
References


BACHD rats expressing full-length mutant huntingtin exhibit differences in social behavior compared to wild-type littermates

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Abstract

Background: Huntington disease (HD) is a devastating inherited neurodegenerative disorder characterized by progressive motor, cognitive, and psychiatric symptoms without any cure to slow down or stop the progress of the disease. The BACHD rat model for HD carrying the human full-length mutant huntingtin protein (mHTT) with 97 polyQ repeats has been recently established as a promising model which reproduces several HD-like features. While motor and cognitive functions have been characterized in BACHD rats, little is known about their social phenotype.

Objective: This study focuses especially on social behavior since evidence for social disturbances exists in human patients. Our objective was to compare social behavior in BACHD and wild-type (WT) rats at different ages, using two different measures of sociability.

Methods: Animals were tested longitudinally at the age of 2, 4 and 8 months in the social interaction test to examine different parameters of sociability. A separate cohort of 7 months old rats was tested in the three chamber social test to measure both sociability and social novelty. Gene expression analyses in 8 months old animals were performed by real time qRT-PCR to evaluate a potential involvement of D1 and D2 dopaminergic receptors and the contribution of Brain-derived neurotrophic factor (BDNF) to the observed behavioral alterations.

Results: In the social interaction test, BACHD rats showed age-dependent changes in behaviour when they were reintroduced to their cagemate after a 24 hours-period of individual housing. The time spent on nape attacks increased with aging, Furthermore, a significant higher level of pinning at 2 months of age was shown in the BACHD rats compared to wild-types, followed by a reduction at 4 and 8 months. On the other hand, BACHD rats exhibited a decreased active social behaviour compared to wild-types, reflected by genotype-effects on approaching, following and social nose contact. In the three chamber social test, BACHD rats seemed to show a mild deficit in preference for social novelty, but no changes in social interest. Molecular analyses revealed that BACHD animals exposed to the social interaction test displayed decreased mRNA levels of the total form of BDNF in ventral striatum and unaltered striatal expression of D1 and D2 dopamine receptors.

Conclusions: Taken together, these results indicate deficits in several parameters representative of sociability. Altered BDNF expression in the ventral striatum may contribute to the deficits in sociability in 8 months old BACHD rats. These data support the validity of the BACHD rat model in mimicking features of certain social deficits that could be relevant to symptoms in patients.
Introduction

Huntington disease (HD) is a dominantly inherited neurodegenerative disorder that is caused by an unstable expansion of a CAG repeat within the coding region of the huntingtin (HTT) gene (Huntington’s Disease Collaborative Research Group, 1993). It is characterized by motor impairment, abnormal choreic involuntary movements and by psychiatric, psychological and intellectual disorders (Kumar et al., 2015). Although more emphasis has been placed on detecting the early cognitive and motor impairments (Aylward et al., 2004; Kirkwood et al., 1999; Lawrence et al., 1998; Paulsen et al., 2006), emotional dysfunction might also precede the clinical HD diagnosis (Duff et al., 2007; Johnson et al., 2007). Thus, the identification of early psychiatric symptoms may be particularly important in HD because of their deleterious effects on everyday functioning and quality of life (Duff et al., 2007; Marder et al., 2000). Characterizing early neuropsychiatric phenotypes in animal models of HD is therefore especially important.

The BACHD rat model of Huntington disease was generated using a human bacterial artificial chromosome (BAC) which contains the full-length HTT genomic sequence with 97 CAG/CAA repeats and all regulatory elements (Yu-Taeger et al., 2012). BACHD rats present motor, cognitive and emotional alterations. Previous characterization studies in these rats showed clasping behavior starting at the age of 3 weeks, robust deficits in the Rotarod task already at 1 month (Yu-Taeger et al., 2012), decreased activity in the Phenomaster from 3 till 6 months (Yu-Taeger et al., 2012) and mild gait alterations in the Catwalk test by 12 months of age (Abada et al., 2013b). In a fear conditioning set-up, BACHD rats exhibited associative memory deficits by 4 months of age while an impairment of their reversal learning performance emerged at 6 months when rats were tested in a cross maze task (Abada et al., 2013a). At this age, also signs of fronto-striatal impairment were observed in different Skinner box tasks for short term memory (Clemensson et al., 2017). Starting at 4 months of age, BACHD rats display also changes in emotionality as suggested by the decreased anxious-like behavior in the elevated plus maze (Yu-Taeger et al., 2012). At a comparable age (between 3 and 5 months), an impulsive-like phenotype was demonstrated in a delayed discounting paradigm and in the Differential Reinforcement of Low Rate of Responding task (Manfré et al., 2016) while later, at 9 months of age, deficits in prepulse inhibition became evident as well (Abada et al., 2013b). In spite of the extensive use of the BACHD rat line in the last years, its psychiatric phenotype has been only partly investigated indicating that the BACHD rat line requires further phenotyping.

In this study, we focused on social behavior of BACHD rats as its changes are an important component of neuropsychiatric symptoms. Some aspects of social behavior have been partly examined in different rodent models of HD, showing altered social interaction (Pietropaolo et al., 2011; Nguyen et al., 2006; Wood et al., 2015) and social preference (Pietropaolo et al., 2011), reduced social memory and recognition (Ciamei and
Morton, 2008; Rudenko et al., 2010) and even increased levels of aggression (Shelbourne et al., 1999), supporting the view of impaired social behavior in HD.

We therefore investigated different parameters of the social behavior repertoire and associated molecular alterations in the BACHD rat model of HD. We performed detailed analyses of social behavior parameters to monitor development of potential deficits over time. To achieve this aim we performed: 1) a social interaction test to investigate different parameters of sociability at different ages; 2) a so-called three chamber social test to assess both sociability and social novelty. To link behavior to molecular correlates of social behavior, we quantified mRNA levels of D1 and D2 dopamine receptors and the total form of BDNF. Subtle impairments in specific aspects of social behavior were found which could be relevant readouts in pre-clinical HD-treatment studies. Additionally, changes in molecular markers were detected and they could underlie the social behavior deficits in HD.

Material and methods

Ethical statement
The experiments were carried out at 2 different locations. The experiments reported here were either approved by the Animal Ethics Committee (Dier Experimenten Commissie, RU-DEC, Nijmegen, The Netherlands) or by the local ethics committee (Regierungspraesidium Tuebingen, Germany), in full compliance with the European Union legislation on the use of animals for scientific purposes (Directive 2010/63/EU). All experimental procedures at Radboudumc (Nijmegen, The Netherlands) were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, CCD, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act, WOD). All experimental procedures at the University of Tuebingen (Tuebingen, Germany) were performed in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations.

Animals
For the social interaction test, fifteen transgenic males were supplied from a BACHD colony (TG5 line, Yu-Taeger et al., 2012) at Charles River (Wilmington, MA, USA) and an in-house breeding colony was preserved and maintained at Radboudumc (Nijmegen, The Netherlands) by cross-breeding these males with wild-type female rats (Charles River, Germany). WT and BACHD animals (N=24/group) were maintained on a Sprague-Dawley (SD) background. Genotyping and determination of BAC transgene integrity were performed via PCR analysis using genomic DNA extracted from ear biopsy tissue at postnatal day (PND) 21. Rats were weaned at PND 21 and test pairs were then group-housed...
two per cage with littermates of the same genotype and sex in a constant temperature (19.5 ± 1°C) and humidity room (55 ± 10%) with a reversed 12 h light/dark cycle with lights on/off at 8:00 P.M. /8:00 A.M.). Housing by test pairs (N=2 siblings per cage) was chosen because two familiar animals were tested for social interaction, on the base of previously published protocols (Peters et al., 2016).

All experimental animals used in the three chamber social test (University of Tuebingen, Germany) were bred on an SD background by pairing heterozygous BACHD males with wild-type females (Charles River, Germany). Rats were genotyped following previously used protocols (Yu-Taeger et al., 2012) at PND 20. At weaning (PND 21), rats were housed in groups of 4 with same sex-littermates of mixed genotype like in previous behavioral characterization experiments in the BACHD rat model to keep conditions as much comparable as possible between experiments in the same facility. All experimental animals were maintained in a room with constant temperature (22 ± 1°C) and humidity (55 ± 10 %) with a regular 12 h light/dark cycle (lights on/off at 6:00 A.M. /6:00 P.M).

All experimental animals in both facilities were provided food and water ad libitum, and behavioural tests were conducted during the active (dark) phase of the cycle.

The BACHD rat colony in Nijmegen, including the animals used for this study, was the F1 generation of animals ordered from the original breeder that were bred in-house with regular SD wild-type females, also specifically ordered from the regular supplier. Since the same procedure of generating animals for sectional and longitudinal studies has been used in Tuebingen and the two colonies have not been bred on different sites for a long period of time, the chance of genetic drift or other breeding/colony-effects is relatively low. Notably, other studies confirmed that the timeline of onset of deficits, such as the impaired Rotarod performance, is comparable between different labs applying different housing conditions (Abada et al., 2013a, 2013b; Yu-Taeger et al., 2012).

Behavioral Procedures

Experiment 1: Social Interaction Test

Apparatus and procedures. A total of 48 male rats (24 WT and 24 BACHD) was used for the study. All animals were longitudinally tested during the dark phase at 2, 4 and 8 months to monitor the progression of deficits and underwent the same schedule. The protocol of the social interaction test (adapted from (Peters et al., 2016)) is based on social interest and interaction with a familiar animal. Table 1 provides a survey of the timeline of experiments, performed as follows: on day 1, the body weight was measured and a first 20 minute habituation session in the test-arena was given to each animal individually. On day 2 cagemates were habituated (i.e. under social conditions) for a second time to the test arena for another 20 minute session. Any initial novelty-induced behavior declines after repeated exposure to the test environment (Rossetti et al., 2016; Schmittgen and Livak, 2008) therefore the effect of repeated testing was considered to be minimized by these 2 days of habituation that were repeated for each test at each age-point. On day 3 animals were
given 1 day-rest and on day 4 cagemates were separated and individually housed for 24 h. During this period of time cagemates were unable to see each other, but could smell and hear all the animals present in the room. Thus, only real active social interaction was prevented. The day of rest was given for practical reasons since the testing required the full day without any chance of separating the animals in between. On day 5 cagemates were brought together and tested for 20 min in the social interaction test.

The arena used in this experiment consisted of an enlarged, customized version of the PhenoTyper® (PhenoTyper 9000, Noldus Information Technology, Wageningen, The Netherlands). The animals were tested in these large chambers since it is argued that the expression of social behavior requires space (Peters et al., 2016; Spruijt et al., 2014). The arenas themselves consisted of a black floor plate (floor dimensions: 90 × 90 cm) and transparent Perspex walls (height: 100 cm) (Figure 1). The large PhenoTypers (PT-900) were equipped with a camera and infrared lighting. PhenoTypers were wiped clean with 70% ethanol between test subjects.

Variables measured. Video recordings were used to score behavior from captured video files using ½ playback speed of the video by using Observer XT 12.5 (Noldus Information Technology, Wageningen, The Netherlands). The time spent in social interactions (see Table 2 for the used ethogram, adapted from (Peters et al., 2016)) was manually scored by one observer blind to the test subjects’ genotype. Since the behavioral elements were scored from the view of one of the animals (focal animal) of a pair, behavioral elements from the category ‘social’ are scored either as receiver or as actor (Peters et al., 2016) and data are thus representative of N=12 per genotype.

Table 1 Timeline of experiment 1.

<table>
<thead>
<tr>
<th>Age</th>
<th>Days</th>
<th></th>
<th>2</th>
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<th>4</th>
<th>5</th>
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<td>Age</td>
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<td>3</td>
<td>4</td>
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<td>Habituation in Pairs</td>
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<tr>
<td></td>
<td>Single Habituation</td>
<td></td>
<td></td>
<td></td>
<td>Rest day</td>
<td>Individual Housing</td>
</tr>
<tr>
<td></td>
<td>Habituation in Pairs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Social Interaction Test</td>
</tr>
<tr>
<td>4 Months</td>
<td>Body Weight</td>
<td></td>
<td></td>
<td></td>
<td>Single Habituation</td>
<td>Habituation in Pairs</td>
</tr>
<tr>
<td></td>
<td>Single Habituation</td>
<td></td>
<td></td>
<td></td>
<td>Rest day</td>
<td>Individual Housing</td>
</tr>
<tr>
<td></td>
<td>Habituation in Pairs</td>
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<td></td>
<td></td>
<td></td>
<td>Social Interaction Test</td>
</tr>
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<td>8 Months</td>
<td>Body Weight</td>
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<td></td>
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<td>Habituation in Pairs</td>
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<tr>
<td></td>
<td>Single Habituation</td>
<td></td>
<td></td>
<td></td>
<td>Rest day</td>
<td>Individual Housing</td>
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<tr>
<td></td>
<td>Habituation in Pairs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Social Interaction Test</td>
</tr>
</tbody>
</table>

Animals sacrificed
Experiment 2: three chamber social test

Apparatus and procedure. A three chamber social test was applied in a cohort of 7 month old rats (17 WT and 15 BACHD) to assess sociability and social novelty using procedures adapted from previously published protocols (Crews et al., 2012; Toth and Neumann, 2013). Behavior was assessed in a black Plexiglas box (40 x 120 x 45 cm) divided in three sub-chambers (or arenas) consisting of a central arena (named neutral arena) and two lateral ones, interconnected by two transparent walls. While the central arena was empty for the whole length of the test, each lateral arena contained a wire mesh box that was either empty or hosted a stranger rat, depending on the testing phase. These stimulus-animals were naïve age- and weight-matched wild-type male rats from the same strain. Restraining the stranger rats in these boxes has the advantage of limiting the mobility of the stranger rat while still allowing visual, olfactory and tactile interaction between the experimental rat and the strangers. By limiting the mobility of the stranger rats, one can examine the social interest of a test animal reducing the influence of the

![Figure 1](image_url) PhenoTyper® 9000 (PT9000) cage setup for testing.

A photo displaying two rats during the social interaction test. Cagemates were brought together after being individually housed for 24h. Animals were marked red or black using a permanent marker in order to distinguish each rat of the couple. In contrast to black marking, the red marking was not visible because of the infrared lighting conditions, and it was used to prevent that the marking could become a confounding factor.
social interaction levels of the strangers in the test performance. In this way, a test animal can choose whether to explore a stranger and whether to explore the familiar or the novel animal that instead serve as social stimuli. Moreover, placing stranger animals in wire mesh boxes prevents the development of possible aggressive responses that may develop between test and stranger animals.

Testing was performed in the dark phase. Animals were allowed to habituate to the testing room for an hour before starting the test that consisted of three consecutive sessions: habituation (5 min), social interaction (10 min, 1 stranger rat present) and social novelty (10 min, 2 stranger rats present). Animals were given a 7 min intertrial interval between test sessions. During the first session (habituation), an experimental rat was placed in the central arena and was allowed to explore the box while each lateral arena contained an empty wire mesh box. In the second session (social interaction), a stranger conspecific rat was placed in the cage of one of the two lateral arenas while the test rat was re-introduced in the neutral arena and let explore the whole box. In this phase the preference of the test rat for exploring the stranger (social stimulus) or an empty box (non-social stimulus) on

<table>
<thead>
<tr>
<th>Behavioral category</th>
<th>Behavioral element</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social contact</td>
<td>Nape attacking</td>
<td>The focal animal attacks the neck area of another with its front part of the body</td>
</tr>
<tr>
<td></td>
<td>Pinning</td>
<td>An animal turns on its back and the focal animal pins the other animal to the ground with its forepaws or its whole body</td>
</tr>
<tr>
<td></td>
<td>Social nose contact</td>
<td>The focal animal establishes contact or near-contact with its nose to the other animal’s body part</td>
</tr>
<tr>
<td></td>
<td>Allogrooming</td>
<td>The focal grooming animal has one or both front paws on top of the other and pulls/licks at its fur</td>
</tr>
<tr>
<td>Social interest</td>
<td>Approaching</td>
<td>The focal animal gets in proximity of another animal, by a directed movement whereby the space between the 2 animals readily decreases</td>
</tr>
<tr>
<td></td>
<td>Following</td>
<td>The focal animal moves/follow the other to maintain a close distance while the other animal is moving around/away</td>
</tr>
<tr>
<td>Social avoidance</td>
<td>Moving away</td>
<td>The focal animal moves away from another animal after being in close proximity</td>
</tr>
<tr>
<td>Non-social</td>
<td>Solitary</td>
<td>The animal performs individual actions such as self-grooming, rearing, exploration (at least 1 body-length away from the other animal)</td>
</tr>
</tbody>
</table>
the opposite side is measured. In the third session (social novelty), a second stranger rat was placed in the cage of the opposite lateral arena. In this phase, the experimental rat can choose whether to explore the rat that was already present in the box in the social interaction phase (familiar rat) or the newly introduced one (novel rat). Social and novelty arenas and strangers were randomized between left and right sides in the box to avoid that a side preference in the box may influence the time spent exploring social/familiar or novelty arenas and relative strangers. The strangers were additionally randomized between WT and BACHD testing animals and between test phases. To prevent that removing animals from a cage could affect the performance of the remaining animals in the same cage, testing of cage mates on the same day was avoided. The stranger animals were not habituated to the wire mesh cages before the test. However, since the order of testing was randomized, as mentioned above, any effect of confinement of the strangers in the boxes is expected to be equally distributed over the groups/genotypes, preventing a strong effect on any genotype-results. The chamber and the wire mesh cages were wiped clean with 70% ethanol between test subjects.

Variables measured. The time spent in each arena and the time spent exploring the wire mesh boxes in each phase were manually scored with The Observer XT 12.5 (Noldus Information Technology, Wageningen, The Netherlands). Wire mesh box exploration was defined as sniffing the box (apparent snout contact), climbing the box, resting on the box with the front limbs, sitting and moving on the box. A rat was considered accessing a chamber when entering inside with half of the body length.

Reliability analysis. Since behavioral variables were scored manually in both social tests, a second observer re-scored part of the social interaction test and the three chamber social test videos in order to assess the reliability of our results. The second scoring was performed using ½ playback speed of the video with Observer XT 12.5 on six videos (three for each genotype) for each test, randomly chosen. By using a built-in reliability analysis feature in The Observer XT, the percentages of agreement between the two observers were calculated and the resulting statistics is presented in the Supporting Information (S1 and S2 tables).

Tissue collection. After the last social interaction test 8 months-old animals were euthanized by intraperitoneal injection of 90 mg/kg pentobarbital. Immediately after death, the animals were decapitated and their brains removed, isolated and frozen in aluminium foil on dry ice and stored at −80°C. In a cryostat (−12°C), the brains were prepared in 300 μm-thick coronal slices in order to obtain punches from dorsal and ventral parts of the striatum (Bregma +3.72 and +3.30 mm). The brain areas were bilaterally punched out with a Miltex 1.0 mm biopsy puncher (Miltex Inc., York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at −80°C.

RNA extraction. Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad, Hercules, USA), according to manufacturer’s instructions. RNA concentrations were measured and RNA purity checked (A260/280 ratio between 1.8 and 2.0) with a NanoDrop 1000 spectrophotometer.
Subsequently, an aliquot of each sample was treated with DNase to avoid DNA contamination to perform gene expression analyses as previously reported (Rossetti et al., 2016).

**Real time qRT-PCR.** The mRNA levels of total BDNF, D1 and D2 dopamine receptors were analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l) using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicate as multiplexed reactions with a normalizing internal control (β-actin). The following primer and probe sequences were used; 

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Probe (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bdnf</td>
<td>5’ AAGTCTGCATTACATTCTCGA-3’</td>
<td>5’ GTTTTCTGAAAGAGGGA-CAGTTTAT -3’</td>
<td>5’ TGTGTTTTGCGTGGCAAG -3’</td>
</tr>
<tr>
<td>D1</td>
<td>5’ ATACGTCTCGCAACCTTG-3’</td>
<td>5’ ACAGTTGCTCACGGTCTCCTC -3’</td>
<td>5’ ACCACTCAAGGGCAACTG -3’</td>
</tr>
<tr>
<td>D2</td>
<td>5’ ACCGATGGGCTACGTACATGG -3’</td>
<td>5’ CACCTCAAGGGCAACTG -3’</td>
<td>5’ CACTTTCTACAATGAGCTCGG -3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’ TGTGTTTTGCGTGGCAAG -3’</td>
<td>5’ CAGTTTCTACAATGAGCTCGG -3’</td>
<td>5’ CTGGATGGGCTACGTACATGG -3’</td>
</tr>
</tbody>
</table>

Thermal cycling was initiated with a 10-min incubation at 50°C during which the reverse transcription of RNA to cDNA took place (RNA retro-transcription), followed by 5 min at 95°C (TaqMan polymerase activation) and 39 amplification cycles with 10s at 95°C and 30s at 60°C. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression versus the control group. Specifically, relative target gene expression was calculated according to the 2^−ΔCt method (Schmittgen and Livak, 2008).

**Statistical analyses**

Two-way repeated measures ANOVAs were used to analyze all behavioral parameters of the social interaction test. The pairs were the statistical unit, with N=24 per genotype resulting in 12 pairs of animals per genotype that were analyzed using age as within-subject factor and genotype as between-subject factor. Bonferroni post-hoc test was used to follow up any significant effects of genotype found in the two-way ANOVAs. The three chamber social test was analyzed with two-way ANOVA, followed by Tukey or Sidak post-hoc test when appropriate. One WT individual was identified as outlier based on the standard deviation of the mean and the residuals of the statistical model and therefore excluded from the statistical analyses. The real time RT-PCR 2^−ΔCt data have been normalized to the average of the wild-type group and have been analyzed using an unpaired t student’s test.

Statistical analyses for the social interaction test and the RT-PCR were conducted using GraphPad Prism v.6.0 (GraphPad Software Inc., San Diego, USA) while analyses of three chamber social test parameters were performed using GraphPad Prism v.7.0. Statistical significance was set at p< 0.05 in all tests.
Results

Behavioral experiments

Experiment 1: social interaction test

Animals of both genotypes showed a significant increase of time spent on “nape attacking” between 2 and 8 months of age (age effect: $F_{(2, 22)} = 7.310, p=0.0037$). When compared to their WT littermates, BACHD animals spent an increased time spent on “nape attacking” showing significant genotype (8 months: $p<0.0001$) and age x genotype ($F_{(2, 22)} = 3.922, p=0.0349$) effects (Figure 2A). Conversely, transgenic animals exhibited a decreased time performing “pinning” between 2 and 8 months of age, showing a significant genotype (2 months $p<0.01$), age ($F(2, 22) = 10.07, p=0.0008$) and age x genotype effects ($F_{(2, 22)} = 4.733, p=0.0195$) (Figure 2B).

“Social nose contact” (sniffing) is the most frequently used parameter to define the interest of an animal in another animal (Himmler et al., 2013). The duration of “social nose contact” increased in both WT and BACHD between 2 and 4 months of age while it remained stable between 4 and 8 months of age, showing a significant age ($F_{(2, 22)} = 65.78, p<0.0001$) effect (Figure 2C). At the three different ages BACHD rats spent significantly less time on “social nose contact” compared to WT rats (genotype effect ($F_{(1,11)} = 6.281, p=0.0292$).

After an initial increase of “allogrooming” in BACHD rats at 2 months of age (post-hoc analyses $p<0.001$) (Figure 2D), with increasing age they exhibited a trend to groom each other less compared to the control rats, showing significant genotype x age effect ($F_{(2, 22)} = 11.79, p=0.0003$). Post-hoc analyses of “approaching” revealed a significant genotype effect in BACHD rats aged 4 and 8 months (Figure 2E), indicating that transgenic rats showed less “approaching” behavior (post-hoc analyses at 4 and 8 months: $p<0.05$). Interestingly, both the “approaching” and “allogrooming” decreased in both BACHD and WT rats between 4 and 8 months of age (significant age effect for approaching ($F_{(2, 22)} = 14.02, p=0.0001$) and for allogrooming ($F_{(2, 22)} =53.28, p<0.0001$). Conversely, the “following” significantly decreased in both groups of animals between 2 and 8 months of age ($F_{(2, 22)} = 55.02, p<0.0001$) and post-hoc analyses showed significant reduction in the time spent on “following” of 2 months old BACHD rats compared to WT (2 months: $p<0.05$) (Figure 2F). BACHD rats seemed to spend less time on following the partner compared to the control group. The “moving away” parameter did not show any significant differences whatsoever (Figure 2G). Concerning non-social behavior, BACHD rats exhibited augmented “solitary” behavior. A significant interaction between age and genotype was found ($F_{(2, 22)} = 8.571, p=0.0018$) (Figure 2H), although a significant genotype effect was only present at 4 months of age (post-hoc analyses $p<0.05$). We further performed a reliability analysis of the present results, and the outcome reported in the Supporting Information (S1 table).
Figure 2  Social interaction test.

(A) Nape Attacking.  (B) Pinning.  (C) Social Nose Contact.  (D) Following.  (E) Approaching.  (F) Allogrooming.  (G) Moving Away.  (H) Solitary. Data are expressed as means + S.E.M. Two-way ANOVA results are displayed above each graph. Results from post-hoc analysis are indicated on the graph in case significant genotype differences were found. N=12 pairs of WT and 12 pairs of BACHD rats.
Experiment 2: three chamber social test

In the habituation phase WT and transgenic animals spent a comparable length of time in the left and right arenas and exploring the empty boxes (Figures 3A, 3B). Within each genotype, no significant preference was detected for left or right arena and the respective empty boxes. Two-way ANOVA showed an arena effect ($F_{(2,87)} = 51.92, p<0.0001$) and no genotype or arena x genotype interaction. Tuckey test revealed only a difference in time spent exploring the neutral arena compared to the lateral compartments (both $p<0.0001$), but no differences between left and right side ($p=0.2281$).

In the social interaction phase, when a conspecific rat was introduced in one of the boxes, both genotypes showed a comparable preference for the social arena and box with conspecific relative to non-social arena and empty box, respectively (Figures 3C, 3D). Two-way ANOVA in this phase showed an arena effect ($F_{(2,87)} = 405.8, p<0.0001$) and an arena x genotype interaction ($F_{(2,87)} = 3.175, p=0.0467$), but no genotype differences ($F_{(2,87)} = 3.719e^{-007}, p=0.9995$). Post-hoc test indicated a preference for the social arena versus both neutral and empty arenas in both genotypes (all $p<0.0001$). ANOVA analyses showed also a box effect ($F_{(1,58)} = 268.6, p<0.0001$), but no genotype ($F_{(1,58)} = 0.2843, p=0.5959$) or box x genotype interaction ($F_{(1,58)} = 2.216, p=0.1420$).

In the social novelty phase, when a novel conspecific was introduced in the previously empty arena, transgenic rats spent a comparable amount of time in the familiar and novel arenas and exploring the boxes with familiar and novel conspecifics, while WT rats stayed longer in the novel than in the familiar arena and showed higher interest in the novel conspecific relative to the familiar one (Figures 3E and 3F). Two-way ANOVA analyses showed an arena effect ($F_{(2,87)} = 36.83, p<0.0001$) and an arena x genotype interaction ($F_{(2,87)} = 4.109, p<0.05$), but no genotype differences ($F_{(2,87)} = 2.549e^{-007}, p=0.9996$). Similarly, ANOVA analyses showed a box effect ($F_{(1,58)} = 4.4665, p=0.0349$) and a box x genotype interaction ($F_{(1,58)} = 4.484, p=0.0385$), but no genotype effects ($F_{(1,58)} = 0.1332, p=0.7164$). Post-hoc analyses revealed a significant difference in time spent between novel arena and neutral arena in both genotypes (both $p<0.0001$) and a difference in time spent between novel arena and familiar arena in WT ($p=0.0028$), but not in transgenic animals ($p=0.9390$). In line with the arena effects, Sidak test also indicated a significant difference in time spent exploring the novel and familiar conspecific in WT ($p=0.0064$) and not in transgenic animals ($p=0.9995$). We further performed a reliability analysis of the present results, and the outcome reported in the Supporting Information (S2 table).

Molecular analysis

Gene expression analysis of dopamine D1 and D2 receptors in the striatum

mRNA levels of D1 and D2 dopamine receptors have been measured in the ventral and dorsal striatum of BACHD rats to evaluate their potential involvement in the previously observed behavioral alterations. However, as shown in Figure 4, we did not find any significant difference in the gene expression of both receptors in the mutant animals in
**Figure 3** Three chamber social test.

The figure shows the time spent in the arenas (A, C, E) and exploring the conspecifics (boxes) (B, D, F) in the three test phases. Data are expressed as means + S.E.M. The p values obtained from the two-way ANOVA analyses, are displayed above each graph. The p values resulting from post-hoc analyses are indicated on the graph for significant differences between genotypes as well as for significant differences among arenas and between boxes within genotype. N=16 WT and 15 BACHD. Abbreviation: Ar. = Arena.
comparison with wild-type rats, neither in the ventral (D1: WT 1 ± 0.11, N=11; BACHD 0.97 ± 0.11, N=9; t(18)= 0.1947, \(p=0.8478\). D2: WT 1 ± 0.09, N=11; BACHD 1.01 ± 0.16, N=9; t(18)= 0.045, \(p=0.9649\)) nor in the dorsal striatum (D1: WT 1 ± 0.081, N=9; BACHD 0.87 ± 0.06, N=10; t(18)= 1.304, \(p=0.2096\). D2: WT 1 ± 0.1, N=11; BACHD 1.15 ± 0.09, N=12; t(21)= 1.175, \(p=0.2531\)).

**Gene expression analysis of BDNF in the striatum**

Alterations of the neurotrophin Brain-derived neurotrophic factor (BDNF) are thought to be relevant for the neurodegeneration observed in Huntington’s disease (Zuccato and Cattaneo, 2007). Therefore, we evaluated the mRNA levels of the total form of BDNF in both ventral and dorsal striatum of BACHD rats with respect to wild-type animals. As shown in Figure 5, we observed a significant and marked decrease of the mRNA levels of the total form of BDNF in the ventral striatum of BACHD rats compared to WT littermates (WT 1 ± 0.21, N=9; BACHD 0.31 ± 0.1050, N=8; t(15)= 2.846, \(p<0.01\)). Conversely, the expression of the neurotrophin was not modulated by the genotype in the dorsal striatum (WT 1 ± 0.14, N=10; BACHD 1.33 ± 0.15 N=10; t(18)= 1.601, \(p=0.1269\)).

**Figure 4** mRNA levels of D1 (A) and D2 (B) receptors in the ventral striatum and mRNA levels of D1 (C) and D2 (D) receptors in the dorsal striatum of WT and BACHD rats.

Data were normalized to the average of the fold changes in the WT group (set at 1.0) and analyzed using unpaired \(t\) student’s tests. Abbreviations: vST = ventral striatum; dST = dorsal striatum.
Discussion

The use of multiple social paradigms permitted us to detect potential HD-related deficits in different specific aspects of social behavior. The social interaction test revealed abnormal social play and aggressive behavior in BACHD animals as well as a trend towards a decreased interaction with conspecifics. It is worth mentioning the fact that we decided to use familiar pairs (cagemates) to have a better translational value of the results since HD causes major disruption in family life (Vamos et al., 2017). The three chamber social test showed mild deficits in social recognition in transgenic animals, providing an estimation of the social interest towards an unknown conspecific as well as of the recognition abilities between familiar and novel conspecifics, with limited physical interaction. Additionally, striatal expression of D1, D2 receptors and BDNF was assessed and related to social contact and social interest in 8 month old animals, reporting a decrease in the expression of BDNF in the ventral striatum and intact dopamine receptor expression.

In rats, one of the most notable social behaviors is play fighting, which involves attack and defense of the nape of the neck, which if contacted, is gently nuzzled with the snout (Himmler et al., 2013). When initiating social play, one animal directs to the neck region of the partner and this can be accompanied with biting and pulling fur in that region. BACHD rats performed more play-fighting compared to their WT littermates, as showed by the increased nape attacking. 8 month old BACHD rats showed a twofold increase in the time spent on nape attacks, and a decreased duration of pinning compared with control rats. Pinning is a commonly used measure for play, which essentially involves the subject positioned supine with its partner standing on top (Himmler et al., 2013; Panksepp, 1981).

Figure 5  mRNA levels of total BDNF in the ventral (A) and dorsal (B) striatum of WT and BACHD rats.

Data were normalized to the average of the fold changes in the WT group (set at 1.0). Data were analyzed using unpaired t student’s tests. Abbreviations: vST = ventral striatum; dST = dorsal striatum.
These rougher and more aggressive play behaviors likely reflect abnormal social play behaviors and might facilitate the development of social and aggressive behaviors (Veenema and Neumann, 2009). Conversely, “following” significantly decreased in both groups of animals between 2 and 8 months of age, suggesting a reduced inclination to search for an interaction over time. The lack of these social behaviors in BACHD rats may suggest a “social deficit” that could be related to coping styles (Van Den Berg et al., 1999; Varlinskaya and Spear, 2008), and considered as a form of apathy (Wood and Morton, 2015) as showed by the R6/2 mouse model of HD.

Although we cannot directly confirm apathy with the parameters we measured, apathy has been previously reported in the BACHD and in z_Q175 mouse models (Oakeshott et al., 2012) and is commonly reported in patients with HD (Naarding et al., 2009). Future studies on other specific aspects of social behavior, e.g., social reward, will be important to define whether or not a social apathy-like phenotype is present in BACHD rats. Alternatively, the deficits in social behaviors described in BACHD rats may represent behavioral changes that depend more specifically on alterations in brain social networks which mechanisms in HD are still mostly unclear.

Interestingly, at 2 months of age the BACHD rats “nape attacking” was significantly increased as well as “pinning”, where we can assume that the rats established the dominance hierarchy, leading to a dominant and a subordinate rat. This disappeared at 4 and 8 months of age, probably because the dominance was already determined.

The lower approaching and following behaviors observed in this study are not likely to be affected by anxiety levels in BACHD rats, as previous studies in this model showed decreased exploratory anxious behavior in the elevated plus maze starting at 4 months of age (Yu-Taeger et al., 2012). If we consider the time spent on nape attacks related to social dominance and dominance to be dependent on anxiety levels (Hollis et al., 2015) with dominant individuals being less anxious than subdominant ones, than the increase in nape attacking in BACHD rats could be facilitated by the lower anxiety levels in these animals. However, one should keep in mind that the decreased anxious-like behavior in BACHD rats was shown only with the elevated plus maze (Yu-Taeger et al., 2012). It is therefore difficult to conclude anything about the anxious phenotype in BACHD rats and to relate it to social behavior parameters.

It is worth noting that these social behavior alterations were not confounded by an overall reduced motor activity, such as levels of general exploration or activity, as BACHD animals reported similar levels of locomotion compared to their wild-type littermates in our previous study (Manfré et al., 2017). Furthermore, during all other social tests, transgenic animals did not differ from wild-types in the expression of non-social activities.

Besides the changes in social interaction parameters, we also detected a mild deficit in social novelty in transgenic animals at 7 months of age, as suggested by a lack of preference for a novel conspecific relative to a familiar one in the three chamber social test. Cognitive deficits have already been described in BACHD rats for different aspects.
not related to social behavior (Abada et al., 2013a; Clemensson et al., 2017; Manfré et al., 2016). The novelty effect in our study is in line with earlier research in HD mouse models showing alterations in social recognition and memory (Ciamei and Morton, 2008; Rudenko et al., 2010) as well as with evidence of disrupted social cognition in HD patients (Larsen et al., 2016; Snowden et al., 2003). The brain changes underlying these symptoms in HD are not well known and it is not clear to which extent deficits in social cognition, mostly described as impaired emotion recognition, may depend on other cognitive and emotional changes and whether they could be the cause of social behavior alterations in HD patients.

While decreased or increased social interaction (Nguyen et al., 2006; Pietropaolo et al., 2011; Wood and Morton, 2015) have been described in other animal models of HD using different behavioral paradigms, we did not observe altered sociability levels in BACHD rats exposed to the three chamber social test. In line with these findings, one recent study showed no changes in the preference for an unknown conspecific relative to an unknown object in BACHD rats (Lamirault et al., 2017). Although unchanged levels of social interaction in the three chamber social test are present, they are not necessarily in contrast with parameters measured in the social interaction test within this study. For example, increased approaching in the social interaction test is present along with an increased nape attacking which may relate to a play fighting-like behavior that is prevented in the other test where limited physical contact is allowed.

Gene expression analyses were carried out in 8 month old animals tested for social interaction, highlighting an altered expression of BDNF in ventral striatum and an intact striatal D1 and D2 receptors expression. The striatum is one of the most affected brain areas in HD where alterations in dopaminergic and BDNF brain systems have been well described previously (Cepeda et al., 2014; Zuccato and Cattaneo, 2007). mRNA levels of striatal BDNF and dopaminergic receptors were therefore examined as potential mechanisms at the base of behavioral abnormalities. Furthermore, we linked social behavior to striatal functionality via D1 and D2 dopamine receptors in 8 month old animals which, along with BDNF, have been proposed as possible underlying regulatory factors (Báez-Mendoza and Schultz, 2013). The lack of changes in mRNA levels of D1 and D2 dopamine receptors in BACHD rats subjected to the social interaction test would not suggest a direct link between the dopaminergic system and the behavioral changes. Measuring protein levels in future social interaction studies may help to understand better whether the dopaminergic system plays a role in the behavioral changes. BDNF mRNA levels were also quantified. We detected a decreased expression in the ventral striatum of BACHD animals and no changes in the dorsal region. Therefore, it is possible that altered BDNF expression in this region may have contributed to the deficits in sociability in 8 months old BACHD rats. Nonetheless, we are aware that the limit of molecular investigations is the restriction of the analyses to the transcript levels. Striatal BDNF transcripts are very low at basal level and usually BDNF protein is not directly transcribed.
in the striatum, but it is produced in the cerebral cortex and anterogradely transported to the striatum (Zuccato et al., 2010). Therefore, we cannot draw any conclusion on the mechanisms underlying the BDNF decrease and its link to social deficits in BACHD rats and it will be assessed in future experiments. Alternative mechanisms that could have contributed to the social deficits and are worthwhile to assess in future studies are the oxytocinergic and vasopressinergic systems, which are interesting in the context of their involvement in social recognition and social behavior (Báez-Mendoza and Schultz, 2013).

The strength of our study is that we performed two different behavior experiments to investigate different aspects of BACHD rats’ social behavior: the social interaction test was performed longitudinally to make the monitoring of behavior across different ages possible; the three chamber social test provided an estimation of the social interest towards an unknown conspecific as well as of the recognition abilities between familiar and novel conspecifics. Additionally, two different observer coded the behaviors in both social tests, strengthening the reliability of our results. Accordingly, the correspondence is in line with inter-observer agreement, i.e., generally acceptable to a level of 70–85% (Martin and Bateson, 2007). On the other hand, we are aware that animals of both experiments had different housing conditions and applying the same housing conditions in the two experiments would have improved the study design since they can affect animals’ behavior. However, this was not the main goal of this chosen setup, and we do not consider our results incompatible, since we should bear in mind that: 1) this study assesses different aspects of social behavior in the same animal model and does not present a direct comparison of the same parameters obtained with two different tests; 2) we do not make any statistical correlation of the changes in parameters obtained in one test with those shown in the other test; 3) we do not think that the results shown in the social interaction test are in contrast to those in the three chamber social test. We do not know to what extent the housing conditions may have affected the results in each experiment of our study because these tests were performed for the first time in the BACHD rat model. Performing both experiments with each of the housing settings would be certainly informative about that although this was not the aim of the present study. In conclusion, this study characterizes social behavior in the BACHD rat model. We report changes in different parameters of social interaction and recognition and potential molecular correlates by using paradigms that are well established in rodents (Silverman et al., 2010; Veenema and Neumann, 2009) and that can be used to study disturbances in this spectrum (Silverman et al., 2010). Being easily measurable, such parameters provide the basis for further social behavior studies and may represent valid readouts for treatment studies.

Conflict of interest
At the time of the studies the authors G. Manfré and Dr. J.E. van der Harst were working for the EU funded “PhenoRat” project of which Noldus Information Technology was an
industrial partner. Dr. J.E. van der Harst was part-time scientific project advisor for “PhenoRat” employed by Noldus Information Technology. Dr. A. Novati was working at the University of Tuebingen for the EU funded “Switch HD” project of which QPS Austria was an industrial partner. Noldus Information Technology and QPS Austria had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. G. Manfré and Dr. J.E. van der Harst’s involvement in the current study should thus be considered the work of independent researchers, rather than representatives of a commercial company. There are no patents, products in development, or marketed products to declare. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Acknowledgments
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References


## Supporting information

### S1 Table  Percentage of scoring agreement – Social Interaction Test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage of agreement ± SEM</th>
</tr>
</thead>
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<tr>
<td>WT</td>
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<tr>
<td>BACHD</td>
<td>82.188 ± 1.042</td>
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Data are expressed as means ± S.E.M.

### S2 Table  Percentage of scoring agreement – Three Chamber Social Test.

<table>
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<th>Genotype</th>
<th>Test Phase</th>
<th>Percentage of agreement ± SEM</th>
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<td>WT</td>
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<tr>
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<td>Social Interaction</td>
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<tr>
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<td>Social Novelty</td>
<td>89.234 ± 1.096</td>
</tr>
<tr>
<td>BACHD</td>
<td>Habituation</td>
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<td></td>
<td>Social Interaction</td>
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<tr>
<td></td>
<td>Social Novelty</td>
<td>92.189 ± 1.722</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.M.
Social behavior differences in BACHD rats
Impulsivity trait in the early symptomatic BACHD transgenic rat model of Huntington Disease

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Highlights

- Different forms of impulsivity were assessed in early symptomatic BACHD rats (line TG5), a transgenic rat model of Huntington’s disease (HD).
- BACHD rats showed high levels of choice impulsivity favoring “smaller, sooner” over “larger/later” rewards (delay discounting task).
- BACHD rats also exhibited a lack of behavioral inhibition indicated by bursts and premature responses in a DRL task assessing action impulsivity.
- Our study is the first to provide evidence of deficits in impulse control in a rodent model of HD.
- These results are relevant to psychiatric alterations in early symptomatic HD patients. They increase the face-validity of the BACHD rat model.

Abstract

Impulsivity trait was characterized in 3-5 months old BACHD rats, a transgenic model of Huntington Disease, using (1) the delay discounting (DD) task to assess cognitive/choice impulsivity, and (2) the Differential Reinforcement of Low Rates of Responding (DRL) task to evaluate motor/action impulsivity. Transgenic animals showed a high level of choice impulsivity and, to a lesser extent, action impulsivity. Our results provide the first evidence that the transgenic BACHD rat line displays changes in impulsivity as early as 3 months old, as described in early symptomatic HD patients, thus adding to the face validity of the rat model.

Keywords: Huntington disease, Impulsivity, Executive functions, Decision making, Transgenic rats
Impulsiveness is a behavioral trait that refers to the tendency to engage in inappropriate or maladaptive behaviors. Impulsivity is not a unitary trait, but embraces two categories of behavior: motor or action impulsivity, defined as the inability to withhold a prepotent motor response (Neill 1976; Sokolowski and Salamone 1994; Uslaner and Robinson 2006), and cognitive or choice impulsivity, which is the inability to weigh and/or to use all consequences of events (Evenden 1999; Arce & Santisteban, 2006). Impulsivity, poor risk assessment and altered behavioral inhibition are frequently encountered personality traits in many neuropsychiatric disorders (Hamilton et al., 2003; Duff et al., 2007) and in neurodegenerative diseases such as Parkinson’s (Antonini et al., 2011; Bugalho & Oliveira-Maia, 2012; Weintraub et al, 2015) and Huntingdon’s diseases (HD; Beglinger et al, 2008; Kalkhoven et al, 2014).

HD is an autosomal dominantly inherited, progressive neurodegenerative disorder caused by an expansion of the polyglutamine repeat of variable length (>38 CAG repeats) in exon 1 of the gene encoding the protein huntingtin (HTT) (Huntington et al., 1993). HD results first in neurodegeneration of medium spiny neurons of the striatum (Vonsattel et al. 1985), and in atrophy of cortical and limbic structures as the disease progresses (Rosas et al. 2005, 2008). Cognitive/psychiatric disorders, including perseveration, lack of insight, distractibility, and impulsivity, affect HD patients before motor symptoms, (Novak & Tabrizi 2010; Rosenblatt, 2007) and have thus an early deleterious impact on the quality of HD patient’s life (Guttman et al., 2003; Rosenblatt, 2007).

Modeling the complexity of the psychiatric symptoms of pre-clinical HD in a rodent model is therefore important for increasing our understanding of the neurobiological mechanisms underlying the HD neuropathology. However, certain behavioral processes (e.g. impulsivity trait) are typically more challenging to evaluate in mice (Tecott & Nestler 2004; Wistantley et al, 2011). Therefore, we aimed at investigating impulse control disorders in a recently generated transgenic rat model of HD, using a human bacterial artificial chromosome (BAC) which contains the full-length HTT genomic sequence with 97 CAG/CAA repeats and all regulatory elements (Yu-Taeger et al. 2012). Primary characterization of BACHD rats demonstrated an early progressive HD-like phenotype with motor dyscontrol and emotional impairment (Yu-Taeger et al., 2012; Abada et al, 2013). To determine impulsivity traits in these rats, we used two different paradigms, the delay discounting task (DD), and the Differential Reinforcement of Low Rates of Responding task (DRL), to assess respectively choice and action impulsivity. Animals were food deprived at 85% of their normal weight and all experiments were performed in accordance with the recommendations of the EEC and the French Ethics Committee for compliance and use of laboratory animals.

Impulsivity in the DD task is characterized by a preference for small, immediate rewards (SS: smaller, sooner) over larger, delayed rewards (LL: larger, later). The task design was modified from Simon et al. (2013) and run in operant chambers with 2 levers each side of a food magazine (Coulbourn Instruments, USA). Three months old naive male WT
(n=16) and BACHD (n=7) rats were trained to lever-press for food on a daily schedule. A 30 min-session of magazine training with 30 pellets (45 mg food pellets, Bioserv) delivered using a VI60 (range from 30 to 90s) was followed by lever-press sessions under a continuous reinforcement schedule (CRF) until 50 reinforcements were earned in 30 minutes, one session for each lever (Coulbourn Instruments, USA). The animals were then trained for 8 sessions to discriminate between a small (1 pellet) and a large (3 pellets) reward associated with the left or the right lever (counterbalanced between rats). Five blocks of 12 trials (2 forced choices and 10 free choices) were run during each session. Each 60-s trial began with a 10-s illumination of the food magazine during which a nose poke into the magazine extinguished the light and triggered extension of either a single lever (forced-choice trials) or both levers simultaneously (free-choice trials) for a maximum of 10 s. Once a lever was pressed and food delivered, both levers were retracted for the remainder of the trial. At the end of training, WT and BACHD animals chose the large reward in more than 90% of free trials, with no genotype difference or genotype x training session interaction (Fs<2.33, ns). Animals were then trained for 12 sessions in the delay discounting task. Each session and trial organization was kept the same except that delays were introduced between lever pressing and the large reward delivery. Each block started with two forced-choice trials to expose the rats with the delays in effect for that block, followed by 10 free-choice trials. The delay duration increased between each block of trials (0, 4, 8, 16, 32 seconds), but remained constant within each block. The averaged choice for the large reward (LL choice) was calculated for each delay across all blocks of the 12 sessions. Contrast analyses of variance (ANOVAs) using VAR3 statistical software (Rouanet et al., 1990) as well as the Fisher Exact Test, with an alpha level of 0.05, were used for statistical assessments. As the delays increased, BACHD and WT rats shifted progressively their choices to the immediate small reward (Figure 1A; F(4,84) = 65.03, P<.001). BACHD rats shifted more rapidly to the immediate small reward than WT rats (genotype: F (1,21)= 6.20, P<.05; genotype x delay interaction: F(4,84)= 5.01, P<.01). The higher proportion of impulsive rats (less than 50% choices for the large delayed reward) in BACHD compared to WT animals was confirmed for delays longer than 8s (Figure 1B).

Impulsivity in the DRL task is characterized by the inability to withhold responses for a required amount of time. If the time between two responses (inter-response time, IRT) is less than 5 seconds, no reward is delivered and the timing contingency reset. WT (n=12) and BACHD (n=11), 5 months old, male naive rats were trained on magazine training (30 min, 30 pellets), and on a CRF session until a criterion of 50 reinforced lever presses earned in 30 minutes. Then, rats started a DRL-5s schedule for five sessions, during which a lever press resulted in a food pellet delivery only if at least 5 seconds had elapsed from the previous lever press. If the rat performed a premature lever press, the 5 s time period was reset. Animals were then trained for ten sessions on a DRL-10s schedule, during which the response had to be withheld for 10 seconds to obtain the reward. Each DRL session ended either after 60 minutes or 200 reinforcements were earned, whichever came first. Several
indexes of performance were calculated and the results are presented in Figure 2, for 5 sessions of DRL-5s (left panel) and 10 sessions of DRL-10s (right panel). First, the efficiency/impulsivity measured the ratio of correct/rewarded responses (>5s or >10s) to total responses. Then, inter-response times (IRTs) were classified according to a ratio of the DRL value as burst responses (responses during 0-1s for DRL-5s and 0-2s for DRL-10s), premature responses (1-4s and 2-8s for DRL-5s and -10s, respectively) and timing error (4-5s and 8-10s for DRL-5s and -10s).

During the DRL-5s, WT rats learnt the task and showed improvement of their efficiency across sessions (Figure 2A; $F_{(4,44)} = 13.02$, $P<.001$) whereas BACHD rats did not ($F_{(4,40)} =$

Figure 1  Delay-discounting task showing higher choice impulsivity in BACHD rats compared to WT.

(A) Mean + S.E.M percentage of choice of delayed reward across different delays. Genotype significant difference is indicated by (*) $P = 0.05$. Significant interaction is indicated by (#) $P<0.01$. (B) Percentage of high impulsive (<50% choice of large reward choices) animals for the different delays (Fisher exact-tests: delay 8s, $P = 0.022$; delay 16s, $P = 0.026$; delay 32s, $P = 0.02$).
2.23, ns); there was a significant genotype x session interaction ($F_{(4,84)} = 4.46, P<.005$) and no genotype difference ($F<1$). The number of bursts responses decreased for both groups with session repetition (Figure 2C; $F_{(4,84)} = 12.99, P<.001$) with no group x session interaction ($F_{(4,84)} = 2.16, ns$) and no genotype difference ($F<1$). Both groups decreased their number of premature responses across sessions (Figure 2E; ($F_{(4,44)} = 19.78, P<.001$ and $F_{(4,40)} = 2.94, p<.05$ respectively) with no genotype difference ($F<1$). However, a significant genotype x session interaction ($F_{(4,84)} = 8.79, p<.001$) indicated that WT rats decreased their premature responses more rapidly than BACHD rats. Finally, BACHD rats increased more their timing errors than WT rats (Figure 2G; $F_{(1,21)} = 4.23, P=.05$) with a significant genotype x session interaction ($F_{(4,84)} = 3.54, P<.05$).

When switching to the DRL-10s, both groups initially dropped their efficiency to then improve over the 10 training sessions (Figure 2B; WT: $F_{(9,99)} = 21.32, p<.001$; BACHD: $F_{(9,90)} = 8.02, p<.001$), with no significant interaction between genotype and session ($F_{(9,189)} = 1.49, ns$) and no genotype effect ($F_{(1,21)} = 1.35, ns$). The number of bursts responses decreased with session repetition (Figure 2H; $F_{(9,189)} = 9.44, P<.001$). However, BACHD transgenic rats exhibited a higher rate of burst responding compared to WT ($F_{(1,21)} = 4.53, P<.05$), with no genotype x session interaction ($F_{(9,189)} = 1.43, ns$). The number of premature responses decreased also significantly (Figure 2F; $F_{(9,189)} = 66.12, P<.001$), but with no group difference and no genotype x session interaction ($F<1$). There was a slight evolution of timing errors for both groups (Figure 2D; $F_{(9,99)} = 2.24, p<.05$ and $F_{(9,90)} = 3.66, p<.01$ respectively), with no genotype difference ($F<1$) and no genotype x session interaction ($F_{(9,189)} = 1.76, ns$). Taken together, these results indicate that BACHD rats were nearly as efficient as WT in completing the DRL-5s and -10s tasks, with a less precise temporal discrimination and a higher rate of burst responses during the very first seconds following a reward.

These results demonstrate that HD pathology in 3 to 5 months old BACHD rats induce a high level of choice impulsivity and, to a lesser extent, action impulsivity. The DD paradigm describes primarily the devaluation of an event as the delay to that event increases; the maintained bias toward larger delayed gratification has been taken to index increased self-control (Isles et al. 2004). Indeed, most decision-making procedures confront the individuals with several alternatives differing in cost and benefit. The increment of costs for the usually more-preferred larger reward leads to a discounting in the value of this option. The DRL paradigm describes the capacity to withhold actions during a fixed amount of time and mostly involves two cognitive/behavioral abilities: (1) behavioral inhibition or self-control (Barkley, 1997) indicated by bursts and premature responses. Burst responses, which follow immediately a rewarded lever-press, indicate perseverative responses induced by the failure to re-obtain an immediate feedback to their responses; (2) the temporal discrimination ability, which allows to know when the time $t$ has elapsed (Kramer and Rilling, 1970), indicated by timing errors. BACHD rats exhibited a steep delay discounting curve in the DD showing a high level of cognitive/choice impulsivity. On the
Figure 2 Temporal characteristics of responding at DRL-5s (left panels) and -10s (right panels) schedules.

(A,B) Percent efficiency (reinforced lever presses/total lever presses x 100) across DRL schedules; (C,D) Burst responses are those occurring <1s (DRL-5s) and <2s (DRL-10s) since the previous response; (E,F) Premature responses are those occurring 1-4s (DRL-5s) and 2-8s (DRL-10s) since the previous response; (G,H) Timing-error responses occurring at 4-5s (DRL-5s) and 8-10s (DRL-10s) since the previous response. Data are expressed as means + S.E.M. Genotype significant differences are indicated by (*) P < 0.05. Significant interactions are indicated by (#) P<0.05.
other hand, they show a nearly normal efficiency in the DRL, but timing errors and the high rate of burst responses when the delay increased indicate signs of motor impulsivity. These results could reflect an impairment in timing and/or working memory. In fact, both procedures (DD and DRL) are dependent upon limits on memory and sustained attention abilities. Deficits of these functions alter the ability to analyze all information about the possible alternatives and thus difficulties to evaluate future options, breaking the possibility to anticipate the consequences of actions and leading to incapacity to plan the most efficient behavior. Working memory deficits have previously been demonstrated in different tasks in early symptomatic HD patients (Harrington et al, 2014; You et al, 2014; Georgiou-Karistianis et al, 2013; Stout et al, 2011). However, the inability of BACHD rats to resist immediate rewards and/or to wait for larger rewards in DD rather suggests an impaired decision process, possibly due to the failure of the autonomous nervous system to mark negative outcomes (Campbell et al, 2004) or to process correctly the cue/outcome contingencies through trial-to-trial feedback processing (Holl et al, 2012).

At the neurobiological level, lack of inhibitory control has been observed in several neuropathological disorders associated with abnormal dopaminergic transmission (deficit/hyperactivity disorder, Parkinson’s Disease, Alzheimer’s Disease and HD (Barkley, 1997; Rosenblatt, 2007; Rochat et al., 2008; Novak & Tabrizi 2010; Nombela et al., 2014)). Furthermore, there is a strong correlation between dopamine receptors availability and the two different types of impulsivity: variation in striatal D2/D3 receptor levels is negatively correlated with impulsive action in rodents (Dalley et al., 2007; Laughlin et al. 2011) and with impulsive choice and action in humans (Lee et al., 2009; Ghahremani et al., 2012), whereas D1-like receptors appear to be most selectively involved in mediating impulsive choice in rats which may be explained by their differing locations within the prefrontal cortex (PFC), an area known to be involved in impulsive choice (Loos et al., 2010; Koffarnus et al, 2011). In BACHD rats, a decreased D2 receptor binding potential has been observed in the striatum, as well as an imbalance between the striatal striosome and matrix compartments (Yu-Taeger et al. 2012). Since these two compartments contain disproportionate amounts of D1 and D2 neurons, the striosome/matrix imbalance may influence the equilibrium of the inhibitory and excitatory output from the striatum to downstream neurons and thus have a behavioral impact that could account for BACHD rats’ impulsivity. Furthermore, impulsivity, as well as compulsivity and flexibility, are closely interrelated executive processes in the context of inhibitory control mediated by the PFC (Bari & Robbins, 2013; Wise, 2008). However, according to the model proposed by Bechara in 2005, willpower emerges from the dynamic interaction of two separate, but interacting, neural systems: an impulsive system, in which the amygdala is a critical neural structure involved in triggering the affective and emotional signals of immediate outcomes, and a reflective system, in which the ventral PFC is crucial in triggering the affective and emotional signals of long-term outcomes. Using a delay discounting paradigm, rats with inactivation and disconnection of the medial PFC and basolateral amygdala become
more impulsive, showing preference for smaller immediate over larger delayed rewards (Churchwell et al, 2009). Similarly, rats with amygdala lesions (basolateral nuclei) are more impulsive when rewards are delayed, preferring the immediate reward more quickly than controls (Winstanley et al., 2004; Cardinal, 2006; Churchwell et al., 2009; Ghods-Sharifi et al., 2009). In HD patients, both the prefronto-striatal circuit and the amygdala system seem to be dysfunctional (Thieben et al., 2002; Rosas et al., 2003; Douaud et al., 2006). In BACHD rats from 3 months of age, a high expression of mutant huntingtin (mhtt) aggregates was found in the neocortex and in limbic areas including the amygdala (Yu-Taeger et al, 2012). A dysfunctional prefronto-striatal/amygdala imbalance could thus account for the high level of choice impulsivity observed in BACHD rats, giving rise to behavioral inadaptability.

In sum, given the differences observed between WT and BACHD rats, it can be assumed that this transgenic rat model recapitulates some of the cognitive/psychiatric impairments already seen in HD patients. To our knowledge, our study is the first to provide evidence of deficits in impulse control in a rodent model of HD. This gap is surprising, due to the relevance of psychiatric alterations in the initial phases of HD and their deleterious impact on the quality of HD patient’s life (Guttman et al. 2003; Rosenblatt, 2007), but may be due to a lack of models until now that would permit testing of self-control. Taken together, our results indicate that early symptomatic BACHD rats express a high level of impulsivity, impairments described in early symptomatic HD patients, which makes this rat model highly suitable for drug discovery purposes.

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References


Impulsivity trait in BACHD rats


Measuring anxiety-like behavior in the BACHD rat model of Huntington Disease: classical and automated phenotyping

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In preparation
Abstract

Background: Huntington disease (HD) is a progressive neurodegenerative disorder characterized by motor, cognitive and neuropsychiatric symptoms. Although anxiety has been under-researched in HD, it is recognized as a common feature of this disorder and has an early deleterious impact on the quality of HD patient’s life. The BACHD transgenic rat is the newest rat model for HD and its psychiatric phenotype has not been fully determined yet.

Objective: The present study aims to characterize risk assessment and anxiety-like traits in BACHD rats, combining classical paradigms with an automated tool for the automatic assessment of anxiety within the home cage, the light spot test (LS).

Methods: Risk assessment and anxiety-like traits were characterized in 2–4–8–12 month-old wild-type and transgenic BACHD rats. To measure anxiety and risk assessment in a novel environment, rats were challenged with classical tests: the elevated plus maze and the light-dark box. Home-cage anxiety-like responses were assessed in an automated home-cage environment, the PhenoType®, applying an adverse stimulus (‘light spot’) directed at the food-hopper during a part of the dark-(active) phase.

Results: BACHD rats did not show any changes in anxiety-like behavior or risk assessment in the elevated plus maze and the light-dark box. However, the light spot test indicated a decreased anxiety-like response in BACHD rats at 2, 8 and 12 months of age, as shown by an increased time spent in the feeder zone while the light spot was on.

Conclusions: Our results did not confirm anxiety-like traits over different ages in the BACHD rat model. Overall, our results do not mimic the generalized anxiety observed in HD patients.

Keywords: Huntington disease, polyglutamine disease, model characterization, anxiety, anxiety-like response, transgenic rat, neurodegenerative disorders, elevated plus maze, light-dark box, automated home-cage monitoring.
Introduction

Huntington disease (HD) is a severe autosomal dominant neurological disorder and the onset of symptoms is during middle age, consisting of a triad of motor, cognitive and psychiatric symptoms (Walker, 2007). HD motor symptoms are varied and include involuntary movements such as chorea as well as impaired voluntary movements, which cause limb incoordination and impaired hand function (Novak and Tabrizi, 2010). These symptoms are worsened by loss of postural reflexes and their pattern tends to change over time, with chorea declining and dystonia, rigidity, and bradykinesia becoming more marked (Nguyen and Cenci, 2015; Novak and Tabrizi, 2010). Typical latency from diagnosis to death is 20 years (Walker, 2007; Watt, 1990). This so-called polyglutamine (polyQ) disease is caused by an expanded CAG mutation in the huntingtin (HTT) gene (Huntington's Disease Collaborative Research Group, 1993), leading to initial atrophy and cell loss in the neostriatum (caudate nucleus and putamen in humans), then spreading to cortical areas and ultimately affecting the whole brain (Vonsattel et al., 1985). Although disease progression is currently untreatable, efforts are aimed at identifying novel symptomatic, neuroprotective and reparative treatments (Frank, 2014). Thus far, the clinical diagnosis of HD is dependent on the motor signs of the disease, involving problems with voluntary and involuntary movements. However, cognitive and psychiatric symptoms may be present many years before the onset of motor problems (Dale and van Duijn, 2015; Videnovic, 2013). The psychiatric component of the disease can involve a number of different complaints including depression, irritability, anxiety, apathy, obsessive-compulsive behaviors (OCBs), and psychosis (Craufurd et al., 2001; van Duijn et al., 2007). Although motor and cognitive symptoms worsen with time, psychiatric symptoms appear to have a more variable course (Paulsen et al., 2005) with the exception of apathy, which is more prevalent in advanced disease stages. A systematic review of psychopathology in HD identified that the prevalence of anxiety among manifest HD participants was between 34% and 61%, depending on the disease stage and the measure used (van Duijn et al., 2007).

Because of the deleterious effects on patients’ lives, studying the psychiatric disorders along with the motor symptoms at both clinical and preclinical level is very valuable. Therefore, including psychiatric symptoms in the modeling of the disease with animals is crucial for the validity of preclinical studies. However, studying the complexity of such symptoms in a fully translational way in animal models may be challenging. The BACHD rat is the latest transgenic rat model of HD expressing full-length human mutant huntingtin (mHTT) and is currently being characterized in order to understand its advantages and limitations concerning modeling of HD.

In this study we focused on anxiety as one of the non-motoric symptoms seen in HD patients, combining the standard behavioral tests elevated plus maze and light-dark box with an anxiety test implemented in the automated home cage PhenoTyper 4500 (Noldus IT, Wageningen, NL) hereafter mentioned as light spot test (LS). Anxiety in the elevated
plus maze is mostly characterized by less time spent in the open arms. In the same set-up, risk assessment, defined as movements of the snout or head out of the closed area with the rest of the body and all four paws remaining in the closed arms (Fernandes et al., 1999; File and Wardill, 1975; Kyriakou et al., 2017a; Wall and Messier, 2001), also contributes significantly to assessing a more complete anxiety-like phenotype. The light-dark box is a broad animal paradigm used in pharmacology to assess anxious behavior in rodents in response to their aversion to light (Bourin and Hascoët, 2003; Kulesskaya and Voikar, 2014; Miller et al., 2011). Variables that are measured are, typically, the percentage of time spent inside the brightly illuminated area and the number of transitions between the two compartments. The light spot test is a relatively recent tool for studying anxiety in rodents by measuring their responses when a white light spot is directed at the feeding area of a home cage environment for a certain amount of time during the animals’ dark (i.e. active) phase (Aarts et al., 2015, Kyriakou, 2017). When a light spot is directed towards the feeding area, where the rats need to go in order to eat, this negative stimulus evokes an approach-avoidance conflict: the first is an exploratory response geared at investigating stimulus change, and the second is to avoid the bright light (Godsil and Fanselow, 2004). The light spot takes advantage of the natural tendency of rodents to become active at the beginning of the dark phase including a clear peak in feeding behavior, thus creating this approach-avoidance conflict in rats.

In order to investigate disease development over time in relation to anxiety-like behavior, in the present study we focused on tasks that may reveal (subtle) disturbances in this behavioral trait related to human HD. To achieve that, we combined classical behavioral paradigms with automated home-cage observations which allow high-throughput testing. The tests were performed at four different ages (2, 4, 8 and 12 months) to assess the onset and progression of anxiety-like behavioral traits.

Material and methods

Ethical statement
All experiments reported here were approved by the Animal Ethics Committee (Dier Experimenten Commissie, RU-DEC, Nijmegen, The Netherlands) in full compliance with the European Union legislation on the use of animals for scientific purposes (Directive 2010/63/EU). All experimental procedures were carried out at Radboudumc (Nijmegen, The Netherlands) and performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, CCD, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act, WOD).
Animals
Fifteen transgenic males were supplied from a BACHD colony (TG5 line, Yu-Taeger et al., 2012) at Charles River (Wilmington, MA, USA) and an in-house breeding colony was preserved and maintained at Radboudumc (Nijmegen, The Netherlands) by cross-breeding these males with wild-type female rats (Charles River, Germany). WT and BACHD animals were maintained on a Sprague-Dawley (SD) background. The BACHD rat colony was the F1 generation of animals ordered from the original breeder that were bred in-house with regular SD wild-type females, also specifically ordered from the regular supplier. Genotyping and determination of BAC transgene integrity were performed via PCR analysis using genomic DNA extracted from ear biopsy tissue at postnatal day (PND) 21. Rats were weaned at PND 21 and test pairs were then group-housed two per cage with littermates of the same genotype and sex in a constant temperature (19.5 ± 1°C) and humidity room (55 ± 10%) with a reversed 12 h light/dark cycle with lights on/off at 8:00 P.M. /8:00 A.M.).

All experimental animals were provided food and water ad libitum, and behavioural tests were conducted during the active (dark) phase of the cycle.

Study design
24 animals (N=12/group) were divided in two groups of 6 WT and 6 BACHD for each group, and longitudinally tested at 2, 4, 8 and 12 months of age according to the following scheme. On day 1, rats were challenged with the light dark box test. On day 2 animals were housed in the PhenoTyper® 4500 cages for seven consecutive days (day 2-9) to assess anxiety with the light spot test after a 5-day habituation period. On day 9 animals were then taken out of the PhenoTyper® and tested in the elevated plus maze. All behavioral tests were performed during the dark phase and carried out by a single experimenter blind to the rats’ genotype.

Behavioral procedures
Light-dark box test
The light/dark box test was used to determine the unconditioned anxiety-like rodent behavior that is based on the innate aversion of rodents to brightly illuminated areas and the conflict between the drive to explore novel areas and the tendency to avoid the unfamiliar (neophobia) (Bourin and Hascoët, 2003). The set-up consisted of two compartments, a dark and an illuminated compartment separated by a connecting gate. Dark and light compartments were of the same size (32.5 x 32.5 x 32 cm) with different brightness conditions: 5.50 lux inside the dark compartment and circa 400 lux inside the light compartment. The rats were initially placed inside the illuminated arena, and the amount of time spent in each chamber and the number of transitions was measured over a 5 minute-session (Bourin and Hascoët, 2003). During each trial two animals were tested and videotaped in two different light/dark boxes. In order to facilitate the scoring, animals’
fur was black painted with a marker. Between trials the chambers were cleaned with 70% ethanol. The following day the rats were placed into the PhenoTypers’

Light spot (LS) test in the PhenoTyper
Rats were individually housed in PhenoTypers 4500 cages (Noldus Information Technology, Wageningen, The Netherlands) with dimensions 45 × 45 × 55 cm for a total duration of 7 days. Each cage has an opaque floor covered with cellulose-based bedding, a water bottle, a shelter at the right corner and a food dispenser. The tracking of the animals was performed by an infrared sensitive video camera installed on the top unit of each cage together with a number of infrared LEDs. The first 5 days were considered habituation days in which the rats were allowed to acclimatize to the new home-cage environment (Kyriakou et al., 2017b).

The rats were placed into the PhenoTyper the day after the light dark box test, during the dark phase. Rats were then housed into the Phenotypers for a total of seven days. After 5 days of habituation in the PhenoTyper 4500, the measurements of the 5th day were taken as a baseline for the normal activity pattern of each animal. On day 6, fifteen minutes after the start of the sixth dark phase, a bright light (the ‘light spot’, 500 lux) was automatically turned on, resulting in the illumination of the feeding dispenser. The 15-minutes delay after the start of the dark phase assured that the shift from light to dark phase and the activation of the light spot do not overlap. Thus, the time of the light beam coincides with the period during which rats are usually active in search of food (Aarts et al., 2015). After three hours the light spot switched off. Moreover, in order to minimize stress and to diminish the influence of humans on the outcome a radio was provided as background noise for the whole duration of the experiment. After the light beam, rats had one day-rest and the next day they were tested in an elevated plus maze.

Elevated plus maze
The elevated plus maze (EPM) is an anxiety test based on the animal’s aversion to open spaces and on the tendency to remain near to vertical surfaces. The test setting consisted of a plus-shaped apparatus with two open arms located perpendicular to two closed arms surrounded by 40 cm-high walls. The four arms (50 cm-long) were connected by a central area (10 x 10 cm) and elevated 50 cm above the floor. At the beginning of the test each animal was placed in the central area, facing one of the open arms. Activity was recorded for five minutes. Room was illuminated by white light of approximately 10 lux in the open arm and 2,5 lux in the closed arm. After each trial the plus maze was cleaned with 70% alcohol solution to prevent transmission of olfactory cues. Animals were videotaped using a camera placed above the maze and the number of entries into the closed and open arms as well as the entries into the central area was tracked with Ethovision 11.5 (Noldus Information Technology, Wageningen, The Netherlands). All animals were tracked with three-point detection (center of gravity, nose and tail base) in
order to have a clear location of the animal in the maze. The analysis includes the traditional anxiety-related behavior parameters measured in the EPM (time spent and number of entries in the open arms) as well as measure of risk assessment behavior (head dips in the open arms). After each testing phase the animals were returned to their home-cages.

**Statistical Analyses**
All statistical analyses were conducted using GraphPad Prism v.6.0 (GraphPad Software, San Diego California USA, http://www.graphpad.com). Two-way repeated measures ANOVAs were used to analyze all parameters. Age was used as within-subject factor, and genotype as between-subject factor. Bonferroni post-hoc test was used to follow up any significant effect of genotype found in the two-way ANOVAs. A \( p \)-value < 0.05 was considered statistically significant.

Between two testing periods, two WT rats had to be sacrificed and removed from the experiment at 11 months of age due to the reaching of the HEP (Humane Endpoint). In both cases, the reason for the HEP concerned tumors. Due to technical problems, the light spot data from two WT and two BACHD rats could not be analyzed.

Thus, the N of the analyses changed as follows: for the light-dark box (WT: 10, BACHD: 12), for the light spot (WT: 10, BACHD: 10), for the elevated plus maze (WT: 10, BACHD: 12). Age development analyses excluded data from animals that were not assessed at all ages. No other exclusion criteria were used.

**Results**

**Light-dark Box**
Figures 1A and 1B highlight that the percentage of time spent in the light and the number of transitions increased slightly in both BACHD and WT rats between 2 and 8 months of age and decreased slightly at 12 months of age. These data show a significant age effect in both parameters (% time spent in the light: \( F_{(3, 60)} = 11.03, p<0.0001 \); number of transitions: \( F_{(3, 60)} = 20.98, p<0.0001 \)). However, there was no significant effect of the genotype (% time spent in the light: \( F_{(1, 20)} = 0.3203, p=0.5777 \); number of transitions: \( F_{(1, 20)} = 0.2075, p=0.6537 \)) or interaction (% time spent in the light: \( F_{(3, 60)} = 0.5937, p=0.6216 \); number of transitions: \( F_{(3, 60)} = 0.7043, p=0.5532 \)) in both parameters. Taken together, these results show that BACHD rats exhibit similar anxiety-like responses compared to WT littermates after exposure to the light-dark box.

**Light Spot in the Phenotyper**
Figure 2 illustrates the frequency of entrances in / visits to the feeder zone during the light spot (A), the time spent in the feeder zone during the light spot normalized for the total time outside the shelter (B), and the time spent outside the shelter during the light spot normalized for the total time (C).
The frequency in the feeder zone and the time spent outside the shelter decreased between 2 and 12 months of age in WT animals, showing significant age effects for the number of entrances / visits ($F(3,27) = 22.49, p<0.0001$), the time spent in the feeder ($F(3,27) = 3.78, p=0.0219$), and for the time spent outside the shelter ($F(3,27) = 7.371, p=0.0009$).

Interestingly, while WT animal presented a strong decrease of the time spent in the light spot /feeder zone with increasing age, BACHD animals spent more time in the light spot / feeder zone at 2, 8 and 12 months of age compared to their WT littermates (genotype effect; $F(1,9) = 5.237, p=0.0479$). Conversely, BACHD entered the light spot / feeder zone with the same frequency as WT littermates (genotype effect; $F(1,9) = 0.2463, p=0.6316$). During the light spot, a decrease over time was shown in the time spent outside the shelter, which was comparable in transgenic and control animals (genotype effect; $F(1,9) = 2.156, p=0.1761$). At all separately analyzed ages, no interactions concerning age and genotype were observed ($p >0.05$). In summary, the light spot test reveals a decreased anxiety-like response in BACHD rats at 2, 8 and 12 months of age, as shown by an increased time spent in the feeder zone while the light spot was on.

**Figure 1** Light Dark Box.

**(A)** Percentage of time spent in the light compartment. *(B)** Number of transitions between the light and the dark compartment. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. N= 10 WT and 12 BACHD rats. (***) $p < 0.001$, (****) $p < 0.0001$, ns (not significant).
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Elevated Plus Maze

As shown in Figure 3A, the percentage of time spent in the open arms decreased in both BACHD and WT over time, showing a significant age effect ($F_{(3, 60)} = 6.86, p=0.0005$) and non-significant effects of genotype ($F_{(1, 20)} = 0.1785, p=0.6771$) as well as no interaction between age and genotype ($F_{(3, 60)} = 0.1592, p=0.9233$). BACHD rats did not seem to have a different frequency of entries in the open arms compared to WT littermates at each age tested ($p >0.05$) (Figure 3B). This trend may suggest a less anxiety-like behavior phenotype of the BACHD rats, although it did not reach statistical significance.

Figure 2 Light Spot in the Phenotyper.

(A) Frequency of entrances/visits to in the light spot / feeder zone when the light spot was on. (B) Mean proportion of time spent in the feeder zone during the light spot. (C) Mean proportion of time spent outside the shelter during the light spot. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. N= 10 WT and 10 BACHD rats. (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$, ns (not significant).
In addition, risk assessment parameters were measured, with both BACHD and WT animals having a significant effect of age on the time spent head dipping ($F(3,60) = 7.266, p=0.0003$). Neither a genotype effect was detected ($F(1,20) = 0.1013, p=0.7535$) nor a genotype x age interaction ($F(3,60) = 0.6936, p=0.5596$) (Figure 3C). Conversely, both BACHD and WT rats showed fluctuations in the frequency of head dips in the open arms over different ages (age effect: $F(3, 60) = 12.34, p<0.0001$) without presenting any genotype effect ($F(1, 20) = 0.2684, p=0.6101$) (Figure 3D).

Figure 3 Elevated Plus Maze.

(A) Percentage of time spent in the open arms. (B) Frequency in the open arms. (C) Time spent head dipping inside the open arms. (D) Head dip frequency in the open arms. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed within each graph. Results from post-hoc analysis are indicated in case significant genotype differences were found. N= 10 WT and 12 BACHD rats. (***) $p < 0.001$, (****) $p < 0.0001$, ns (not significant).
In contrast to the light spot test, the outcome of EPM exposure did not disclose BACHD rats’ significant anxiety-related changes by measuring the EPM classical parameters and the risk assessment parameters.

**Discussion**

In this study we focused on the development of the anxiety-like responses of the BACHD rat model over four different ages (at 2, 4, 8 and 12 months), attempting to have a comprehensive picture of BACHD rats’ repertoire of anxiety-like behavior. Although HD is a neurodegenerative disorder best known for its effect on motor control, mood disturbances such as depression, anxiety, and irritability have a high prevalence in patients with HD, and often start before the onset of motor symptoms (Pla et al., 2014). The BACHD transgenic rat is the newest rat model for HD and its psychiatric phenotype has not been fully determined yet, with previous studies reporting decreased anxiety-like behavior in the elevated plus maze (Clemens et al., 2015; Yu-Taeger et al., 2012) and increased impulsivity (Manfré et al., 2016) in the delay discounting and in the Differential Reinforcement of Low rate of responding (DRL) tasks. Modeling the complexity of the psychiatric symptoms of pre-clinical HD in a rodent model is therefore important for increasing our understanding of the neurobiological mechanisms underlying the HD neuropathology. To our knowledge our study is the first one to explore the anxiety-like phenotype of BACHD rats by challenging them with a battery of tests, not only classical ones in a novel environment, but also including an automated test in the home-cage. Our data demonstrated a very subtle decrease of anxiety-like behavior in BACHD transgenic rats across 4 different developmental stages of the disease. Although not striking, there were still some behavioral differences in the three tests.

In the light–dark box BACHD rats showed similar anxiety-like responses when compared to control animals. The results of the light-dark box test are in line with BACHD rats’ spending, in comparison with control animals, the same amount of time and having the same frequency of visits in the open arms of the EPM. In a previous study it has been reported that BACHD rats spent significantly more time in the open arms of the EPM compared to WT animals (Clemens et al., 2015; Yu-Taeger et al., 2012) starting at 4 months of age. Our BACHD animals did not replicate the same EPM phenotype, likely because of repeated testing. A subtle change in risk assessment behavior, as reflected by a slight increase in time spent head dipping in the open arms, was detected in BACHD rats at the age of 4 months, although it did not reach statistical significance. The employment of the ethological measurement of risk assessment in this study confirms a unaltered coping strategy of BACHD animals. Overall, the outcome of both traditional test might not allow us to draw final conclusions on anxiety in BACHD rats.
Quantification of anxiety levels for the purpose of behavioral characterization of an animal model often contains the application of a battery of tests in order to capture the whole phenotype (Steimer, 2011). We implemented the light spot test since it is based on the rodents’ natural aversion for brightly lit areas and the assurance that a covered shelter is offered. Therefore, using a mild aversive stimulus in a home-cage environment circumvents the stress of handling, transportation and novelty, and also offers the opportunity for continuous automated quantification of behavior (Aarts et al., 2015). No prior manipulations such as food restriction or deprivation or any kind of training is needed to stimulate the animal’s participation to the light spot test. The lack of a need for prior training and food or water deprivation suggest to use the LS in the same way as described for the light-dark box test (Bourin and Hascoët, 2003): the time spent in the feeder zone is indicative of the approach response to the stimulus and it could be directly compared to the time spent in the light compartment during the light-dark box. In the case of the light-dark box, the animal can choose in which of the two sides it will stay without being forced to get exposed into the bright light (Ennaceur, 2014). Instead, in the case of the LS, the animal has to face its natural instinct of feeding whereas in the light-dark box there is the instinct of exploration of the novel box (Bourin and Hascoët, 2003). In addition, in the light-dark box the animal is directly placed in the light compartment whereas the animal is placed in the home-cage environment during the dark phase. As well as in traditional tests, in the LS test there is the element of curiosity for exploring the new situation presented in the home cage and assessing the potential threat. Another advantage of the LS test is that it is conducted in a home-cage environment after a full 5 days of habituation. The continuous monitoring and high sampling-rate provides great opportunities with respect to the within-group comparison to baseline to optimally use the data acquired. BACHD rats spent significantly more time in the feeder zone at 2, 8 and 12 months of age, suggesting reduced anxiety-like behavior. However, no differences were found at 4 months of age. Interestingly, BACHD rats’ time spent in the feeder zone during the light spot remains fairly constant from 4 to 12 months of age, demonstrating the same coping strategy with an adverse or unexpected situation (the ‘approach-avoidance’ conflict). In addition, future research should aim at investigating even older animals to establish if the phenotype remains stable over time.

One might argue that altered anxiety-like responses might be related to changes in locomotion (Pla et al., 2014). However, in our study it is unlikely that BACHD rats’ increased time spent in the feeder zone is influenced by locomotor alterations, given that our previous study showed an intact locomotor activity of BACHD animals during their active phase (Manfré et al., 2017).

The reliability and robustness of our study is the fact that the battery of behavioral experiments, combining novel and classical test setups, was conducted under well controlled environments (temperature, humidity, food), allowing the monitoring of behavior across different ages. Rodents, and especially rats, are highly suitable animals for
preclinical studies and in the last decade there has been a concerted effort towards automating methods for continuous automated home-cage assessment (Bains et al., 2017; Chort et al., 2013; Vandeputte et al., 2010), embracing rodent models of polyglutamine diseases (Kyriakou et al., 2015, 2017a; Manfré et al., 2017; Portal et al., 2013). Different technologies and paradigms have been developed to capture a wide range of behaviors through the possibility to house rodents in automated home-cage environments for extended periods of time and to measure voluntary activity without interference from the investigator (Schaefer and Claridge-Chang, 2012). Moreover, automated home cage observation allows data collection 24 h a day, 7 days a week, and it eliminates the handling and transportation of animals from home cage to test apparatus (de Visser et al., 2006; Kas and Van Ree, 2004). Several options for home cage monitoring are commercially available for rodents. PhenoTyper home cages but also many other automated home cages are used for the measurement of a broad variety of parameters, including cognition, locomotor activity, velocity, food and water consumption (Spruijt and DeVisser, 2006). Therefore, we decided to employ an automated test in the home-cage besides the traditional tests. Our results support the notion that going beyond the classical behaviors provides a more comprehensive analysis, thus adding value to the EPM and light-dark box behavioral patterns. This was shown by the fact that 2-, 8- and 12-month old BACHD animals seem to exhibit a decrease in their anxiety-like response in the light spot task, indicated by a statistically significant decrease compared to WT. By definition, anxiety is a complex biological phenomenon as a response to an unknown danger or threat stimulus or even an internal conflict that in turn triggers the appropriate response. The responses can vary depending on several factors (such as the type of the signal, environmental factors or contextual clues and even individual differences). Therefore, at this point we cannot exclude that the increased time spent in the feeder zone during the light spot may be related to a decreased eye-sight, or to a slower feeding speed. In previous studies, WT and BACHD rats deprived to 85% of their free-feeding body weight did not seem to differ in their interest in consuming 100 reward pellets, although BACHD rats needed more time to eat all pellets (Jansson et al., 2014). It may be that BACHD rats need more time to consume the same amount of food of WT animals, thus spending more time in the feeder zone.

On the other hand, it has to be noted that we performed multiple behavioral tests with the same group of rats. Repeated testing might have influenced the outcome of the tests (carry-over effect), although such an approach offers a better possibility to investigate phenotype onset and development than in separate cohorts of rats. It should be also noted that the LS test is a rather recent test which to our knowledge has only been reported once in mice and once for rats thus far (Aarts et al., 2015; Kyriakou, et al., 2017). As the light-dark box test has been repetitively described in literature being sensitive to strain, weight and age differences (Hascoët et al., 2001), the LS test may also be influenced by those variables. Therefore, further research may be needed (possibly using different
mouse and rat strains) to validate this test and verify its robustness under different circumstances, although the option to use a within-group baseline offers a solid basis for the interpretation of the data.

Conclusion

Taken together, our results indicate that the BACHD rat model does not display clear-cut anxiety-like responses in the classical paradigms over different ages. Interestingly, BACHD rats seemed to show reduced anxiety-like behavior when tested in an automated home-cage setting when an aversive stimulus (light spot) was offered. In translation to a patients’ situation, our results are not in line with findings of mood changes including anxiety in early- and late-symptomatic patients. Overall, our results do not mimic the generalized anxiety as seen in case reports, making the BACHD rat, as tested thus far, a less suitable model for therapeutic studies in the domain of anxiety.

Conflict of interest
At the time of the studies, the authors G. Manfré, E.I. Kyriakou and Dr. J.E. van der Harst were working for the EU funded “PhenoRat” project of which Noldus Information Technology was an industrial partner. Dr. J.E. van der Harst was part-time scientific project advisor for “PhenoRat” employed by Noldus Information Technology.

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References


General discussion
Thesis objective

Understanding strengths and weaknesses of the various animal models of Huntington Disease (HD), as well as knowledge of their phenotypes and end points, should guide researchers in the selection of the right animal model for a particular purpose and research question.

The aim of this thesis was to investigate aspects of the different psychiatric and motor phenotypes of the BACHD rat model of Huntington disease and parallel them to patients’ symptoms. To this purpose, the studies described in this thesis aimed at detecting phenotypes that resemble HD-like symptoms, as well as at monitoring their development by investigating the animals at different ages. We therefore selected different behavioral domains and designed different behavioral experiments, employing a specific battery of tests, to acquire more knowledge about certain aspects of the disease-phenotype that can be utilized for the identification of candidate compounds with therapeutic promise.

In chapter 2, we set out to investigate how different aspects of motor activity could influence the poor rotarod performance BACHD rats exhibited in previous studies. In accordance with these previous findings, our transgenic animals exhibited the same performance, confirming that BACHD animals present behavioral anomalies in motor performance in comparison with their WT littermates. We further investigated other aspects of this motor phenotype, testing rats for fine motor control by using the pasta handling and the pellet reaching tests. These tests did not reveal fine motor control deficits in BACHD animals at 2, 7 and 12 months of age. However, when WT and BACHD rats were assessed for gross motor function in the holding bar test, transgenic animals exhibited impairments indicative of decreased muscle endurance. Conversely, transgenic animals showed no impairments in performance in the grip strength test when compared to control animals, indicative of normal muscle strength. Interestingly, when tested in an automated home-cage environment, BACHD rats showed a decreased locomotion during the light (inactive) phase only, indicated by a reduced distance moved, velocity and time spent on the shelter.

Other than motor symptoms, psychiatric disturbances are quite common in manifest HD, although less is known about these symptoms in the earliest phase of the illness. Therefore, in chapter 3 we decided to perform a longitudinal study to establish the onset of social behavior disturbances in HD animals. BACHD rats showed age-dependent changes in behavior when they were re-introduced to their cagemate after a 24 hours-period of individual housing. In the social interaction test, BACHD rats showed abnormal social play and decreased active social behavior compared to their WT littermates. In the three chamber social test, BACHD rats seemed to show a mild deficit in preference for social novelty without any changes in social interest. We also carried out molecular analyses,
revealing that BACHD animals exposed to the social interaction test displayed decreased mRNA levels of the total form of BDNF in the ventral striatum and unaltered striatal expression of D1 and D2 dopamine receptors.

In chapter 4 we subjected control and BACHD rats to two Skinner box tests to assess their impulsivity trait. Impulsivity, poor risk assessment and altered behavioral inhibition are frequently encountered personality traits in HD so we explored two different aspects of impulsive behavior, namely choice and action impulsivity. When tested on the delay discounting (DD) task, BACHD rats showed high levels of choice impulsivity favoring “smaller, sooner” over “larger, later” rewards. Additionally, BACHD rats exhibited a lack of behavioral inhibition as indicated by bursts and premature responses in a DRL-5 and -10 (Differential Reinforcement of Low Rate of Responding task).

In spite of being under-researched, the prevalence of anxiety in manifest HD ranges from 13% to 71%, thus being an important clinical feature of HD. As HD patients may suffer from anxiety, in chapter 5 we conducted a longitudinal study to assess anxiety-like behavior in BACHD and WT rats at 2, 4, 8 and 12 months of age. In this chapter, classical and novel behavioral tests were used to assess different traits of anxious-like and risk assessment behavior, using the elevated plus maze, the light-dark box and the light spot (LS) test in the Phenotyper. In all tests, BACHD rats showed a trend towards decreased anxiety, making this model less suitable for finding potential treatments of anxiety-related disorders in HD-patients.

**BACHD rats present muscle endurance but not fine motor control deficits on a test battery of motor function**

Previous experiments have shown a strong motor coordination deficit in the Rotarod test (Abada et al., 2013b; Clemens et al., 2015; Yu-Taeger et al., 2012), mild gait impairments and altered open field activity (Abada et al., 2013b) in BACHD rats. In order to further dissect the motor phenotype of transgenic animals, fine motor skills, muscle strength and endurance have been assessed, as well as several parameters of activity-related motor function in a home-cage-like environment.

With respect to fine motor control, BACHD rats did not present any significant impairment. Although HD patients are significantly impaired in fine motor skills (Klein et al., 2011), skilled reaching and manual dexterity appeared to be intact in BACHD rats when tested in the pasta handling and the pellet reaching tests. However, our results are in line with previous observations in the transgenic Huntington disease (tgHD) rat strain (Fielding et al., 2012), which expresses a truncated N-terminal fragment of mHTT. We could conclude that fine motor skills did not play a role in the poor rotarod performance previously reported in these rats.
Concerning muscle strength, transgenic rats exhibited a significant impairment in the holding bar task at 2 months of age, suggesting reduced muscle endurance. Nevertheless, no differences were found at 7 and 12 months of age, as WT rats' performance dropped down to BACHD rats level at these ages. The difference at 2 months of age is perhaps related to a reduced amount of muscle mass and increased amount of fat mass carried by BACHD rats, as reported in a previous study (Jansson et al., 2014). Accordingly, a reduced performance in the holding bar test could be expected, as transgenic animals have to sustain the same body weight with a significantly lower amount of muscle mass, when compared to their WT littermates. On the other hand, the drop that WT animals exhibited at 7 and 12 months of age might be either due to repeated testing or due to the large increase in body weight from 2 to 7 months of age, which might have made it impossible for the rats to hang longer than a certain minimum amount of time. Thus, the test might not allow us to draw final conclusions on muscle endurance in older rats.

Conversely, the grip strength test did not show an indication that BACHD rats’ “passive” forelimb grip strength was reduced, even at the youngest age. A possible explanation could be that in the holding bar test the rats have to work against gravity and their own weight, while this does not take place in the grip strength. Due to the abnormal body composition (reduced muscle and increased fat mass), BACHD rats’ workload during the holding bar test is higher than that of WT rats. In contrast, WT and BACHD rats are likely exposed to a more comparable workload during the grip strength test.

Our study revealed specific motoric impairments in the BACHD rats although it is hard to draw a final conclusion on what caused the strong rotarod impairment. Even though there is a motor component, the deficit may also be related to non-motor aspects, like motivation or increased fat mass, as such differences have been reported in previous studies (Clemensson et al., 2017; Jansson et al., 2014). Future research will be therefore needed to understand the underlying mechanisms contributing to this motor deficit.

Automated home-cage monitoring revealed subtle differences in locomotor and activity patterns in BACHD animals

Automated methods for continuous automated home-cage assessment and for measuring motor function have been extensively used in the last years (Bains et al., 2017; Chort et al., 2013; Vandeputte et al., 2010). A comprehensive range of behaviors, free from experimenter bias, can be captured through the possibility to house rodents in automated home-cage environments for extended periods of time and to measure voluntary activity (Schaefer and Claridge-Chang, 2012).

To expand the repertoire of meaningful motor function tests in our rat model, we investigated general aspects of motor function related to rats’ baseline behavior through a video-based observation system in a home-cage like setting, namely the Phenotype®. In this setup, BACHD rats exhibited a generally reduced number of jumps on the shelter, while the time spent on the shelter was similar to WT rats. This suggests a
specific motoric difficulty to reach the top of the shelter rather than a reduced motivation to use it. Interestingly, this phenotype was present at all investigated ages. Furthermore, we found BACHD rats to have a generally reduced locomotor activity, as became apparent by a lower distance moved, velocity and time spent and number of jumps onto the shelter, specifically restricted to the light phase. These data suggest that, in our setting, the overall walking ability and activity of the rats was relatively preserved, as it was unaffected during their active phase. This is in line with previous findings in BACHD rats of the same age, which showed only mild gait abnormalities in both static and dynamic parameters in the Catwalk system (Abada et al., 2013b). Conversely, the results are in contrast with a lower rearing and ambulatory activity observed with the PhenoMaster set-up at 3 and 6 months of age (Yu-Taeger et al., 2012) and with an initial hyperactivity followed by hypoactivity starting at 4 months of age showed by BACHD rats in an open field test-like setup (Abada et al., 2013b). The different outcome could be due to the different test protocols and test setups used. Most prominently, we screened the animals for 6 consecutive days, while the other two studies screened for 22 hours and 1 hour, respectively. Thus, those results will probably reflect the rats’ response to novelty, while we have investigated their baseline behavior.

In conclusion, the reduction in activity during the light phase might suggest the existence of an altered activity pattern, which would be in line with the disrupted circadian rhythmicity HD patients as well as the BACHD and the R6/2 mouse models exhibited in previous studies (Kuljis et al., 2012; Morton et al., 2005). As circadian sleep disturbances are an important pathological feature of HD, further investigations may reveal insights in the underpinnings of an abnormal circadian activity.

Abnormal social play and diminished active social behavior in BACHD animals

The complex disease symptoms result in loss of independence, eliciting devastating personal and social consequences in HD patients. Since HD causes major disruption in family life (Vamos et al., 2017) there is an urgent need to use a family perspective when assessing the necessity for psychosocial intervention in HD. Thus, we decided to use familiar pairs (cagemates) in the social interaction test in order to have a better translational value of our results. By using two different social paradigms we aimed to detect potential HD-related deficits in social behavior.

The social interaction test revealed abnormal social play in BACHD animals as well as a trend towards a decreased interaction with conspecifics. Play fighting is one of the most notable social behaviors in rats, involving attack and defense of the nape of the neck, which if contacted, is gently nuzzled with the snout (Himmler et al., 2013). When initiating social play, one animal directs to the neck region of the partner and this can be accompanied with biting and pulling fur in that region. BACHD rats performed more play-fighting compared to their WT littermates, as showed by the increased nape
attacking. 8 month-old BACHD rats showed a twofold increase in the time spent on nape attacks, and a decreased duration of pinning compared with control rats. Pinning is a commonly used measure for play, which essentially involves the subject positioned supine with its partner standing on top (Himmler et al., 2013; Panksepp, 1981).

Social play has been suggested to represent a separate category of behavior (Baenninger, 1967; Hole, 1991b; Panksepp et al., 1984) and considerable differences between social play and aggressive behavior have been found (Hole, 1991a). Nevertheless, such behaviors are likely to represent more than just precursors of adult sexual or aggressive behaviors (Vanderschuren et al., 1997). We can therefore presume that these rougher and more aggressive play behaviors might facilitate the development of aggressive behaviors (Veenema and Neumann, 2009), which has been listed as psychiatric manifestation in patients who carry the HD mutation (Epping et al., 2016; Rosenblatt, 2007). More specific tests are anyway needed to verify the occurrence of aggressive behavior in adult BACHD animals.

Furthermore, the following and approaching parameters significantly decreased in BACHD animals at different age points, suggesting a reduced inclination to search for an interaction. The lack of these social behaviors in BACHD rats may suggest a “social deficit” that could be related to coping styles (Van Den Berg et al., 1999; Varlinskaya and Spear, 2008), and considered as a form of apathy, as showed by the R6/2 mouse model of HD (Wood and Morton, 2015). Among HD’s psychiatric manifestations, apathy has an extremely high prevalence and, as a psychiatric symptom distinct from depression, has been described as a combination of diminished motivation, reduced goal-directed behavior, lack of interest in new experiences, and diminished emotional responsivity (Starkstein and Leentjens, 2008). Although we cannot directly confirm its occurrence with the parameters we measured, apathy has been previously reported in the BACHD, in the z_Q175, and in the HttQ111/+ mouse models (Minnig et al., 2017; Oakeshott et al., 2012) and is commonly reported in patients with HD (Naarding et al., 2009). Changes in instrumental motivation may serve as a translatable readout of basal ganglia dysfunction in rodent models of HD. Future studies on other specific aspects of social behavior, e.g., social reward, will be important to define whether or not a social apathy-like phenotype is present in BACHD rats. Alternatively, the deficits in social behaviors described in BACHD rats may represent behavioral changes that depend more specifically on alterations in brain social networks which mechanisms in HD are still mostly unclear.

The lower approaching and following behaviors observed in this study are not likely to be affected by anxiety levels in BACHD rats, as in chapter 5 we reported unchanged anxiety-like responses in the elevated plus maze, in the light-dark box and a slightly decreased anxiety-like response in the light spot test. Furthermore, it is worth noting that these social behavior alterations were likely not confounded by an overall reduced motor activity, such as levels of general locomotion or activity: BACHD animals display similar levels of locomotion compared to their wild-type littermates as shown in chapter 2.
In summary, these results indicate deficits in several parameters representative of social play and active social behavior, thus replicating the disrupted social behaviors seen in HD.

**BACHD rats exhibit mild deficits in preference for social novelty, but no changes in social interest**

Cognitive deficits have already been described in BACHD rats for different aspects not related to social behavior (Abada et al., 2013a; Clemensson et al., 2017).

While decreased or increased social interaction (Nguyen et al., 2006; Pietropaolo et al., 2011; Wood and Morton, 2015) have been described in other animal models of HD using different behavioral paradigms, we did not observe altered sociability levels in BACHD rats exposed to the three chamber social test. In line with these findings, one recent study showed no changes in the preference for an unknown conspecific relative to an unknown object in BACHD rats (Lamirault et al., 2017). Although unchanged levels of social interaction in the three chamber social test are present, they are not necessarily in contrast with parameters measured in the social interaction test within this study. For example, increased approaching in the social interaction test is present along with an increased nape attacking which may relate to a play fighting-like behavior that is prevented in the other test where limited physical contact is allowed.

The novelty effect in our study is in line with earlier research in HD mouse models showing alterations in social recognition and memory (Ciamei and Morton, 2008; Rudenko et al., 2010) as well as with evidence of disrupted social cognition in HD patients (Larsen et al., 2016; Snowden et al., 2003). The brain changes underlying these symptoms in HD are not well known and it is not clear to which extent deficits in social cognition, mostly described as impaired emotion recognition, may depend on other cognitive and emotional changes and whether they could be the cause of social behavior alterations in HD patients. In conclusion, we were also able to detect a mild deficit in social novelty in BACHD animals at 7 months of age, as suggested by a lack of preference for a novel conspecific relative to a familiar one in the three chamber social test. These data, along with the changes in social interaction parameters, support the validity of the BACHD rat model in mimicking features of certain social deficits that could be relevant to symptoms in patients.

**Possible involvement of BDNF in transgenic animals’ decreased sociability**

Gene expression analyses were carried out in 8 month-old animals tested for social interaction, highlighting an altered expression of BDNF in ventral striatum and an intact striatal D1 and D2 receptors expression. The striatum is one of the most affected brain areas in HD where alterations in dopaminergic and BDNF brain systems have been well described previously (Cepeda et al., 2014; Zuccato and Cattaneo, 2007). We therefore investigated mRNA levels of striatal BDNF and dopaminergic receptors as potential mechanisms at the base of behavioral abnormalities. Furthermore, we linked social
behavior to striatal functionality via D1 and D2 dopamine receptors in 8 month-old animals which, along with BDNF, have been proposed as possible underlying regulatory factors (Báez-Mendoza and Schultz, 2013). The lack of changes in mRNA levels of D1 and D2 dopamine receptors in BACHD rats subjected to the social interaction test would not suggest a direct link between the dopaminergic system and the behavioral changes. However, this result is in line with unpublished studies of our collaborators that showed unaltered density of D2 receptors in the striatum of 3 and 9 months old BACHD rats. Nonetheless, measuring protein levels in future studies may help to understand better whether the dopaminergic system plays a role in the behavioral changes. BDNF mRNA levels were also quantified and we detected a decreased expression in the ventral striatum of BACHD animals and no changes in the dorsal region. Therefore, it is possible that altered BDNF expression in this region may have contributed to the deficits in sociability in 8 months old BACHD rats. One should keep in mind that striatal BDNF transcript levels are very low at basal level and that usually BDNF protein is not directly transcribed in the striatum, but it is produced in the cerebral cortex and anterogradely transported to the striatum (Zuccato et al., 2010). In light of this, we cannot draw any conclusion on the mechanisms underlying the BDNF decrease and its link to social deficits and it should be the aim of future experiments, possibly using different age cohorts of BACHD rats.

**BACHD animals exhibit high levels of choice and action impulsivity**

Impulsivity, poor risk assessment and altered behavioral inhibition are frequently encountered personality traits in Huntington disease (Duff et al., 2007; Weintraub et al., 2015). Cognitive and psychiatric disorders affect HD patients before motor symptoms (Novak & Tabrizi 2010; Rosenblatt, 2007) and have a detrimental impact on the quality of HD patients’ life (Guttman et al., 2003; Rosenblatt, 2007).

To determine impulsivity traits in these rats, we used two different paradigms to assess choice and action impulsivity. During the delay discounting task (DD), BACHD and WT rats shifted progressively their choices to the immediate small reward as the delays increased. BACHD rats shifted more quickly to the immediate small reward than WT rats, thus showing high levels of choice impulsivity. In the DRL-5s and -10s (Differential Reinforcement of Low Rate of Responding task) BACHD rats were almost as efficient as WT animals, with a less precise temporal discrimination and a higher rate of burst responses during the very first seconds following a reward. In addition, BACHD rats exhibited a lack of behavioral inhibition as indicated by bursts and premature responses in the DRL task.

These results demonstrate the occurrence of a high level of choice impulsivity and, to a minor extent, action impulsivity in BACHD rats. The DD paradigm describes primarily the devaluation of an event as the delay to that event increases: the maintained bias towards larger delayed gratification has been taken to index increased self-control (Isles et al., 2004). The DRL paradigm depicts the capacity to withhold actions during a fixed amount of time. Burst responses, which follow immediately after a rewarded lever-press,
indicate perseverative responses induced by the failure to re-obtain an immediate feedback to their responses. Timing errors define the temporal discrimination ability, which allows assessment of when the time \( t \) has elapsed (Kramer and Rilling, 1970). BACHD rats exhibited a steep delay discounting curve in the DD showing a high level of cognitive/choice impulsivity. On the other hand, they show a nearly normal efficiency in the DRL, but the timing errors and the high rate of burst responses when the delay increased indicate signs of motor impulsivity.

At the neurobiological level, lack of inhibitory control has been observed in several neuropathological disorders associated with abnormal dopaminergic transmission such as deficit/hyperactivity disorder (ADHD), Parkinson disease, Alzheimer disease and Huntington disease (Barkley, 1997; Rosenblatt, 2007; Rochat et al., 2008; Novak & Tabrizi 2010; Nombela et al., 2014). Furthermore, there is a strong correlation between dopamine receptors availability and the two different types of impulsivity: variation in striatal D2/D3 receptor levels is negatively correlated with impulsive action in rodents (Dalley et al., 2007; Laughlin et al. 2011) and with impulsive choice and action in humans (Lee et al., 2009; Ghahremani et al., 2012). On the other hand, D1-like receptors appear to be most selectively involved in mediating impulsive choice in rats which may be explained by their different locations within the prefrontal cortex (PFC), an area known to be involved in impulsive choice (Loos et al., 2010; Koffarnus et al, 2011). Furthermore, impulsivity, as well as compulsivity and flexibility, are closely interrelated executive processes in the context of inhibitory control mediated by the PFC (Bari & Robbins, 2013; Wise, 2008). However, according to the model proposed by Bechara (Bechara, 2005), willpower emerges from the dynamic interaction of two separate, but interacting, neural systems: an impulsive system, in which the amygdala is a critical neural structure involved in triggering the affective and emotional signals of immediate outcomes, and a reflective system, in which the ventral PFC is crucial in triggering the affective and emotional signals of long-term outcomes. Using a delay discounting paradigm, rats with inactivation and disconnection of the medial PFC and basolateral amygdala become more impulsive, showing preference for smaller immediate over larger delayed rewards (Churchwell et al., 2009). In HD patients, both the prefronto-striatal circuit and the amygdala system seem to be dysfunctional (Rosas et al., 2003). Starting from 3 months of age, BACHD rats present a high expression of mutant huntingtin (mhtt) aggregates in the neocortex and in limbic areas including the amygdala (Yu-Taeger et al., 2012). Although further studies are needed, we assume that a dysfunctional prefronto-striatal/amygdala imbalance might contribute to the high level of choice impulsivity observed in BACHD rats. Similar to the impulsive and perseverative behaviors described in early symptomatic HD patients, BACHD rats resembles the same features, making this rat model highly suitable for drug discovery purposes.
BACHD animals show unchanged anxiety-like responses in classical but not automated tests

Although chorea is a prerequisite for formal clinical diagnosis (Loy and McCusker, 2013; Reilmann et al., 2014) and the most recognized HD-related deficit (Papoutsi et al., 2014), psychiatric (and cognitive) symptoms often appear during the prodromal phase of the disease, as many as 10 years prior to the onset of motor dysfunctions (Epping et al., 2016; Tabrizi et al., 2013). Anxiety may be an early feature of HD and it may coexist with depression (Craufurd et al., 2001) and these aspects are often described as being the most burdensome symptoms to both HD patients and their families (Paulsen, 2011).

Our study attempted to explore the anxiety-like phenotype of BACHD rats by challenging them with a battery of tests, not only classical ones in a novel environment, but also including an automated test in the home-cage. Our data did not demonstrate marked differences in anxiety-like behavior in BACHD transgenic rats across 4 different developmental stages of the disease.

The time spent and the number of entries in the light compartment of the light-dark box did not reveal any significant differences in any age group. Similarly, in the elevated plus maze test, the time spent and the number of entries into the open arms did not differ between the two groups at any age, which indicates a similar level of anxiety in the two groups. The outcome of both test is in contrast with previous findings in patients and in different rodent models of HD. Increases in anxiety-like behavior have been shown in BACHD mice using the open-field test, the elevated zero maze, the elevated plus maze, the light-dark box test and a fear conditioning test (Carter et al., 1999; Menalled et al., 2010). Conversely, some studies have shown that R6/2 mice exhibit a decrease in anxiety-like behavior in the EPM (File et al., 1998). Similarly, a decrease in anxiety-like behavior has been shown in the R6/1 mouse (Naver et al., 2003) and in our BACHD rat model in the EPM (Yu-Taeger et al., 2012), where a reduced level of anxiety was observed. Additionally, we employed the ethological measurement of risk assessment. A subtle change in risk assessment behavior, as reflected by a slight increase in time spent head dipping in the open arms, was detected in BACHD rats at the age of 2 months, although it did not reach statistical significance.

In the last decade, the continuous monitoring and high sampling-rate made possible by automated systems provides great opportunities with respect to the within-group comparison to baseline to optimally use the data acquired. We decided to combine the light-spot and the light-dark box tests as the lack of a need for prior training and food or water deprivation suggest to use the LS in the same way as described for the light-dark box test (Bourin and Hascoët, 2003): the time spent in the feeder zone is indicative of the approach response to the stimulus and it could be directly compared to the time spent in the light compartment during the light-dark box. BACHD rats spent significantly more time in the feeder zone at 2, 8 and 12 months of age, suggesting reduced anxiety-like behavior. However, no differences were found at 4 months of age. Interestingly, BACHD
rats’ time spent in the feeder zone during the light spot remains fairly constant from 4 to 12 months of age, demonstrating the same coping with an adverse or unexpected situation (the ‘approach-avoidance’ conflict). In addition, future research should aim at investigating even older animals to establish if the phenotype remains stable over time. Furthermore, one has to keep in mind that an altered anxiety-like behavior might be related to changes in locomotion (Pla et al., 2014). It is unlikely that BACHD rats’ increased time spent in the feeder zone is influenced by locomotor alterations, given that in chapter 2 we show an intact locomotor activity of BACHD animals during their active phase. Overall, the outcome of traditional and automated tests investigated within the thesis would suggest that the BACHD rat is not a reliable model of the anxious behavior exhibited by HD patients, thus making BACHD rats a less suitable model for therapeutic studies in the domain of anxiety.

Concluding remarks

Based on the findings presented in this thesis we proposed several interesting directions for future research, as summarized above. These future perspectives stress the importance of modeling the complexity of motor and psychiatric symptoms of Huntington Disease in a pre-clinical setting to increase our understanding of the neurobiological mechanisms underlying the HD neuropathology, and develop and validate robust disease models for therapeutic efficacy studies.

In our motor control study we dissected the motor phenotype of BACHD rats looking at different aspects of motor control, namely fine and gross motor control. We provided evidence of BACHD rats’ impairment in muscle endurance that can be possibly linked to a reduced amount of muscle mass and/or to an increased amount of fat mass as showed in previous experiments (Jansson et al., 2014). Furthermore, previous studies provided evidence of peripheral pathology such as weight loss and muscle wasting in HD patients (Cepeda et al., 2007; Imarisio et al., 2008; Miller and Bezprozvanny, 2010), progressive skeletal muscle atrophy in HD patients and in the R6/2 mouse model for HD (She et al., 2011; Zielonka et al., 2014). Another possibility is that this impairment might be related to deficits in the joints, ligaments and tendons, which are structures that are compromised in HD patients and in the BACHD mouse model (De Aragão et al., 2016; Nguyen and Cenci, 2015). In an automated home-cage setting the global walking ability and activity of BACHD rats appeared to be preserved, as it was unaltered during the dark phase, the major activity phase. Interestingly, BACHD rats exhibited a generally reduced locomotor activity during the light phase only. These findings are interesting in the context of previous studies in HD patients and in the BACHD mouse model that reported alterations in the circadian rhythm (Kuljis et al., 2012; Morton et al., 2005). Since our findings indicate
Figure 2 Overview of the behavioral phenotypes investigated within this thesis.
that BACHD animals exhibited a reduced activity during the light phase, that could be indeed indicative of an altered night-day activity pattern. Therefore, a direction for follow-up research should be towards a detailed assessment of circadian behavior in order to investigate the mechanism underlying this behavioral alteration, as it can very well be related to the reduction in muscle endurance that we found, resulting in exhaustion and a higher need to rest or sleep during the inactive phase.

Overall, the results from the motor phenotyping study are not robust enough to justify, at this stage, using this line for therapeutic studies to test efficacy of pharmacological compounds, although the literature suggests that the histopathology of this model is quite robust.

By using two different social paradigms we detected the occurrence of HD-related deficits in specific aspects of social behavior. The social interaction test highlighted abnormal social play and social dominance behaviors in BACHD animals as well as a trend towards a decreased interaction with conspecifics. Furthermore, the three chamber social test disclosed mild deficits in social recognition in transgenic animals. Due to the dramatic impact HD has on family life on a daily basis, we decided to use familiar pairs (cagemates) in the social interaction test in order to have a better translational value of our results. Additionally, it would be interesting to implement the resident-intruder test in order to assess the occurrence of aggressive behavior in BACHD animals after exposure to a naive animal.

In our study we have also investigated potential molecular correlates of social behavior. We did not detect any changes in the mRNA levels of D1 and D2 dopamine receptors in BACHD rats subjected to the social interaction test, thus excluding a direct link between the dopaminergic system and the behavioral changes. We disclosed a decreased expression of BDNF mRNA in the ventral striatum of BACHD animals and no differences in the dorsal region. Therefore, it is possible that altered BDNF expression in this region may have contributed to the deficits in sociability in 8 months old BACHD rats. Alternative measurement techniques should be employed to assess the oxytocinergic and vasopressinergic systems in future studies, which are interesting in the context of their involvement in social recognition and social behavior (Báez-Mendoza and Schultz, 2013). A battery of classical and novel paradigms has been employed to characterize anxiety-like behaviors in BACHD animals. The light-dark box and the elevated plus maze did not highlight any changes in anxiety-like responses, in contrast with HD patients who appear to have a more anxious behavior. Interestingly, the light spot test in an automated home-cage system showed a decrease of anxious behavior in presence of a mild stimulus such as the light into the food hopper. The outcome of our battery of tests evidences that the BACHD rats are not suitable for the investigation of molecular markers of the generalized anxiety seen in HD patients in case reports.
To summarize the observations within the psychiatric domain, the BACHD animals resemble selected neuropsychiatric symptoms of HD, namely alterations of social behaviors and impulsivity. Although being a complex trait, we shed light on the psychiatric manifestations shared with the human patients. The BACHD rat appeared to be not appropriate for the anxiety-like profiling and highly suitable for investigations of the social and impulsive behavior domains, making them valuable markers of the pre-motor phase of HD pathology.

Future studies should then unravel the mechanisms underpinning impulsive and social behavior, guiding us in advancing promising therapeutic agents to deliver effective treatments for HD patients.

In conclusion, the question of which species and which particular model to choose for studying HD is dependent on the purpose of the research and the question of interest. Different species of animals suit better for modelling certain aspects of HD and for different applications. Rodent models of HD are versatile, especially due to the presence of test batteries available for the assessment of cognitive, psychiatric and motor-like features of the disease.

With this thesis we bring new information about the appearance and the progression of different motor and psychiatric phenotypes of the BACHD rat model. The outcome of our studies should be used to address specific questions about possible phenotype manifestations and their course in order to design novel and targeted interventional studies. Hopefully, the collective efforts of the field will lead to the achievement of the ultimate goal: effective disease-modifying treatments for HD.


English summary
Nederlandse samenvatting | Dutch summary
Sommario | Italian summary
English Summary

Neuropsychiatric and neurological disorders constitute a major health problem in Europe, and their impact on public health and society is increasing with the aging of the population. In 2006, the World Health Organization estimated that neurological disorders, ranging from epilepsy to Alzheimer disease, from stroke to headache disorders, affect up to one billion people worldwide.

One particularly relevant neurological disorder in the framework of the foregoing concerns is Huntington disease (HD). HD is an autosomal-dominantly inherited, fatal, neurodegenerative disorder. It is caused by an unstable, expanded CAG base triplets in the coding region of the Huntingtin gene (HTT), resulting in an abnormal polyglutamine sequence in the huntingtin protein (htt). HD patients typically suffer from a triad of movement, psychiatric and cognitive symptoms. The onset of the symptoms, that can vary between individuals and affected members of the same family, usually occurs in mid-adulthood (30-40 years old), but the onset of disease may be earlier or later in life. The diagnosis of adult-onset HD typically involves involuntary motor abnormalities and chorea that often decrease in a late stage, when parkinsonism, dystonia and rigidity dominate. In the early stages patients have deficits in executive system functioning, short-term memory and visuospatial functioning. The later stages are characterized by bradykinesia, spasticity, dysarthria, dysphagia and incontinence. In addition to motor abnormalities, cognitive decline and personality changes are part of HD, and, like motor impairments, they deteriorate gradually. Additionally, cognitive deficits progress to more widespread global subcortical dementia with apathy, impulsiveness and depression, resulting in a negative impact in social life and interpersonal relations. Less common are delusional depression or schizophrenia-like-psychosis, which might require psychiatric treatment.

Since the discovery of the HTT mutation 25 years ago, more than 13,000 papers have been published on HD, approximately half of which relate to attempts to model various aspects of the disease. Thus, despite the considerable progress in our understanding of the disease, an effective treatment that either prevents or slows the pace of HD remains out of reach. The slow rate of progression of HD often necessitates lengthy and therefore costly clinical trials. In addition, the number of patients with HD who are available to participate in such trials is limited, which means that a relatively small number of compounds can be tested every time.

Therefore, one of the most important challenges for finding more effective drugs for brain disorders is the development of model systems that translate to human pathology and are predictive of clinical efficacy. Accordingly, part of that research effort involves identifying suitable animal models that provide a valid representation of the pathological and behavioral profile of the human disease and that can serve for the identification of therapeutic candidates and novel approaches to therapy.
Several animal models of Huntington disease have been established following the discovery of the Huntingtin gene and the disease-causing mutation. Each model has strengths and weaknesses, and their combined use is of importance for preclinical research concerning disease mechanisms and potential therapeutics. Thorough characterization of a given animal model is important in order to understand to what extent it models the actual disease and how to work with it in an appropriate way. In particular, rodent models of HD are versatile, especially when considering the high number of established batteries of tests available that allow assessment of motor, cognitive and psychiatric-like features of the disease. Studies in rodents that emphasize careful behavioral analysis are being developed as effective and inexpensive models that complement clinical studies. The BACHD rat is the latest rat model of HD, and expresses the full length human huntingtin (fl-mhtt) with 97 CAG-CAA repeats under the control of the human HD promoter gene and all its regulatory elements to determine its similarity to the human condition (construct validity). The BACHD rat model is particularly relevant since it presents motor, emotional and cognitive impairments resembling symptoms seen among HD patients (face validity). Furthermore, neuroanatomically and histologically, the models would display selective, age-dependent striatal and cortical neuronal loss and atrophy, nuclear inclusions and neuropil aggregates. Its predictive validity is currently impossible to establish, as for diseases such as HD there is no effective treatment available yet. Despite the extensive use of the BACHD rat line in the last years, the studies performed so far did not cover fine and gross motor function as well as psychiatric endophenotypes such as social behavior, impulsivity and anxiety. In this thesis the focus is on the investigation of these domains, which is necessary for the early detection of the first core symptoms of the disease and for the monitoring of its development so that possible therapies can be more effective in preventing further development of neurodegeneration.

Patients and several HD rodent models have been found to present cognitive symptoms already before the start of any motor symptoms. Conversely, BACHD transgenic rats have repeatedly been found to show early impairments in the Rotarod test which progressively worsen over time. Altered activity in the PhenoMaster and in an open field test-like setup as well as abnormalities in unhindered walking gait have also been reported. These findings suggested that a more in-depth investigation of various aspects of motor function in BACHD rats was needed. In chapter 2, we set out to investigate how different aspects of motor activity could influence the poor Rotarod performance BACHD rats exhibited in previous studies. In accordance with these previous findings, our transgenic animals exhibited the same performance, confirming that BACHD animals present behavioral anomalies in motor performance in comparison with their wild-type (WT) littermates. We further investigated other aspects of this motor phenotype, testing rats for fine motor control by using the pasta handling and the pellet reaching tests. These tests did not reveal fine motor control deficits in BACHD animals at 2, 7 and 12 months of age. However, when WT and BACHD rats were assessed for gross motor function in the holding
bar test, transgenic animals exhibited impairments indicative of decreased muscle endurance. Conversely, transgenic animals showed no impairments in performance in the grip strength test when compared to control animals, indicative of normal muscle strength. Interestingly, when tested in an automated home-cage environment, BACHD rats showed a decreased locomotion during the light (inactive) phase only, indicated by a reduced distance moved, velocity and time spent on the shelter.

Cognitive and psychiatric symptoms, including perseveration, lack of insight, distractibility, impaired social life, anxiety and impulsivity, affect HD patients before motor symptoms and have thus an early deleterious impact on the quality of HD patients’ life. The psychiatric phenotype of BACHD rats has been only partly investigated indicating that the BACHD rat line requires further phenotyping to test the parallels of their phenotypes to patients’ symptoms. Therefore, in chapter 3 we decided to perform a longitudinal study to establish the onset of social behavior disturbances in HD animals. BACHD rats showed age-dependent changes in behavior when they were re-introduced to their cagemate after a 24 hours-period of individual housing. In the social interaction test, BACHD rats showed abnormal social play and decreased active social behavior compared to their WT littermates. In the three chamber social test, BACHD rats seemed to show a mild deficit in preference for social novelty without any changes in social interest. We also carried out molecular analyses, revealing that BACHD animals exposed to the social interaction test displayed decreased mRNA levels of the total form of BDNF (brain-derived neurotrophic factor) in the ventral striatum and unaltered striatal expression of D1 and D2 dopamine receptors.

In chapter 4 we subjected control and BACHD rats to two Skinner box tests to assess their impulsivity trait. Impulsivity, poor risk assessment and altered behavioral inhibition are frequently encountered personality traits in HD so we explored two different aspects of impulsive behavior, namely choice and action impulsivity. When tested on the delay discounting (DD) task, BACHD rats showed high levels of choice impulsivity favoring “smaller, sooner” over “larger, later” rewards. Additionally, BACHD rats exhibited a lack of behavioral inhibition as indicated by bursts and premature responses in a DRL-5 and -10 (Differential Reinforcement of Low Rate of Responding task).

In spite of being under-researched, the prevalence of anxiety in manifest HD ranges from 13% to 71%, thus being an important clinical feature of HD. As HD patients may suffer from anxiety, in chapter 5 we conducted a longitudinal study to assess anxiety-like behavior in BACHD and WT rats at 2, 4, 8 and 12 months of age. In this chapter, classical and novel behavioral tests were used to assess different traits of anxious-like and risk assessment behavior, using the elevated plus maze, the light-dark box and the light spot (LS) test in the Phenotyper. In all tests, BACHD rats showed a trend towards decreased anxiety, making this model less suitable for finding potential treatments of anxiety-related disorders in HD-patients.
In chapter 6, we provided an extensive discussion of the research findings summarized above and we proposed directions for future research.

In conclusion, this thesis includes a series of studies focusing on the characterization of different psychiatric and motor phenotypes of the BACHD rat model of Huntington disease in order to parallel them to patients’ symptoms. To this purpose, the studies described in this thesis aimed at detecting phenotypes that resemble HD-like symptoms, as well as at monitoring their development by investigating the animals at different ages. The outcome of our studies should therefore be used to address specific questions about possible phenotype manifestations and their course in order to design novel and targeted interventional studies.
**Nederlandse samenvatting**

Neuropsychiatrische en neurologische aandoeningen vormen een groot gezondheidsprobleem in Europa, en hun invloed op de volksgezondheid en de samenleving neemt toe met de vergrijzing van de bevolking. De World Health Organization schatte in 2006 neurologische aandoeningen, variërend van epilepsie tot de ziekte van Alzheimer, van beroerte tot hoofdpijn stoornissen, wereldwijd circa 1 miljard mensen treffen.


Sinds de ontdekking van de HTT-mutatie 25 jaar geleden, zijn meer dan 13.000 artikelen op de zvH gepubliceerd, waarvan ongeveer de helft betrekking heeft op pogingen om verschillende aspecten van de ziekte te modelleren. Dus, ondanks de aanzienlijke vooruitgang in ons begrip van de ziekte, blijft een effectieve behandeling die het tempo van de zvH vertragen of voorkomen buiten bereik. De trage progressie van zvH maakt vaak lange en daarom dure klinische onderzoeken noodzakelijk. Bovendien is het aantal patiënten met zvH dat beschikbaar is om deel te nemen aan dergelijke onderzoeken beperkt, wat betekent dat een relatief klein aantal verbindingen elke keer kan worden getest.

Daarom is de ontwikkeling van modelsystemen die zich vertalen in menselijke pathologie en de klinische werkzaamheid voorschijnen een van de belangrijkste uitdagingen voor het vinden van effectievere geneesmiddelen voor hersenstoornissen. Dienovereenkomstig
omvat een deel van die onderzoeksinspanning het identificeren van geschikte diermodellen die een geldige weergave geven van het pathologische en gedragsprofiel van de menselijke ziekte en die kunnen dienen voor de identificatie van therapeutische kandidaten en nieuwe behandelingsbenaderingen.

Verschillende diermodellen voor de ziekte van Huntington zijn vastgesteld na de ontdekking van het Huntington-gen en de ziekte-veroorzakende mutatie. Elk model heeft sterke en zwakke punten, en hun gecombineerde gebruik is van belang voor preklinisch onderzoek met betrekking tot ziektemechanismen en potentiële therapieën. Grondige karakterisering van een bepaald diermodel is belangrijk om te begrijpen in welke mate het de werkelijke ziekte modelleren en hoe ermee op een geschikte manier te werken. Met name zijn de ZvH-modellen van ZvH veelzijdig, vooral als wordt gekeken naar het grote aantal gevestigde batterijen van beschikbare tests die de beoordeling van motorische, cognitieve en psychiatrieke eigenschappen van de ziekte mogelijk maken.

Studies bij knaagdieren die de nadruk leggen op zorgvuldige gedragsanalyse, worden ontwikkeld als effectieve en goedkope modellen die een aanvulling vormen op klinische studies. De BACHD-rat is het nieuwste ratmodel van de ZvH en drukt het menselijke huntingtine van de volledige lengte (fl-mhtt) uit met 97 CAG-CAA-repeats onder de controle van het menselijke ZvH-promotorgen en alle regulerende elementen om de gelijkenis met de mens te bepalen voorwaarde (constructvaliditeit). Het BACHD-rattenmodel is bijzonder relevant omdat het motorische, emotionele en cognitieve stoornissen vertoont die lijken op symptomen waargenomen bij ZvH-patiënten (gezichtsvaliditeit). Bovendien zouden de modellen neuroanatomisch en histologisch selectief, leeftijdsafhankelijke striatal en cortex neuronale verlies en atrofie, nucleaire insluitels en neuropeilaggregaten vertonen. Het is momenteel onmogelijk om het voorspellend vermogen vast te stellen, wanneer voor ziekten zoals ZvH er geen effectieve behandelingen beschikbaar zijn. Ondanks het uitgebreide gebruik van de BACHD-ratlijn in de afgelopen jaren hebben de tot nu toe uitgevoerde onderzoeken geen betrekking op fijne en grove motorische functies en op psychiatrieke endofenotypen zoals sociaal gedrag, impulsiviteit en angst. In dit proefschrift ligt de focus op het onderzoek van deze domeinen, wat nodig is voor de vroege detectie van de eerste kernsymptomen van de ziekte en voor het monitoren van de ontwikkeling ervan, zodat mogelijke therapieën die effectiever kunnen zijn in het voorkomen van verdere ontwikkeling van neurodegeneratie.

Het is gebleken dat patiënten en verschillende ZvH-knaagdieren reeds vóór het begin van motorische symptomen cognitieve symptomen vertonen. Omgekeerd is er herhaaldelijk gevonden dat BACHD transgene ratten vroege stoornissen in de Rotarod-test vertonen die na verloop van tijd geleidelijk verslechteren. Veranderde activiteit in de PhenoMaster en in een open veld testachtige opstelling, evenals afwijkingen in een ongehinderd lopen gang zijn gemeld. Deze bevindingen suggereren dat een meer diepgaand onderzoek van de verschillende aspecten van de motorische functies in BACHD ratten nodig was. In hoofdstuk 2 hebben we onderzocht hoe verschillende aspecten van motorwerking de
slechte Rotarod-prestaties konden beïnvloeden die BACHD-ratten vertoonden in eerdere studies. In overeenstemming met deze eerdere bevindingen vertoonden onze transgene dieren dezelfde prestatie, hetgeen bevestigt dat BACHD-dieren gedragsafwijkingen vertonen in motorprestaties in vergelijking met hun wild-type (WT)-nestgenoten. We onderzochten verder andere aspecten van dit motorfenotype door ratten te testen op fijne motorische controle door het gebruik van de pasta handling and the pellet reaching testen. Deze testen toonden geen fijne motorische controle-tekortkomingen bij BACHD-dieren op de leeftijd van 2, 7 en 12 maanden. Echter, wanneer WT en BACHD ratten werden beoordeeld op de grove motorische functie in de holding bar test, vertoonden transgene dieren stoornissen die indicatief zijn voor verminderd spieruithoudingsvermogen. Omgekeerd vertoonden transgene dieren geen stoornissen in de prestaties bij de grip strength test vergeleken met controledieren, hetgeen indicatief is voor normale spiersterkte. Interessant genoeg toonden BACHD-ratten bij een test in een geautomatiseerde thuiskooiomgeving een verminderd motoriek tijdens de lichte (inactieve) fase alleen, wat wordt aangegeven door een verminderde afstand, snelheid en tijd besteed aan de shelter.

Cognitieve en psychiatrische symptomen, waaronder doorzettingsvermogen, gebrek aan inzicht, afleidbaarheid, verminderd sociaal leven, angst en impulsiviteit, beïnvloedden ZvH-patiënten vóór motorische symptomen en hebben dus een vroeg schadelijk effect op de kwaliteit van het leven van de ZvH-patiënt. Het psychiatrische fenotype van BACHD-ratten is slechts gedeeltelijk onderzocht, wat aangeeft dat de BACHD-ratlijn verdere fenotypering vereist om de parallelle fenotypen te testen en met die van patiënten. Daarom, hebben we in hoofdstuk 3 een longitudinale studie uitgevoerd om het ontstaan van sociaal gedragsstoornissen bij ZvH-patiënten vast te stellen. BACHD-ratten vertoonden leeftijdsafhankelijke gedragsveranderingen toen ze opnieuw werden geïntroduceerd in hun cagemate na een periode van 24 uur van individuele huisvesting. In de sociale interactietest toonden BACHD-ratten een normaal sociaal spel en verminderde actief sociaal gedrag in vergelijking met hun WT-nestgenoten. In de sociale test met drie kamers leken BACHD-ratten een mild tekort te vertonen in de voorkeur voor sociale nieuwigheid zonder enige verandering in maatschappelijk belang. We voerden ook moleculaire analyses uit, waaruit bleek dat BACHD-dieren die werden blootgesteld aan de sociale interactietest, verminderde mRNA-niveaus vertoonden van de totale vorm van BDNF in het ventrale striatum en onveranderde striatale expressie van D1- en D2-dopaminereceptoren.

In hoofdstuk 4 onderwierpen we controle en BACHD-ratten op twee Skinner-box testen om hun impulsiviteitskenmerken te beoordelen. Impulsiviteit, slechte risicobeoordeling en veranderde gedragsinhibitie zijn vaak aangetroffen persoonlijkheidskenmerken in de ZvH. Daarom hebben we twee verschillende aspecten van impulsief gedrag onderzocht, namelijk keuze- en actie-impulsiviteit. Bij het testen op de delay discounting (DD) toonden, BACHD-ratten een hoge mate van keuze-impulsiviteit, waarbij "kleinere,
eerder” dan “grotere, latere” voordelen werden bevoordeeld. Bovendien vertoonde de BACHD-rat een gebrek aan gedragsinhibitie zoals aangegeven door bursts en premature responsen in een DRL-5 en -10 (Differential Reinforcement of Low Rate of Responding task).

Ondanks het feit dat het te weinig onderzocht is, varieert de prevalentie van angst in manifeste ZvH van 13% tot 71%, wat dus een belangrijk klinisch kenmerk van ZvH is. Omdat ZvH-patiënten last van angst kunnen hebben, hebben we in hoofdstuk 5 een longitudinale studie uitgevoerd om angstachtig gedrag bij BACHD- en WT-ratten op 2, 4, 8 en 12 maanden oud te beoordelen. In dit hoofdstuk werden klassieke en nieuwe gedragstests gebruikt om verschillende kenmerken van angstachtig en risicobeoordelingsgedrag te beoordelen, met behulp van het elevated plus maze, de light-dark box en de light spot (LS) -test in de Phenotyper. In alle testen vertoonden BACHD-ratten een trend naar verminderde angst, waardoor dit model minder geschikt was om potentiële behandelingen van angstgerelateerde stoornissen bij ZvH-patiënten te vinden.

In hoofdstuk 6 hebben we een uitgebreide bespreking gegeven van de onderzoeksresultaten die hierboven zijn samengevat en hebben we richtingen voor toekomstig onderzoek voorgesteld.

Concluderend, deze proefschrift omvat een reeks studies gericht op het karakteriseren van verschillende psychiatrische en motorische fenotypen van de BACHD-ratmodel, parallel aan symptomen van patiënten met de ziekte van Huntington. Voor dit doel zijn de studies beschreven in dit proefschrift gericht op het opsporen van fenotypen die lijken op HD-achtige symptomen, evenals op het toezicht op hun ontwikkeling door het onderzoeken van de dieren op verschillende leeftijden. De resultaten van onze studies moeten daarom worden gebruikt om specifieke vragen over mogelijke fenotype-manifestaties en hun cursus aan te pakken om nieuwe en gerichte interventionele studies te ontwerpen.
I disturbi neuropsichiatrici e neurologici costituiscono un grave problema di salute in Europa e il loro impatto sulla salute pubblica e sulla società è in continuo aumento con l'invecchiamento della popolazione. L'Organizzazione Mondiale della Sanità ha stimato nel 2006 che i disturbi neurologici, che vanno dall'epilessia alla malattia di Alzheimer, dall'ictus alle cefalee, colpiscono fino a un miliardo di persone in tutto il mondo.

Una malattia neurodegenerativa di rilevante importanza è la malattia di Huntington, o còrea di Huntington. La malattia di Huntington è una malattia genetica neurodegenerativa causata da una mutazione autosomica dominante in una delle due copie (alleli) di un gene codificante una proteina chiamata huntingtina. La malattia di Huntington è una delle malattie ereditarie da espansione di triplette: esse sono causate dall'allungamento, in misura superiore al normale, di una sezione ripetuta di un gene. Il gene, chiamato HTT, contiene una sequenza di tre basi di DNA - citosina-adenina-guanina (CAG) - ripetuta più volte, nota come espansione di triplette. Nel codice genetico, CAG rappresenta l'amminoacido glutammina, per cui una serie di queste triplette porta alla produzione di una catena di glutammina nota come tratto poliglutamminico (o tratto polyQ), mentre la parte ripetuta del gene prende il nome di regione polyQ. Generalmente le persone sane presentano meno di 36 ripetizioni CAG nella regione polyQ. Al contrario, una sequenza di 36 o più triplette comporta la produzione di una proteina che ha caratteristiche diverse.

I pazienti affetti da malattia di Huntington soffrono tipicamente di sintomi fisici, psichiatrici e cognitivi. L'insorgenza dei sintomi della malattia si può verificare in qualsiasi età (esiste anche una forma giovanile della malattia), ma più frequentemente in età adulta tra i 30 e i 40 anni. Se i movimenti appaiono bruschi e con una distribuzione casuale (còrea), essi suggeriscono una diagnosi di malattia di Huntington. Questi movimenti anomali tendono a peggiorare nel tempo. In uno stadio avanzato si assiste ad una loro diminuzione e alla chiara apparizione di altri sintomi quali parkinsonismo, distonia e rigidità. Nelle prime fasi, i pazienti presentano anche un deficit del sistema delle funzioni esecutive, della memoria a breve termine e dell'abilità visuo-spaziale. In questa fase si riscontrano anche cambiamenti di personalità e nelle facoltà cognitive che, come nel caso dei disturbi motori, si deteriorano gradualmente. Le disabilità cognitive, inoltre, tendono a peggiorare nel tempo, fino a portare ad uno stato di demenza. Sono state anche riportate manifestazioni neuropsichiatriche come apatia, ansia, impulsività e depressione, con un conseguente impatto negativo sulla vita sociale e sulle relazioni interpersonali. Le fasi successive della malattia sono invece caratterizzate da bradicinesia, spasticità, disartria, disfagia ed incontinenza. Le complicanze, come la polmonite, le malattie cardiache, e i danni fisici da cadute, riducono l'aspettativa di vita di circa 20 anni a partire dall'esordio dei sintomi.

Dal 1993, anno della scoperta della mutazione del gene HTT, sono stati pubblicati più di 13.000 articoli scientifici allo scopo di comprendere i meccanismi fondamentali della
malattia. Nonostante i notevoli progressi nello studio della malattia di Huntington, ad oggi non esiste ancora una cura che prevenga o rallenti i suoi sintomi. La lentezza della progressione della malattia di Huntington richiede spesso studi clinici lunghi e costosi. Inoltre, il numero di pazienti disponibili a partecipare a tali studi è limitato, traducendosi in un numero circoscritto di trattamenti testabili di volta in volta.

Pertanto, una delle sfide più importanti per la ricerca scientifica è lo sviluppo di farmaci più efficaci al fine di ridurre la gravità di alcuni dei suoi sintomi. I modelli animali sono pertanto indispensabili per la comprensione dei meccanismi che causano la malattia e al fine di sostenere le prime fasi di sviluppo dei farmaci.

Diversi modelli animali transgenici sono stati sviluppati in seguito alla scoperta del gene HTT e della mutazione che causa la malattia. Sono stati sviluppati numerosi modelli animali tra cui vermi nematodi, Drosophila melanogaster, topi, ratti, maiali, pecore e scimmie, che esprimono il gene mutante e presentano una progressiva neurodegenerazione e sintomi simili alla condizione. Ogni modello ha punti di forza e punti deboli e il loro uso combinato è importante per la ricerca preclinica focalizzata sui meccanismi della malattia e sulle potenziali terapie. In particolare, i modelli di ratto della malattia di Huntington sono versatili, specialmente se si considera l'elevato numero di test disponibili che consentono la valutazione delle caratteristiche motorie, cognitive e psichiatriche della malattia. Gli studi sui ratti che enfatizzano un'attenta analisi comportamentale sono stati sviluppati come modelli efficaci ed economici che completano e si aggiungono ai risultati ottenuti dagli studi clinici. Il ratto transgenico BACHD è l’ultimo modello di ratto di malattia di Huntington ed esprime l’intero gene dell’huntingtina umano con 97 ripetizioni CAG-CAA sotto il controllo del gene del promotore umano e di tutti i suoi elementi regolatori. Tale costrutto è specificatamente valido in quanto identico a quello umano (validità di costrutto). Il modello di ratto BACHD è particolarmente rilevante in quanto manifesta profili comportamentali (fenotipi) motori, psichiatrici e cognitivi simili a quelli visti nei pazienti con la malattia di Huntington (validità di aspetto). Inoltre, dal punto di vista neuroanatomico e istologico, i modelli animali mostrano atrofia e perdita neuronale selettiva e dipendente dall’età, inclusioni nucleari e citoplasmatiche. La sua validità predittiva è attualmente impossibile da stabilire in quanto non esiste ancora una cura per la malattia di Huntington.

Nonostante l’ampio utilizzo della linea di ratti BACHD negli ultimi anni, gli studi condotti finora non hanno indagato i movimenti fini e grossolani e gli endofenotipi psichiatrici come comportamento sociale, impuslività ed ansia. Lo scopo di questa tesi è l’indagine di questi domini comportamentali che sono necessari per la diagnosi precoce dei primi sintomi della malattia e per il monitoraggio del suo sviluppo, in modo che eventuali terapie possano essere più efficaci nel prevenire la progressiva neurodegenerazione.

Spesso i pazienti e diversi modelli di topi transgenici presentano problemi con la cognizione ancora prima dell’inizio di qualsiasi problema motorio. Viceversa, i ratti
transgenici BACHD mostrano un progressivo peggioramento dell’attività motoria nel test Rotarod già in giovane età. Sono state riportate anche attività alterate nel PhenoMaster, nel test open field e nell’andatura. Questi risultati suggeriscono come sia necessaria un’indagine più approfondita dei vari aspetti della funzione motoria nei ratti BACHD. Nel capitolo 2 è stata studiata la relazione tra movimenti fini e grossolani e il peggioramento dell’attività motoria dei ratti BACHD nel test Rotarod. I nostri animali transgenici hanno esibito le stesse prestazioni viste in studi precedenti, confermando come questo modello presenti anomalie motorie rispetto al gruppo controllo (genotipo wild-type, WT). Nei nostri studi abbiamo valutato i movimenti fini e grossolani dei ratti tramite il pasta handling test (test di manipolazione della pasta) e del pellet reaching test (abilità di raggiungimento del pellet). Questi test, eseguiti su animali BACHD a 2, 7 e 12 mesi di età, non hanno evidenziato deficit nel controllo motorio quando paragonati agli animali controllo. Tuttavia, quando i ratti WT e BACHD sono stati valutati per la funzione motoria grossolana nell’holding bar test (test di resistenza della presa di una sbarra), gli animali transgenici hanno mostrato una diminuzione della resistenza muscolare. Al contrario, gli stessi animali non hanno mostrato alcuna alterazione delle prestazioni nel grip strength (test della forza di presa) rispetto agli animali controllo, suggerendo la presenza di una forza muscolare inalterata. È interessante sottolineare come, quando testati in una gabbia automatizzata, i ratti BACHD abbiano registrato, esclusivamente durante la fase di luce (fase di inattività), una minore distanza percorsa, una minore velocità e un minor tempo speso sul rifugio, risultante in una minore attività locomotoria.

I sintomi cognitivi e psichiatrici, tra cui disturbo ossessivo-compulsivo, irritabilità, distrastibilità, impatto negativo sulla vita sociale, ansia e impulsività, influenzano i pazienti ancor prima della comparsa dei disturbi motori, e hanno quindi un impatto deleterio preconcetto sulla qualità della vita del paziente. Fino ad ora, il profilo psichiatrico dei ratti BACHD è stato esaminato solo in parte, suggerendo come tale linea richieda un’ulteriore caratterizzazione per poter paragonare il loro comportamento ai sintomi dei pazienti. Pertanto, nel capitolo 3 è stato condotto uno studio longitudinale per identificare l’insorgenza di disturbi del comportamento sociale degli animali BACHD a 2, 4, 8 e 12 mesi di età. I ratti BACHD hanno mostrato cambiamenti nel comportamento sociale dipendenti dall’età quando sono stati riuniti con il compagno di gabbia dopo un periodo di isolamento di 24 ore. Nel test di social interaction (interazione sociale), i ratti BACHD hanno mostrato un play behavior (comportamento giocoso) anormale e una riduzione del comportamento sociale attivo rispetto agli animali controllo. Nel three-chamber test (test sociale con tre camere), i ratti BACHD hanno esibito un lieve deficit di preferenza per la novità sociale contrariamente ad un interesse sociale invariato. Sono state anche condotte indagini molecolari, rivelando come gli animali BACHD esposti al test di interazione sociale mostrino una diminuzione dell’espressione dell’mRNA del fattore neurotrofico cerebrale BDNF (brain-derived neurotrophic factor) nello striato ventrale nonché un’espressione striatale inalterata dei recettori della dopamina D1 e D2.
Nel capitolo 4 i ratti controllo e BACHD sono stati testati nella camera di Skinner per valutare la loro impulsività. Alta impulsività, scarsa valutazione del rischio e mancata inibizione di azioni appropriate sono spesso associate a cambiamenti della personalità riscontrabili nei pazienti affetti da malattia di Huntington. Di conseguenza, abbiamo indagato due diversi aspetti del comportamento impulsivo che sono la scelta impulsiva e l’azione impulsiva. Quando sono stati sottoposti al test del delay discounting (sconto del ritardo, DD), i ratti BACHD hanno mostrato alti livelli di scelta impulsiva a favore di ricompense “smaller, sooner” (“più piccole ma prima”) rispetto a ricompense “larger, later” (“più grandi ma più tardi”). Inoltre, i ratti BACHD hanno esibito un’assenza di inibizione comportamentale, come indicato da risposte immediate e premature nell’ottenere le ricompense quando sottoposti al “Differential Reinforcement of Low Rate of Responding task” (DRL-5 e -10).

Una delle manifestazioni neuropsichiatriche meno studiate della malattia di Huntington è l’ansia, nonostante essa si manifesti in un rilevante numero di pazienti. Nel capitolo 5 abbiamo condotto uno studio longitudinale per valutare il comportamento ansioso nei ratti BACHD e WT a 2, 4, 8 e 12 mesi di età. In questo capitolo, sono stati utilizzati test comportamentali classici e innovativi per valutare i diversi tratti di comportamento ansioso tra cui la valutazione del rischio. I ratti sono stati soggetti ai test di elevated plus maze (labirinto elevato), light-dark box (test di luce-buio), e il light spot (test del punto di luce, LS) nel Phenotyper, una gabbia automatizzata. Contrariamente ai pazienti, i ratti BACHD hanno mostrato una leggera diminuzione dell’ansia in tutti i test, suggerendo come questo modello si presti meno alla ricerca di potenziali trattamenti dei disturbi correlati all’ansia.

Nel capitolo 6 abbiamo discusso i risultati della ricerca condotta e abbiamo proposto diverse direzioni per future ricerche.

In conclusione, questa tesi include una serie di studi incentrati sulla caratterizzazione di diversi aspetti del profilo psichiatrico e motorio di questo modello animale di malattia di Huntington, al fine di associarli e paragonarli ai sintomi dei pazienti, nonché a monitorare la loro progressione studiando animali di età diverse. I risultati dei nostri studi potranno essere utilizzati al fine di progettare studi interventistici mirati che possano portare allo sviluppo di trattamenti migliori per le persone affette da malattia di Huntington.
Appendices

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Do. Or do not.
There is no try.
— Master Yoda - Star Wars: Episode V – The Empire Strikes Back

Fare, o non fare.
Non c'è provare.
— Maestro Yoda - Star Wars: Episodio V – L'Impero Colpisce Ancora

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List of publications


About the author

Giuseppe Manfré was born on May 19th, 1987 in Palermo, Italy. In 2005 he obtained his Diploma of Secondary School at Liceo Classico ‘Giuseppe Garibaldi’ in Palermo (Italy) and subsequently studied Biological Sciences at the Università degli Studi di Palermo. Afterwards, he carried out a Master of Science (MSc.) in Cell and Molecular Biology, also at the Università degli Studi di Palermo. After being awarded the Erasmus European Exchange Program scholarship in 2011, he spent his second year at Lund University in Sweden, where he was introduced to the field of neuroscience. During the 10-month internship at the CNS Gene Therapy Group he acquainted himself with many experimental techniques used in the field of experimental neuroscience. Under the supervision of Dr. Luis Quintino and Prof. dr. Cecilia Lundberg he applied microRNA-regulated lentiviral vectors to target genetic modification of resident microglia in the rodent brain. Furthermore, he investigated the expression and functional activity of a regulated glial cell line-derived neurotrophic factor (GDNF) and its potential application to regulate gene expression in the brain of a Parkinson’s disease rat model. The results he obtained were included in two articles in peer-reviewed journals and one book chapter.

In 2013 he obtained his Master’s degree (summa cum laude) and started his PhD project at the Institute of Medical Genetics and Applied Genomics in Tübingen (Germany), under the supervision of Dr. med. Hoa Huu Phuc Nguyen. Phenorat, the European-funded project he was involved in, was an intersectoral PhD programme (Marie Curie Initial Training Network). The consortium included the University of Tübingen, Noldus IT and Radboud University. During his first year, he spent 4 months at the Institut des Neurosciences Paris-Saclay in France, under the supervision of Dr. Nicole El Massiou and Dr. Valérie Doyère, resulting in the lead authorship on the publication presented in chapter 4. In May 2015 he continued and completed his research at the Department of Cognitive Neuroscience at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen, under the supervision of Prof. dr. Benno Roozendaal, Dr. Judith Homberg and Dr. Johanneke van der Harst.

Overall, the research was conducted between October 2013 and December 2017, and resulted in all publications that be found within this thesis. The findings described in this thesis have been presented at different national and international meetings including the Measuring Behavior conferences in Wageningen and Dublin, the Dutch Neuroscience Meeting in Lunteren and the European Huntington Disease Network Plenary Meeting in Barcelona.
Donders Graduate School for Cognitive Neuroscience

For a successful research Institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognised as a national graduate school in 2009. The Graduate School covers training at both Master’s and PhD level and provides an excellent educational context fully aligned with the research programme of the Donders Institute.

The school successfully attracts highly talented national and international students in biology, physics, psycholinguistics, psychology, behavioral science, medicine and related disciplines. Selective admission and assessment centers guarantee the enrolment of the best and most motivated students.

The DGCN tracks the career of PhD graduates carefully. More than 50% of PhD alumni show a continuation in academia with postdoc positions at top institutes worldwide, e.g. Stanford University, University of Oxford, University of Cambridge, UCL London, MPI Leipzig, Hanyang University in South Korea, NTNU Norway, University of Illinois, North Western University, Northeastern University in Boston, ETH Zürich, University of Vienna etc.. Positions outside academia spread among the following sectors: specialists in a medical environment, mainly in genetics, geriatrics, psychiatry and neurology. Specialists in a psychological environment, e.g. as specialist in neuropsychology, psychological diagnostics or therapy. Positions in higher education as coordinators or lecturers. A smaller percentage enters business as research consultants, analysts or head of research and development. Fewer graduates stay in a research environment as lab coordinators, technical support or policy advisors. Upcoming possibilities are positions in the IT sector and management position in pharmaceutical industry. In general, the PhDs graduates almost invariably continue with high-quality positions that play an important role in our knowledge economy.

For more information on the DGCN as well as past and upcoming defenses please visit: http://www.ru.nl/donders/graduate-school/phd/