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Unravelling the behavioural and molecular hallmarks of Spinocerebellar Ataxia type 17 (SCA17)

Studies on a transgenic rat model

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Unravelling the behavioural and molecular hallmarks of Spinocerebellar Ataxia type 17 (SCA17)
Studies on a transgenic rat model

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General introduction and thesis outline
GENERAL INTRODUCTION AND THESIS OUTLINE

Trinucleotide repeat disorders

‘Neurodegenerative disease’ is a broad term used to describe disorders that afflict the nervous system. Their common characteristic is the progressive death of neurons and their inability to be reproduced or replaced by the body. Depending on the area of the brain where the nerve cell death takes place, different diseases develop and neurodegeneration can cause motoric, psychiatric and/or cognitive dysfunction.

Trinucleotide repeat disorders are a group of genetic neurodegenerative diseases caused by trinucleotide repeat expansion. At present, there are several documented trinucleotide-repeat disorders that affect humans. The first one was discovered in the early ‘90’s when a new type of mutation mechanism was found, which was an expansion of an unstable trinucleotide repeat that may be located in both the coding and non-coding DNA regions. First, the unstable, expanded (CGG)n region was found in the untranslated 5’ region (UTR) of the gene responsible for fragile X syndrome (Fu et al., 1991). Subsequently, an unstable expanded region was found in the region of the gene for the androgen receptor. This region consists of (CAG)n triplets and is responsible for the spinobulbar muscular atrophy type Kennedy (SBMA) (Spada et al., 1991). In the meantime, these triplet repeats have been found in at least 16 neurodegenerative diseases, nine of which show an accumulation of glutamines (CAGs) in the protein in question (Cummings and Zoghbi, 2000; Orr and Zoghbi, 2007). Trinucleotide diseases are commonly classified in two groups; (i) those with moderate CAG expansions that result in a polyglutamine stretch in gene sequence, hence termed as ‘polyglutamine disorders’, and (ii) those with very long expansions (usually non-CAG repeats) known as ‘non-polyglutamine disorders’ (Cummings, 2000; Shetty and Christopher, 2000). In the group of non-polyglutamine diseases, at least seven neurodegenerative diseases have been identified, such as the fragile X syndrome, Friedreich’s ataxia, myotonic dystrophy type 1, SCA8, SCA12 und HDL2 (Huntington’s Disease-like 2). All of these diseases have in common that the triplet repeats region is mostly in a non-coding region of the gene of interest, occurring in the 5’ or 3’ UTR, but also in introns (Orr and Zoghbi, 2007). The polyglutamine (polyQ) diseases group include diseases such as Huntington’s disease (HD) and Spinocerebellar ataxia (SCA) type 1, 2, 3, 6, 7, 17, as well as Dautatorubral-pallidoluysian atrophy (DRPLA) and spinobulbar muscle atrophy type Kennedy (SBMA) (Riley and Orr, 2006; Zoghbi and Orr, 2000). These are in all cases trinucleotide repeat diseases in which the disease-causing protein has a prolonged CAG range (polyQ), which leads to characteristic symptoms in every disease. These proteins are often expressed ubiquitously but lead to progressive neurodegeneration. Studies in affected patients as well as in animal models suggest that the prolonged CAG region leads to a so-called “gain of function”, which plays a general role in the pathogenesis of this group of diseases. Although the key protein in all these diseases has been identified, their function is known in only a few of these cases. An
estimated prevalence is 7-10/100,000 population for HD (Harper, 1992), 1/33,000-1/50,000 for SCAs (Whaley et al., 2011), 1/50,000 for FA (Bayot and Rustin, 2013) and 1/8000-10,000 population for MD (Suominen et al., 2011). Another prominent feature of all polyQ diseases is the so-called genetic anticipation. This phenomenon is based on the instability expanded CAG which tend to expand even further, leading to an earlier age of onset and a more severe disease course in the next generations (Figiel et al., 2012; Schöls et al., 2004).

Spinocerebellar ataxias usually end in early death and the majority of them are inherited forms with only about 30% of SCA patients showing no inherited form of ataxia. These families show no further cases of the disease occur and the genetic cause of their disease is usually unclear and are therefore called ‘sporadic’ or ‘idiopathic’ ataxias. The hereditary spinocerebellar ataxias are divided into X-linked, autosomal recessive and autosomal dominant forms. X-linked ataxias are recessively inherited with variable symptoms that usually occur early in life, including hypotonia, developmental delay, intellectual disability, ataxia, and other cerebellar signs (Bertini et al., 2000; Zanni et al., 2008; Zanni and Bertini, 2018). Autosomal recessive ataxias (ARCA) form an extensive group of clinically heterogeneous disorders that can occur at any age but whose onset is typically prior to adulthood. In addition to ataxia, patients often present with polynephropathy and clinical symptoms outside the nervous system (Fogel, 2018).

The most common of these diseases is Friedreich ataxia with symptoms that include progressive gait ataxia, lack of muscle tone in the legs, dysarthria and, in almost all cases, cardiomyopathy (Campuzano et al., 1996). Autosomal dominant cerebellar ataxias (ADCA) (also referred to as spinocerebellar ataxias (SCAs)), are far more rare than sporadic cases of cerebellar ataxia which are further divided into three subgroups: Type I comprises syndromes such as SCA1- SCA4, SCA8, SCA10, SCA12 - SCA23, SCA25, SCA27, SCA28 and DRPLA. Type II comprises syndromes associated with pigmentary maculopathies and SCA7. Finally, type III comprises pure cerebellar syndromes and includes SCA5, SCA6, SCA11, SCA26, SCA29, SCA30 and SCA31 (Harding, 1982; Whaley et al., 2011). The prevalence of ADCA (all types) is estimated at 1/37,000 worldwide. The most common ADCA type I is SCA3 followed by SCA2, SCA1, and SCA8, in descending order. Without doubt founder effects contribute to the variable prevalence between populations (Whaley et al., 2011). In general, they are characterized by progressive degeneration of cerebellum, brainstem, and spinal cord (Durr et al., 1993; Harding, 1993).

In addition to ataxia, which is the most frequent sign (68%), symptoms such as dysphagia, optic nerve atrophy, parkinsonian symptoms such as akinesia and stiff muscles, sensory disorders, muscle spasms or muscle atrophy, as well as occasional dementia, are seen in the type I group. In the type II group, a combination of ataxia and retina migration is often observed and gait impairments, ataxia of the extremities, speech disorders and eye movement disorders in the group III respectively (Rossi et al., 2014). Consequently, without proper treatment the ADCAs, polyglutaminopathies, and thus SCA17 also, can
lead to dramatic neurological dysfunction and ultimately to death – approximately within 10 to 30 years (Alendar et al., 2004).

**History of Spinocerebellar ataxia type 17 (SCA17)**

Spinocerebellar ataxia type 17 (SCA17) was first identified in 1999 in the transcription factor TATA-binding protein (TBP) in a patient with ataxia with negative family history (Koide et al., 1999). Two years later, in 2001, this syndrome was mapped to chromosome 6 and secondary CAG/CAA repeat expansions in the TBP gene were identified in families with spinocerebellar ataxia (SCA), establishing this repeat expansion as the underlying mutation in SCA type 17 (SCA17) (Bruni et al., 2004; Nóbrega, 2018). The SCA17 phenotype is particularly complex and variable with clinical symptoms which overlaps with many neurodegenerative syndromes and Huntington disease specifically. For this reason, it is also called Huntington’s disease-like 4 (HDL4; OMIM #607136) (Toyoshima et al., 2004). Compared to the other SCA subtypes, anticipation in SCA17 kindreds is rare because of the characteristic structure of the TBP gene. Additionally, diagnosis in SCA17 patients is often challenging due to non-penetrance, while the narrow gap between normal and abnormal repeat numbers, makes it difficult to determine a cutoff value for pathologic CAG repeat number in SCA17 (Toyoshima and Takahashi, 2018).

**Genetics and pathology**

SCA17 is caused by a polyglutamine expansion in the TATA-box binding protein with a widely variable prevalence among different regions and populations. Although the exact worldwide prevalence is still unknown, until 2012, fewer than 100 families with SCA17 were reported. Interestingly, due to the phenotypic similarities with Huntington’s disease (HD), accurate diagnosis can be challenging, resulting in a possible underestimation of the exact prevalence of SCA17 (Toyoshima et al., 1993). SCA17 is caused by a relatively small pathogenic range of CAG/glutamine repeats (43–66 units) in both sporadic and hereditary cases. The long expanded CAG repeat of TBP domain responsible for SCA17 has been found to be located in the N-terminus of TBP, which normally regulates the DNA-binding activity of the C-terminus of the protein (Imbert et al., 1994). In literature it has been reported that in humans, the polyQ tract normally contains 25–42 glutamine residues (Gostout et al., 1993). Expanded repeats of >42 glutamines generally result in SCA17 (Fujigasaki et al., 2001; Nakamura et al., 2001; Rolfs et al., 2003; Zühlke et al., 2001), although reduced penetrance has been observed in the range of 43–49 glutamines (Oda et al., 2004; Stevanin, 2003; Stevanin and Brice, 2008; C. Zühlke et al., 2003; Zühlke et al., 2005). Additionally, mutable normal alleles have not been reported to date (Toyoshima et al., 1993). Full penetrance alleles have been reported for 49 or greater CAG/CAA repeats, while the largest repeat size reported to date is 66 (Maltecca et al., 2003). Despite this general categorization, exceptional cases have been reported recently with one symptomatic individual having 41 repeats and four symptomatic persons having 42
repeats (Nanda et al., 2007; Nolte et al., 2010). Additionally, in a few other cases, a minimally prolonged polyglutamine range of 43 repeats has led to clinical symptoms such as mild ataxia and dementia (Silveira et al., 2002), whereas 44 CAA/CAG repeats have also been shown to induce ataxic gait and behavioral changes at a relative young disease onset (Juvenen et al., 2005; Stevanin, 2003). In individuals who are homozygous for an expanded allele in the full-penetrance range, nuclear polyQ pathology involves other brain areas including the cerebral cortex, thalamus, and brain stem (Toyoshima et al., 2004). Up to date, four homozygous individuals (with 47 and 48 CAG/CAA repeats and onset in the fourth decade) and one compound heterozygous individual have been reported showing severe and rapidly progressive symptoms, suggesting that the presence of two expanded alleles influences the severity and rate of progression of symptoms (Hire et al., 2011; Toyoshima et al., 2004; C. H. Zühlke et al., 2003). The age at disease onset can vary significantly and thus, in principle the disease can manifest at all ages. Literature suggests a mean age of ~30 years old as disease onset with an age-margin ranging from 19 to 48 years old (Nakamura et al., 2001). The most extreme age-range of SCA17 cases reported is 3-75 years old (Stevanin and Brice, 2006). The cases where SCA17 symptoms have been manifested in childhood are very few, such as the case of a 9-year old Japanese girl showing obvious ataxic gait, spasticity and muscle weakness (Koide et al., 1999), the case of a 3-year-old child that showed growth retardation, fast progression, and early death (Maltecca et al., 2003), or the youngest of three sisters which had an early onset at 8 years and symptoms such as mental retardation, ataxia and aggregative behavior (Rolfs et al., 2003). In relation to the disease onset, several groups in literature have shown a strong negative correlation between the length of polyQ and the age of onset of the disease (Nakamura et al., 2001; Rolfs et al., 2003; Stevanin and Brice, 2006; Toyoshima et al., 2004). However, although the clinical symptoms correlate with the length of the polyglutamine expansion, they are not absolutely predictive of the clinical course. Conversely, despite this clear correlation shown, in SCA17 patients a meiotic instability has been rarely been observed (Maltecca et al., 2003). That said, the significant variability in the age at onset observed within SCA17 families is most likely attributed to other markers or to the variable phenotype for which an age of onset is sometimes difficult to be predicted (Schöls et al., 2004).

**Symptomatology**

The clinical symptoms of SCA17 patients can be broad and variable, presenting a spectrum of prominent symptoms in an age-dependent manner. Common symptoms include ataxia (~90%) (manifested as gait instability, and slurred speech), involuntary movements (~70%) including chorea and dystonia, seizures, as well as parkinsonism.
Cognitive dysfunction and memory disturbance (such as dementia) have also been recognized as an initial and second most common symptom (~90%) (Nóbrega, 2018). Psychiatric symptoms, pyramidal and extrapyramidal signs, and rigidity are common in combination with occasional epilepsy (20%) and mild sensorimotor axonal neuropathy (Cellini et al., 2004; Toyoshima et al., 2004). More specifically, psychiatric symptoms frequently reported in SCA17 patients include insomnia, restlessness, nervous tics, personality changes, mood swings (including depression) (Herrema et al., 2014; Mariotti et al., 2007) and euphoria (Lin et al., 2007), aggressive behavior (Nielsen et al., 2012), self-mutilation, hypersexuality, paranoia (Fujigasaki et al., 2001), hallucinations, and psychosis (De Michele et al., 2003; Maltecca et al., 2003; Zühlke et al., 2001). Less common symptoms reported are autonomic symptoms (Doherty et al., 2014; Lin et al., 2007; Rolfs et al., 2003; Zühlke et al., 2001) (9%), apraxia (Rolfs et al., 2003) (7%) and peripheral nerve symptoms (Herrema et al., 2014; Maltecca et al., 2003; Mariotti et al., 2007) (3%). Clinical heterogeneity can be observed even within the same family (Koutsis et al., 2014). In childhood, mental deterioration may occur instead of dementia. Behavior or personality changes as initial symptoms may indicate the presence of psychiatric disorders. As patients exhibit the combination of movement disorder, psychiatric manifestations and cognitive impairment, the clinical phenotype sometimes overlaps that of Huntington’s disease (HD) making the proper diagnosis rather challenging. Such individuals are characterized to have HD phenocopy syndromes or HD-like disorders.

Neuropathologically, marked cerebral and cerebellar atrophy as well as Purkinje cell loss are typical in humans with SCA17; with less pronounced neurodegeneration occurring also in other brain regions in the cerebrum (Lasek, 2006). In addition, loss of basket and stellate cells, but also axonal torpedoes and conspicuous structures of Purkinje cell dendrites are frequently observed.

In order to map the progressing atrophy in certain areas of the brain, imaging techniques such as magnetic resonance imaging (MRI) or positron emission tomography (PET) are used in SCA17 patients. Brain MRI studies in patients have shown variable atrophy of the cerebrum, brain stem, and cerebellum. Eventually, due to the atrophy of cerebellum and cortex, the total volume of the brain gets significantly reduced (Brockmann et al., 2012; Bruni et al., 2004; Rolfs et al., 2003). In chronic cases, atrophy is prominent in the cerebellum, mild in the brainstem, and occasionally generalized cortical atrophy most obvious in the parietal lobe (Craig et al., 2005; Hagenah et al., 2004). Neuroradiological examinations in patients have shown a reduction in the availability of presynaptic dopamine transporters in the striatum as well as in glucose metabolism in the basal ganglia, whereas postsynaptic dopamine D2 receptor binding capacity was only slightly reduced (Günther et al., 2004). It is known that the formation of neuronal intranuclear inclusions (NIIs) is a common hallmark of the CAG repeat diseases (Yamada et al., 2000). The wide neuronal intranuclear distribution of the mutant proteins in the CNS, is important when considering the clinical and pathological correlations in polyQ...
diseases, including SCA17 (Sato et al., 2009; Yamada et al., 2001b, 2001a). The molecular mechanisms responsible for the pathogenesis of the polyQ diseases in general but also SCA7 specifically have not yet been completely explained (Lupton et al., 2015; Xu et al., 2015). Although at the beginning it was thought that mutant polyQ aggregates were responsible for neurodegeneration, several studies have supported the disassociation between these two (Cummings et al., 1999; Klement et al., 1998; Saudou et al., 1998). The last few years, data have been published indicating a protective role of the inclusions most probably as a result of the sequestration of the mutant protein (Arrasate et al., 2004; Bowman et al., 2007). That said, despite the similarities in the pathogenesis between SCA17 and other polyQ diseases, it remains still unclear in what way the polyQ track affects the functionality of the TBP protein. Positron emission tomography (PET) analyses have also been performed in SCA17 patients. The first patients scanned had symptoms such as progressive ataxia and dementia, exhibited both cerebellar atrophy and global cerebral atrophy together with reduced glucose metabolism, but not basal ganglia atrophy (Minnerop et al., 2005). Brain-specific atrophy has not only been observed in symptomatic patients but also in pre-symptomatic mutation carriers. Since neurodegeneration could already be detected in pre-symptomatic mutation carriers, these imaging techniques could be used in the future to monitor the disease from an early stage.

**The TBP protein**

The molecular weight of the human TBP protein (339 amino acids) is ~38 kDa and the protein contains a unique long stretch of glutamines (Hsu et al., 2014). TBP, encoding the TATA-box-binding protein, is the only gene in which a mutation is known to cause SCA17 and the gene is located on chromosome 6q27 near the telomere (Imbert et al., 1994). The **TBP gene** has two transcript variants encoding different protein isoforms. The polyglutamine-encoding DNA sequence of the wildtype alleles is divided into several areas. First, there is the highly conserved sequence, which encodes the 180 amino acids located at the C-terminal of TBP, which is responsible for binding to the target DNA. The other is a less conserved region that contains the glutamine stretch, which can be found at the N-terminal end of the TBP (Gostout et al., 1993; Hoffmann et al., 1990; Kao et al., 1990; Peterson et al., 1990; Polymeropoulos et al., 1991). This region of DNA comprises two polymorphic (CAG)n regions and the allele basic structure is (CAG)₃(CAA)₃(CAG)n CAA CAG CAA (CAG)n CAA CAG. Although the CAA CAG CAA middle part is present in all expanded alleles that are stably transmitted (i.e., the allele size is unchanged during meiosis), it was reported to be absent in two families with allele size instability. This suggests that this middle part of the repeat sequence may be a prerequisite of instability in SCA17 (C. Zühlke et al., 2003; Zühlke et al., 2005).

**TBP protein** is a general important transcription factor that binds to DNA -30 base pairs upstream of the promoter and associates with other TBP-associated factors (TAFs) to form the RNA polymerase II preinitiation complex (Stevanin and Brice, 2006) (Figure 1).
Unlike other SCA causative proteins, the function of TBP has been well studied. TBP is nuclear-localized and is the DNA-binding subunit of the RNA polymerase II transcription factor D (TFIID), which is essential for the expression of most protein-encoding genes. Thus, polyQ expansion results in transcriptional dysregulation by affecting the interaction of TBP with its binding partners (Friedman et al., 2008, 2007). It has been shown that the mutant TBP displays reduced binding to TATA box DNA in vitro, suggesting that the mutant TBP can induce neurotoxicity independent of its association with DNA (Friedman et al., 2008). That said, it is clear that in the case of SCA17 the CAG expansion affects an ubiquitous transcription initiation factor which as a component of the transcriptional complex, activates the expression of most genes (Bauer and Nukina, 2009; Koide et al., 1999; van Roon-Mom et al., 2005). As TBP is such a critical transcription factor expressed ubiquitously in all organs including the CNS, the question of whether loss of TBP function plays a role in the pathogenesis of SCA17 remains to be addressed. On the other hand, it can be argued that considering the ubiquitous presence of TBP, the selective neuronal degeneration suggests no significant loss of protein function in individuals with SCA17. It remains therefore unclear why only specific neuronal cells are prone to cell death.

Figure 1  Eukaryotic transcription initiation (© Jared Schneidman, “Molecular Machines that Control Genes,” Scientific American, figure used with permission)
Treatment of SCA17

To this day, there is no known effective treatment or cure for SCA17. Therapeutic strategies followed at this moment are designed mainly in order to ameliorate the primary and secondary symptomatology, aiming at improving the quality of life of the patients. Treatment is therefore essentially palliative. In order to improve the physical and mental condition of SCA17 patients, the current therapeutic strategies aim at treating the psychiatric problems by using the appropriate psychotropic medications, as well as by using antiepileptic drugs (AEDs) for treatment of seizures. Psychological support and monitoring are also frequently used, as well as environment adaptation and proper care based on the level of dementia shown. Dystonia can also be handled with the administration of local injections of botulinum toxin. However, psychotropic medications and AEDs can have several side effects that can burden the patients’ life (such as depression, sedation, nausea, restlessness, headaches, neutropenia, and tardive dyskinesia). For this reason, treatment may sometimes require total or intermittent discontinuation or adjustments in dose levels. In general, patients are advised to avoid the use of sedative or hypnotic agents, as well as the use of alcohol, as these may exacerbate incoordination (Toyoshima et al., 2004, 1993; Toyoshima and Takahashi, 2018).

So far, little progress has been made regarding the discovery of promising or effective disease-modifying compounds, despite the remarkable efforts that have been devoted to tackle this devastating disorder. Several ideas and therapeutic strategies have been proposed, such as rectifying the altered level of downstream transcripts or increasing mesencephalic astrocyte-derived neurotrophic factor (MANF) (Yang et al., 2016). Additionally, gene silencing techniques (such as antisense oligonucleotide, microRNA and shRNA) are being actively pursued as an option to treat polyQ diseases, with promising effectiveness results in preclinical studies for several different SCA types (Cunha-Santos et al., 2016; Evers et al., 2018; Fiszer and Krzyzosiak, 2014; McLoughlin et al., 2018; Ramachandran et al., 2014; Rodríguez-Lebrón et al., 2013; Scholefield and Wood, 2010; Toonen et al., 2017). Considering the essential functions of TBP, it is highly desirable that mutant allele-specific silencing is used when testing the efficacy for SCA17 treatment. Another approach is based on the belief that aggregates including polyQ protein fragments cause neuronal death. Accordingly, reducing the amount of aggregates is an important therapeutic strategy. However, preclinical studies using modulation of the chaperone system have yielded controversial results (Friedman et al., 2009; Weber et al., 2014; Yang et al., 2014).

SCA17 modelling: cell & animal models

Over the past 15 years, several attempts have been made to create suitable SCA17 models to explore the phenotypic features and pathogenesis of SCA17, and subsequently test potential therapies. To investigate the mechanism of neurodegeneration in SCA17, cellular models have been created including cells expressing full-length TBP with a range of polyQ expansions (Reid et al., 2003), neuroblastoma cells (Schaffar et al., 2004) and
cellular model expressing mutant TBP with 105Q (Shah et al., 2009). PC12 cell lines that stably expressed TBP containing 13Q (TBP-13Q) or 105Q (TBP-105Q) show that mutant TBP: (1) inhibits neurite outgrowth, (2) reduces the expression of TrkA (a receptor for nerve growth factor (NGF) that promotes neuronal differentiation and survival), (3) binds more tightly to the transcription factor Sp1 and reduces the association of Sp1 with the TrkA promoter. This suggests that polyQ expansion alters the association of TBP with Sp1 to down-regulate the expression of TrkA, which can contribute to the selective neuropathology of SCA17 (Shah et al., 2009). Additionally, a cellular model expressing full-length TBP with a range of polyQ expansions showed (1) insoluble intracellular inclusions in a repeat-length-dependent manner, (2) interactions of the expanded TBP polyQ tract with other overexpressed polyQ-containing proteins, (3) overexpression of expanded TBP resulting in increased Cre-dependent transcriptional activity. The latter may indicate a mechanism for aberrant polyQ gain of function, since TBP is required for transcription by all RNA polymerases, (Reid et al., 2003). Another model using neuroblastoma cells was used to investigate the interaction of polyQ-expanded Htt exon1 constructs with TBP, showing that (1) mutant Htt deactivates the transcription factors by a polyQ-mediated interaction, independent of the formation of insoluble co-aggregates, (2) interaction of toxic Htt with the benign polyQ repeat of TBP structurally destabilizes the transcription factor, (3) Hsp70/Hsp40 chaperones interfere with the conformational change in mutant Htt and inhibit the deactivation of TBP. The latter suggests a beneficial effect of molecular chaperones (Schaffar et al., 2004).

However, to understand the disease pathomechanisms on organismic level, animal models of SCA17 have been established. Small invertebrates such as flies have also been used to study the disease progression and the molecular mechanisms underlying SCA17 pathogenesis, as well as larger mammalian species such as mice and rats as they provide the benefit of investigating the systemic anomalies and the interaction between different cell populations in the body. Some of the models including their (dis)advantages are outlined in the sections below.

Several *Drosophila melanogaster* models have also been generated by overexpressing polyQ-expanded TBP using a full-length human TBP with 36 (wild-type, TBP-36Q) and 109 (pathogenic allele, TBP-109Q) glutamine residues that recapitulate pathological features such as aggregate formation, mobility defects and premature death (Hsu et al., 2014), or a mutant hTBP with a tract of 80 glutamines (hTBP80Q) that exhibit progressive neurodegeneration, late-onset locomotor impairment and shortened lifespan (Ren et al., 2011). In these Drosophila models for SCA1, neurotoxic aggregates were demonstrated, while the mutant TBP sequestered the wild-type TBP in the neuroblasts of the flies and the normal function of wild-type TBP was impaired. Additionally, Drosophila mutants with loss of Drosophila TBP confirmed that loss of Drosophila TBP function caused age-associated neurodegeneration, suggesting that dysfunction of TBP may play a universal role in polyQ-induced neurodegeneration (Hsu et al., 2014).
To date, several SCA17 rodent models have been established, including transgenic mouse and rat models (Chang et al., 2011; Friedman et al., 2007; Kelp et al., 2013), as well as conditional knock-in mouse models (Huang et al., 2015, 2011; Yang et al., 2014). All transgenic rodent models carry a human TBP cDNA with either a mouse prion promoter or a Pcp2/L7 promoter with a range of 64 to 109 CAG repeats. Conversely, all knock-in mouse models carry a floxed stop codon that prevents the expression of hTBP exon 2 with 105 CAG with different Cre-promoters (nestin-Cre, Tamoxifen-induced Cre-ER, Ella-Cre or muscle-Cre (CKmm-Cre)) with 105 CAG repeats. TBP-71Q and TBP-105Q transgenic mice can recapitulate SCA17 both genotypically and phenotypically in a repeat length dependent manner, as TBP-105Q mice show more severe genotype than TBP-71Q mice. In both mouse lines mutant TBP formed aggregates in an age-dependent manner, with decreased expression of soluble mutant TBP and a decreased lifespan and body weight loss, together with spontaneous seizures and tremor phenotypes. The L7-hTBP transgenic mouse model shows that expression of mutant TBP occurred mainly in the cerebellum and brainstem of the mice. These mice showed a less severe phenotype with no hunchback. They also displayed gait disturbance abnormalities as assessed by a foot printing assay. For the motor deficits, all the mouse models showed poor performance in the rotarod test, as well as a typical clasping phenotype when suspended from the tail. Further neuropathological characterization of these transgenic models revealed gliosis and cerebellar degeneration, degenerated Purkinje cells and axons and prominent nuclear inclusions. On the other hand, knock-in mouse models had a mutant TBP in the nuclei of the cortex, striatum, brain stem and cerebellum, with only small nuclear aggregates found in Purkinje cells. Compared with the transgenic models, knock-in mouse models displayed milder neurological symptoms and neurodegeneration. All TBP knock-in mice showed SCA17 related phenotypes, including hunchback or kyphosis, body weight loss, deceased lifespan and motor deficits. In addition, the significant muscle degeneration in SCA17 knock-in mice seemed be polyQ repeat length dependent. For a thorough description and comparison of all rodent SCA17 models, see the review published by Cui et al., 2017. As the one transgenic SCA17 rat model available in literature thus far is the main model investigated in this thesis, a detailed description of it can be found below in the following paragraphs.

The importance of a valid animal model for SCA17

All these findings support the notion that dysfunction of TBP may play an universal role in polyQ-induced neurodegeneration, allowing the clarification of the causes of other polyglutamine diseases (Toyoshima and Takahashi, 2018). To that extent, animal models enable us to understand the disease mechanisms, and bring valuable insights into the development of potential therapeutic strategies for SCA17 treatment. However, in order to ensure that the disease phenotypes occur within the life span of the mice, an extremely high number of CAG repeats is used, which may not fully recapitulate the conditions of
most SCA17 patients with moderate repeat numbers. Therefore, despite the considerable progress in our understanding of the disease, an effective cure remains out of reach. For this reason, animal models will continue to play an important role at preclinical level by helping identifying and testing promising compounds that will either prevent or slow the pace of SCA17 disease progression. Due to the importance of TBP as a transcription factor, the generation of a good animal model for the study of SCA17 has the potential to provide mechanistic insights into other polyQ diseases that share a number of common phenotypic and pathological aspects (Cui et al., 2017; Marsh et al., 2009). Such a model should comply to the three proposed criteria to define the translational potential of an animal model: construct, face and predictive validity (Belzung and Lemoine, 2011). Between the different species that are being used in research, the rat provides several advantages as a model for human disease; they are big enough in size which facilitates imaging, surgery and tissue collection, live longer and most importantly exhibit a more complex behavioural repertoire (Homberg et al., 2017; Yvonne K Urbach et al., 2010; von Horsten et al., 2003). More specifically, motor and movement analysis rats and primates show a high analogy for several motor patterns such as akinesia, tremor, postural deficits and dyskinesia, which are common characteristics of several neurological diseases (Cenci et al., 2002).

The validity of the transgenic SCA17 rat model
Concerning the three core criteria with which animal models are usually discussed, i.e. face, predictive and construct validity, the transgenic rat model of SCA17 (also known as TBPQ64 rats) covers these aspects as follows. Face validity corresponds to the observable behavioral (ethological validity) outcomes and how these resemble the symptoms and phenotypes observed in the human patients. As already mentioned, the transgenic SCA17 rats present motor and emotional deficiencies that mirror the symptoms seen among patients (Kelp et al., 2013). In the same study it was shown that neuropathologically, this rat model would display neuronal loss and degeneration mainly in the cerebellum. Predictive validity corresponds to the identity of the relationship between the triggering factor and the outcome (induction validity) and between the effects of the treatments on the animal model (remission validity). For neurodegenerative diseases like SCA17 that still remain untreated, the assessment of the predictive validity of an animal model is not applicable yet. Construct validity corresponds to the extent that the pathological features of the disease being modeled are reproduced by the animal model. Since SCA17 is a genetic disease, an animal model would have to express full-length human mutant TBP under the control of the gene’s promoter to have a high construct validity. The heterozygous transgenic SCA17 rats model used in this thesis, carries a full human cDNA fragment of the TBP gene with 64CAA/CAG repeats (TBPQ64), expressed under the control of the murine prion promoter (Prp) (Kelp et al., 2013).
The transgenic SCA17 rat model is a unique animal model as it is the first transgenic rat model for any inherited spinocerebellar ataxia. In this thesis, only heterozygous SCA17 rats were used as in work preceding this thesis (performed by Kelp and colleagues in Tuebingen University) it was found that transgenic homozygous animals do not survive until birth. As mentioned above, in SCA17 patients, homozygosity has been only in four cases reported so far, as the majority of the diagnosed patients are heterozygous (Hire et al., 2011; Toyoshima et al., 2004; C. H. Zühlke et al., 2003). This supports the use of the heterozygous transgenic SCA17 rats in this thesis as appropriate. Additionally, only male rats were used in all experiments presented in this thesis. Based on literature, no specific sex-dependent differences have been reported between human SCA17 patients. SCA17 rats have been reported to show a progressive, severe neurological phenotype including ataxia and impaired postural reflexes already from the age of 5 months. Additionally, reduced activity, loss of body weight as the disease progresses, and early death were observed (although early death was not confirmed as the animals reached the humane endpoint (HEP) at the age of 10 months; an early death of the animals is suspected given the clinical appearance of the animals at the HEP age). In detail, the SCA17 rats show a decreased body weight from ~4 months of age and a severe neurological phenotype (cachectic appearance, kyphosis, tremor, poor grooming). Young animals have been shown to be hyperactive in an automated homecage system, although there is a transfer to hypoactivity at older ages. This method also revealed differences in rearing and ambulatory behaviour as well as in food consumption. Furthermore, increased anxiety and motor abnormalities have also been reported in the SCA17 rats, shown by the significant genotype differences in the latency to fall from the rod during the RotaRod test. Differences in activity using the Phenomaster system (TSE Systems, Germany) and in an open-field setup as well as in freely walking gait test have also been reported. These differences showed an increase compared to the wildtype littermates during the pre-symptomatic age of 3 months and a decrease in the activity at the age of 9 months. This severe phenotype seen in the SCA17 rats was neuropathologically associated with neuronal loss, especially in the cerebellum. Clear degeneration of Purkinje, basket, and stellate cells, changes in the morphology of the dendrites, nuclear TBP-positive immunoreactivity, and axonal torpedos were seen using light and electron microscopy (Kelp et al., 2013). While some of these changes were successfully outlined in other existing mouse models for SCA17, evidence was provided that some crucial characteristics of SCA17 are better mirrored in SCA17 rats. That said, these findings make this SCA17 rat model a promising tool for further research and therapeutic approaches. As its validities have been minimally confirmed so far, there is need for further face validation which will hopefully help to reach a predictive validity in the future.
AIMS AND OUTLINE OF THIS THESIS

Thus far, neuropathological changes detected in the SCA17 rats recapitulate several characteristics of human SCA17 disease. However, only one study has studied the suitability of the transgenic SCA17 rats as an animal model so far (Kelp et al., 2013), and therefore little is still known about fine and gross motor function, and nothing about the effect of this genetic manipulation on other behavioural endophenotypes, which may include effects on social interest, social interaction and anxiety. There is little information about the development of a clear behavioural phenotype preceding the onset of symptoms in the SCA17 rats. Detection of the first core symptoms of the disorder timely and monitor the development of these across time remains to be investigated. This will enable detection of early onset of specific symptoms and provide valid read-out parameters for future therapeutic studies which is a main objective of this thesis. Secondary objectives were the use of a combination of behavioural observations in the period before the onset and during progression of SCA17, to correlate the observed changes in behaviour with SCA17 progression. This will extend previous findings regarding the motor disturbances with more automated and sophisticated tests. The identification of potential non-motor symptoms in SCA17 rats such as anxiety and alterations in social interaction which are also reported in patients suffering from this fatal disorder is also one of the objectives of this thesis. The obtained data will contribute to the validation of read-outs obtained by automated behavioural observation equipment such as the PhenoTyper and Catwalk, which may be of interest for future application to detect and monitor symptoms of other similar neurodegenerative diseases. Finally, I aimed at linking relevant behavioural responses with immunohistochemical and several different biochemical analyses for measuring neuronal activity and different protein and neurotransmitter levels in relevant brain areas. A schematic summary of the information already known and the aspects investigated in this thesis is presented in Fig. 2.

Up until now, the SCA17 rat model was shown to have some motor impairments such as increased ataxia scores and significant genotype differences in the RotaRod test (Kelp et al., 2013). In the same study, using an automated homecage environment, differences in activity were reported, showing an increase during the pre-symptomatic age of 3 months and a decrease in the activity at the age of 9 months. Based on these findings, a more in-depth analysis of the different aspects of the motor function in the SCA17 rats was set as one of the objectives of this thesis. To this end, the study presented in Chapter 2 was designed to further investigate the different aspects of motor phenotype of this SCA17 rat model by using a combination of traditional and automated behavioural tests. The motor aspects that were chosen to be investigated are the fine and gross motor control, muscle strength, gait and locomotion.

Subsequently, in Chapters 3 and 4, the identification of potential non-motor symptoms in the SCA17 rat model, such as anxiety and social interaction, were investigated
as part of the objectives of this thesis, since alterations in these behaviours are also reported in SCA17 patients suffering from this disorder. Anxiety is not only one of the most frequent symptoms reported in human SCA17 patients (Bruni et al., 2004; Rolfs et al., 2003), but it is also an important aspect of neurological phenotyping of animal models in behavioural neuroscience (Yvonne K Urbach et al., 2010). Therefore, due to the deleterious effects on patients’ lives, the importance of studying the psychiatric disorders along with the motor symptoms at both clinical and preclinical level is clearly important. In Chapters 3 and 4, anxiety-like and risk assessment traits were investigated longitudinally in the SCA17 rats, using both classic and novel automated paradigms such as the elevated plus maze, the open field and an approach-avoidance task for assessing anxiety within a homecage environment called ‘light-spot’ (LS) test. Additionally, the relevant behavioural responses were also linked to c-Fos neuronal activity in the amygdala after exposure to the elevated plus maze.

Chapter 5 focuses on social behaviour in the SCA17 rats to evaluate potential deficits that recapitulate the neuropsychiatric symptoms seen in patients. Psychiatric symptoms, behavioural abnormalities, personality changes, social isolation, and marked deficits in understanding social cues are observed frequently in SCA17 patients (Pollack et al., 1995; Riva and Giorgi, 2000; Tavano et al., 2007). Behavior or personality changes as initial symptoms may indicate the presence of psychiatric disorders. To investigate the social behaviour repertoire and social interest of SCA17 rats, we performed detailed analyses of social behaviour and examined several different parameters to monitor the development of potential deficits over time. Testing was performed from pre-symptomatic age (3 months) until a fully-developed phenotype age (9 months).

Another objective of the work presented here was to evaluate the changes of different proteins and neurotransmitters in the SCA17 rat model using different biochemical analyses. SCA patients have been shown to have nuclear accumulation of mutant proteins and inclusions, as well as disturbance of dopamine (DA) and/or serotonin (5-HT) metabolism in different brain areas. Additionally, recent studies have demonstrated a strong link between neurodegeneration and chronic inflammation. Chapter 6 shows the development of the accumulation of TBP aggregates and soluble mutant and endogenous TBP, and the metabolic levels of DA and 5-HT in the SCA17 rat model in different brain areas and ages throughout disease development. Additionally, signs of neuroinflammation and gliosis were investigated in different brain areas and age points.

Last, Chapter 7 provides a general discussion on the results presented in this thesis and suggests directions for future research related to the validation and application of the SCA17 rat model.
Figure 2 Overview of known information and aspects investigated in this thesis

Known:
- Hyperactivity
- Lack in basket & stellate cells
- Body weight loss
- BeamWalk impairments
- Increased anxiety

Investigated in this thesis:
- Fine motor control
- Muscle strength
- Gross motor control
- Social interaction
- Anxiety-like behavior
- TBP aggregation
- Mutant & endogenous soluble TBP levels
- Purkinje-cell loss & microglia and astrocyte activation

- Hyperactivity
- Neurodegeneration, TBP & reduced calbindin immunoreactivity in cerebellum

- High ataxia score
- RotaRod impairments

- Hypoactivity
- Neurodegeneration, TBP & reduced calbindin immunoreactivity in cerebellum

- Body weight loss
- BeamWalk impairments
- Increased anxiety

- HEP
- Degeneration of Purkinje, basket, and stellate cells
- DTI imaging monitors neuropathology

- Fine motor control
- Muscle strength
- Gross motor control
- Social interaction
- Anxiety-like behavior
- TBP aggregation
- Mutant & endogenous soluble TBP levels
- Purkinje-cell loss & microglia and astrocyte activation

- Hypoactivity
- Neurodegeneration, TBP & reduced calbindin immunoreactivity in cerebellum

- Body weight loss
- BeamWalk impairments
- Increased anxiety

- HEP
- Degeneration of Purkinje, basket, and stellate cells
- DTI imaging monitors neuropathology

- Fine motor control
- Muscle strength
- Gross motor control
- Social interaction
- Anxiety-like behavior
- Monoamine analysis

- TBP aggregation
- Mutant & endogenous soluble TBP levels
- Purkinje-cell loss & microglia and astrocyte activation

- Body weight loss
- BeamWalk impairments
- Increased anxiety

- HEP
- Degeneration of Purkinje, basket, and stellate cells
- DTI imaging monitors neuropathology

- Fine motor control
- Muscle strength
- Gross motor control
- Social interaction
- Anxiety-like behavior
- Monoamine analysis

- TBP aggregation
- Mutant & endogenous soluble TBP levels
- Purkinje-cell loss & microglia and astrocyte activation
Ethical statement: all experiments performed in this thesis using the transgenic SCA17 rats were performed after approval of the Ethical Committee for Animal Experiments of the Radboud University Nijmegen Medical Center for compliance to ethical standards and use of laboratory animals according to EU-guidelines. The inherent development of discomfort and the specific criteria for the humane endpoints were thoroughly and repeatedly monitored and discussed with the Animal Welfare Officers of Radboud University.
General introduction and thesis outline
The SCA17 transgenic rat model exhibits motor-related impairments in a battery of motor function tests

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ABSTRACT

Background: SCA17 is a rare autosomal dominant neurodegenerative disease with a complex and variable phenotype characterized by a broad array of motor and non-motor deficiencies (such as psychiatric symptoms and seizures). Patients show symptoms such as ataxia, dystonia and parkinsonism, resulting in impaired grasping, gait abnormalities and concurrent chorea. The SCA17 transgenic rat model is based on development of several disease symptoms in heterogeneous animals over the course of 3 to 9 months. This transgenic model is relatively new and offers the opportunity for a comprehensive characterization of the development of the motor phenotype.

Objective: This study aims to characterize several motor skills in the SCA17 transgenic rat model using both traditional and automated behavioural paradigms.

Methods: Gross and fine motor control was assessed longitudinally at 3, 6 and 9 months of age in SCA17 transgenic male rats and their wildtype littermates. Gross motor function was evaluated using the CatWalk gait analysis system and the grip strength and beam walk tests. Fine motor control was measured using the pellet reaching test.

Results: SCA17 rats showed strong impairment in pellet reaching already at 3 months of age, which persisted at the subsequent time points tested. Ataxic and uncoordinated gait were also observed in transgenic animals together with an affected muscle strength, which was most prominent at the last stage of the disease.

Conclusions: The SCA17 rat model recapitulates several features of the SCA17 patients such as reduced muscle force, ataxic uncoordinated gait and loss of hand dexterity. These clear deficits observed in several aspects of the motor function indicates that the SCA17 transgenic rat model could be a valid model for future treatment studies.
INTRODUCTION

Spinocerebellar ataxia type 17 (SCA17), is a rare autosomal dominant cerebellar ataxia (ADCA) caused by a heterogenous expansion of CAG/CAA trinucleotide repeats within the coding gene of the TATA box binding protein (TBP) (Hsu et al., 2014; Whaley et al., 2011). The onset of SCA17 is commonly during the second or third decade in life, but there are cases in which the onset occurs in childhood or old age. SCA17 is characterized by progressive cerebellar ataxia, psychiatric disorders, pyramidal and extra-pyramidal signs, involuntary movement, such as chorea and dystonia, and weight loss (Nakamura et al., 2001). So far, no disease-modifying therapy exists for any of the SCAs due to the limited knowledge of the dysfunctional pathways. Although disease progression is currently untreatable, efforts are made to identify novel symptomatic treatments (Fan et al., 2014). To this extent, identifying suitable animal models that recapitulate the pathological and behavioural profile of the human patient situation which can serve for the identification of novel therapeutic approaches is an essential research goal (Marsh et al., 2009). Between the different species that are being used in research, the rat provides several advantages as a model for human diseases. Rats (1) are big enough in size which facilitates imaging, surgery and tissue collection, (2) live longer and most importantly (3) exhibit a complex behavioural repertoire (Homberg et al., 2017; Yvonne K Urbach et al., 2010). Additionally, concerning motor and movement analysis, rats and primates show a high analogy for several motor patterns such as akinesia, tremor, postural deficits and dyskinesia (Cenci et al., 2002). These are common characteristics of several neurodegenerative diseases including SCA17, which confirms the suitability of the rat for modelling motor neurodegenerative diseases.

The SCA17 rat is a recently generated transgenic rat model that carries the full-length human TBP-cDNA with an abnormal CAG/CAA repeat of 64 codons which is currently being characterized in order to understand the advantages and limitations. SCA17 rats show a wide variety of behavioural characteristics mimicking the emotional and motor alterations observed in the SCA17 patients (Kelp et al., 2013; Kyriakou et al., 2017, 2016). Primary characterization of the SCA17 rats showed a severe neurological phenotype including ataxia, impairment of postural reflexes and hyperactivity in early stages, followed by reduced activity, loss of body weight, early death at a later stage of the disease. Other characterization studies of this rat model showed emotional alterations, as suggested by a higher level of risk assessment and an increase in anxiety-related behaviour in the elevated plus maze (Kyriakou et al., 2017). SCA17 rats also exhibited a strong approach-avoidance conflict and shelter seeking behaviour in a novel, fully automated home-cage implemented anxiety test called ‘light spot’ reflecting again increased risk assessment behaviour (Kyriakou et al., 2018). More specifically, our SCA17 rat model has shown impairments associated with increased ataxia scores starting from the age of 5 months and significant genotype effects in the latency to fall from the rod.
during the RotaRod test. Differences in activity using the Phenomaster system (TSE Systems, Germany) and in an open-field setup as well as in freely walking gait have also been reported, showing a decrease in the activity at the age of 9 months and a respective increase during the pre-symptomatic age of 3 months (Kelp et al., 2013; Kyriakou et al., 2016). Based on these findings, it becomes clear that a more in-depth analysis of the different aspects of the motor function in the SCA17 rats is needed. To this end, the present study was designed in order to further investigate the motor phenotype of this SCA17 rat model by focusing on the different aspects of motor function and by using a combination of traditional and automated behavioural tests. The motor aspects that were chosen to be investigated are the fine motor control, muscle strength, balance and gait.

One important aspect of motor control is the gross motor function. SCA17 patients are known to show impaired gross motor functions manifested in several different ways such as chorea, gait and/or limb ataxia, intention tremor/parkinsonism (Rolfs et al., 2003). Translating these symptoms to an animal model and identifying these aspects during the characterization process is therefore crucial and requires the use of a battery of different tests. For this, classical as well as novel behavioural paradigms are available in literature. Previous studies in SCA rodent models using different setups have shown consistent motor phenotypes (Hübener et al., 2012; Portal et al., 2013). Additionally, several automatization efforts have been done in the neuroscience field in order to increase experimental throughput and accelerate the phenotyping process using a wide range of methods from continuous automated home cage assessment to automated quantitative gait analysis systems etc. (Bains et al., 2018; Schaefer and Claridge-Chang, 2012; Vandeputte et al., 2010). These efforts aim at delivering more meaningful interpretation of specific behavioral elements and therefore at increasing the reproducibility and reliability of the results. Here we used CatWalk® (Noldus Information Technology, Wageningen, The Netherlands) to generate a gait analysis profile over the three age points tested.

Another important part of the motor pathway is the control of skilled voluntary movements such as reaching and grasping (Alstermark and Pettersson, 2014). Single handed prehension in humans is an advanced normal skill described as conjoint changes in digit forces and moments during multi-finger grasping tasks (Latash and Zatsiorsky, 2016). Such tasks involve also the capability to perform fractionated finger movements, representing the exquisite behavioral attribute of manual dexterity (Schmidlin et al., 2011). These functions are also to some extent homologous in rat’s forepaws (Cenci et al., 2002), providing once more evidence about the suitability and advantages of the rat for modeling motor functions (Klein and Dunnett, 2012). Rats use their forepaws for reaching and grasping stuff and especially for the so-called ‘reach-to-eat’ behaviour, which can be used for assessing hand use in skilled reaching by the rat (Alaverdashvili and Whishaw, 2013). Patients with cerebellar degeneration have been reported to have impaired prehension kinematics and grasping forces, thus, the investigation of these aspects in
our animal model is of great importance for the use of these potential read-outs in future treatment studies (Brandauer et al., 2008).

The work described in this chapter aimed to identify subtle motor impairments as early as possible during disease progression. In order to achieve a more holistic characterization as well as to provide potential readout parameters for future treatment of SCA17, we applied both classical behavioural assays and automated systems with high-throughput output. The transgenic (heterogenous) SCA17 rats and their wildtype littermates were tested longitudinally at 3 different age points (3, 6 and 9 months old) in order to capture as much information as possible about the onset of the different deficiencies and how these progress in time.

MATERIALS AND METHODS

Animals
To minimize the effect of extensive testing to its minimum, two cohorts of naïve male rats were used (i.e. four different groups: two SCA17 groups and two groups of wildtype littermates for the CatWalk, beamwalk and grip strength test experiments: SCA17 n=11 and WT n=132; for the pellet reaching task: SCA17=10 and WT=10). The SCA17 rats were the transgenic heterozygous TBPQ64 rats carrying the full-length human cDNA of the TBP gene with 64 CAG repeats under the control of the murine prion protein promoter (Prp-TBPQ64) and an N-terminal myc-tag. All transgenic animals used in this study were heterozygous and had a Sprague Dawley background. Animals were genotyped using ear tissue collected at postnatal day 21, during weaning, according to transgene-specific PCR protocol as described by Kelp et al. (2013). All animals were socially housed by 3 or 4 of the same sex and genotype, in Makrolon-IVS polycarbonate cages (Techniplast, Italy) except during designated experimental procedures (described in the sections below). Food (standard rat chow: Ssniff, Germany) and municipal water were available ad libitum at all times and reversed day-light conditions (dark: 08:00-20:00) were used in the housing and experimental room with dim red-lights used throughout the dark-cycle. All experiments were performed in compliance to the EU guidelines for the use of animals for scientific purposes and evaluated and approved by an external independent Ethics Committee.

For all experiments, transgenic animals were obtained from an in-house colony preserved and maintained at Radboud Medical Center (Nijmegen, The Netherlands) by crossbreeding transgenic males with wildtype female rats (Charles River, Sulzfeld, Germany). Pups were weaned at postnatal day 21 and were group housed per 3 or 4 with littermates of the same sex and genotype. For this study, male rats were selected and used. Female

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2 The uneven number of animals per genotype and age group is due to the variation in the litter sizes.
littermates of both genotypes were kept for other experiments (see Lorbach et al. (2018)), or for other experiments (not shown in this thesis). Body weights of all animals were measured on a weekly basis throughout the duration of the study in order to monitor at first the growth and subsequently the health status of the SCA17 rats as it is known that SCA17 rats lose a significant amount of weight already from the age of 5 months (Kelp et al., 2013).

**Study Design**

Two cohorts were used for this study, each cohort consisted of two groups: one SCA17 group and one wildtype control group. Cohort 1 was subjected to the gross motor control tests and cohort 2 was subjected to the fine motor control tests. This design was chosen to avoid the effect of extensive testing of the animals and due to logistic reasons since the pellet reaching task requires a long training schedule and several testing days at each age point tested. All experimental procedures described here were performed during the dark phase of the animals.

**Cohort 1: Gross motor control**

Animals were divided into two groups (control and SCA17) and were tested longitudinally at the ages of 3, 6 and 9 months old following the schedule described below in table 1. The protocol is described in detail by Kyriakou et al. (2016). The reason why there are additional days at the 3 months age point, for both CatWalk and beam walk tests is explained below. The CatWalk and beam walk runs were respectively captured and recorded automatically via the CatWalk software, or video recorded via MediaRecorder® software (Noldus Information Technology, Wageningen, The Netherlands) and subsequently scored blindly to the rats’ genotype via Observer® XT 12.5 (Noldus Information Technology, Wageningen, The Netherlands).

**Table 1** Experimental schedule for gross motor control tests

<table>
<thead>
<tr>
<th>Time point tested/Weeks</th>
<th>3 months old</th>
<th>6 months old</th>
<th>9 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>Day 1-2-3-4: CatWalk</td>
<td>Day 1: CatWalk</td>
<td>Day 1: CatWalk</td>
</tr>
<tr>
<td>Week 2</td>
<td>Day 1-2-3-4: Beam Walk Day 5-6: Grip strength</td>
<td>Day 1: Beam Walk Day 5-6: Grip strength</td>
<td>Day 1: Beam Walk Day 5-6: Grip strength</td>
</tr>
</tbody>
</table>
CatWalk test

Gait analysis was performed using the CatWalk® system (Noldus Information Technology, Wageningen, The Netherlands). The apparatus consists of an enclosed corridor (width 8 cm to prevent the animals turning which would interrupt their straight movement) with a glass plate floor (L × W × H: 130 × 68 × 152 cm³), and a goal-box at the end containing the home-cage of the animals. The runway is illuminated from the ceiling with a red light and from the long edge of the glass plate with a green light (green intensity threshold 0.12 and camera gain 15.84). When the animals’ paws make contact with the glass plate, the light is internally reflected and captured by a high-speed video camera which is fixed 60 cm below the corridor (Kyriakou et al., 2016). In this study, CatWalk® was used to analyze the gait, motor function and coordination in the SCA17 rats. The CatWalk has been found to be sensitive to a wide range of injuries and impairments, as it is an automated gait analysis technique that allows objective and rapid quantification evaluated so far for use in several animals models of pain (Gabriel et al., 2009, 2007), sciatic nerve injury (Bozkurt et al., 2008; Deumens et al., 2007), spinal cord injury (Hamers et al., 2006), arthritis (Ångeby et al., 2008; Ferreira-gomes et al., 2008), and CNS based movement disorders such as Parkinson’s, Huntington’s, stroke and spinocerebellar ataxias (Abada et al., 2013; Kyriakou et al., 2016; Vandeputte et al., 2010). In several animal models the CatWalk analysis has been proven to be more sensitive that other standard tests such as the well-known RotaRod since it provides more detailed information on specific gait parameters (Vandeputte et al., 2010). Most importantly, CatWalk has the advantage of provided both static gait parameters as well as time-based information and pressure parameter against other systems (Noldus Information Technology b.v., 2015).

Because data acquisition is dependent upon the animals walking across the runway, one issue we had to take into account in the present study is the altered mobility or motivation to walk in the SCA17 rats across the different age points we tested as the disease symptoms progress over time. The entire training and testing protocol and the definition and significance of changes for each parameter has been described here: Kyriakou et al., (2016). The training protocol is applicable only during the first age point tested (i.e. 3 months old). Regarding the testing procedures, we noticed that the animals of both genotypes were able to remember or recognize the concept of the test during the 2 age points that followed and performed adequate for acquiring satisfactory CatWalk runs. Therefore, for the 3 months testing we used day 4 (i.e. testing day as described in our protocol) in our analysis and for the 6 and 9 months testing we used day 1 (prior habituation of repeated training was not considered necessary). This method was chosen in order to minimize as much as possible any session effects that may possibly occur for some gait parameters as we have shown in the following chapter. A minimum of five good runs were recorded for each rat and test age. Several static (e.g. base of support, print width, max contact area, stride length), and dynamic (e.g. swing duration) gait parameters were measured from the analysis of the CatWalk, as well as
several speed and coordination parameters (e.g. swing speed, stand index, phase dispersions) 
(swing duration and max contact are described in the supplementary material). The primary 
readouts concerned the rats’ gait, balance and coordination. For all parameters that 
CatWalk analysed all 4 paws separately we averaged the 2 front paws and 2 hind paws 
together resulting in one front- and one hind-paw result per parameter per animal. Since 
our focus is on a neurodegenerative animal model which doesn’t show any limb-specific 
deficits, the analysis of each paw separately was considered irrelevant. For the acquisition, 
scoring and analysis of all runs, the software CatWalk v. XT 10.6 (Noldus IT) was used.

**Grip strength test**

With this test functional performance and strength of the forelimbs was assessed in 
order to evaluate the rodent muscle force *in vivo*. The test is based on the tendency of a 
rodent to instinctively grasp a grid when suspended by the tail or the body. The apparatus 
used (grip strength meter, Ugo Basile, Italy) was equipped with a T-shaped horizontal 
holding bar connected to a force transducer indicating the maximum force applied by 
the subject at each grasp and pull attempt. Force was indicated as force to grams and 
each animal was suspended by the base of the tail with the nose pointing towards the 
puling bar allowing the animals to detect and grasp the bar by themselves. When this was 
grabbed, the body was lowered till it was horizontal and then the rat was slowly pulled 
away from the apparatus with relative minimum force possible until the animals let the 
bar go. All animals were trained on one day and tested the following day, on the same 
time of the day, using a slightly adapted protocol as described by Aartsma-Rus and van 
Putten (2014), until the necessary number of pulls was obtained. Each rat was let to 
perform five series of pulls, each followed by a short resting period resulting into a total 
of 15 pulls (3 pulls x 5 times = 15 pulls). Gram-to-force results were subsequently also 
corrected for the body weight of the animal to correct for possible confounding effects 
due to differences in the body weight especially during the 6 and 9 months of testing. 
For analysis, the mean value of the five highest values scored per animal was used 
(Aartsma-Rus and van Putten, 2014).

**Beam walk test**

We used the beam walk test to examine the motor coordination and balance in the 
SCA17 rats as compared to their wildtype littermates. The beam walk provides a well-es-
tablished method of monitoring motor coordination and balance in rodents (Buddeberg 
et al., 2004; Metz et al., 2000). The method has been commonly used to test mostly 
sensorimotor function in the rat, for example to assess sensorimotor coordination 
following injury (Starkey et al., 2005) or in CNS based movement disorders such as 
Parkinson’s, Huntington’s, stroke and spinocerebellar ataxias (Allbutt and Henderson, 
2007; Kelp et al., 2013; Nguyen et al., 2006; Schaar et al., 2010). During the task the 
animals must traverse a 165 cm long narrow beam (see Fig. 3D) which in our case was
flanked by a ledge of 2 cm on both sides in order to prevent the animals from falling off the beam entirely when foot slipping, potentially creating a negative association with the test. The upper level on which the rats have to walk gets narrower towards the end of the beam, starting at 6 cm and getting progressively narrower until only 1.5 cm wide. The whole beam is elevated at a height of 96 cm, high enough to avoid animals from willingly jumping off the beam. A goal box was located at the end of the beam on the ‘safety platform’ where similarly to the CatWalk protocol, a small amount of chocolate sprinkles was placed as a motivator. The beam walk apparatus was custom made used the specifications were based on the beam walking apparatus of OpenSciences Ltd, Russia. This ledged tapered version of the beam walk we used in our study has been described as a more hindlimb-oriented testing in rats as it can detect the displacement dysfunction of the hindlimbs in rats. Foot faults made with the hindlimbs are characterized as deficits in hindlimb dysfunction. For this task, pre-training is required until the animals learn to walk across the beam without turning around and without making any faults. A short break between trials was allowed to prevent over-training. Although it has been argued that hindlimb testing is difficult to assess in rodents, the ledged tapered beam task is able to detect deficits that rats might normally make compensatory adjustments (Schaar et al., 2010). In that case, deficiencies can be concealed if tests such as the CatWalk are used, where animals are let to walk freely on the glass plate. At the age of 3 months, all animals were trained for 3 consecutive days and tested on the fourth day. At the age of 6 and 9 months, the animals were only tested for one day, as both genotypes seemed to remember the apparatus and could transverse the beam without interruptions or turns. For each rat at least 3 runs per day were recorded with a resting period of 10 minutes in between consequent runs to prevent overexposure to the test. All runs were recorded from two different sides using 2 infrared or night vision cameras and the MediaRecorder® software and were scored using The Observer v. XT 12.5 software (Noldus Information Technology, The Netherlands). For our experiment we scored the front and hind foot slips regardless of the side of the paw which slipped. Just as in the case of CatWalk such a distinction was not considered important for the motor assessment of our SCA17 rat model. The front and hind foot slips made during each run were measured by the observer and averaged per day. For the statistical analysis only the results of day 4 (i.e. the testing day) were used for the 3 months old age point and day 1 for the ages of 6- and 9-months old testing.

**Cohort 2: Fine motor control**

**Pellet Reaching test**

The pellet reaching task (also known in literature as reaching chamber or pellet retrieval task (Schaar et al., 2010)) was used in this study to assess accurate forepaw reaching and forelimb motor control of the SCA17 rats. This test has been shown to be sensitive to
motor functioning deficits after brain damage, stroke, and neurodegenerative diseases such as Parkinson’s or Huntington’s disease (Cirillo et al., 2018; Karl and Whishaw, 2011; Klein et al., 2012; Klein and Dunnett, 2012; Manfré et al., 2017; Schaar et al., 2010). For our experiments a transparent plexiglass chamber (35 cm L x W x H) with 2 slit vertical openings in each side of 1 cm each was used (see Fig. 4B). The Plexiglas chamber was placed into a wooden frame, with a 3 cm platform above the floor upon which the pellets were placed in front of the slit openings, allowing the animals to reach for the 20 mg sugar precision pellets (Bio-Serv, Dustless Precision Pellets). At first, the rats were habituated to the sugar pellets for 2 consecutive days by placing a spoonful into their home cages and monitoring if they consumed the pellets. After this, the animals were habituated to the chamber for 3 consecutive days for a short period of time (approximately 10 min per animal per day). During these sessions, a total of +/- 20 sugar pellets were placed randomly on the floor and animals were let to explore the chamber and the presence of the pellets in it. The next phase contained the shaping of the animals to the test. During this phase the animals were tested with one session per day for a total of 5 days. During these 5 days 2 goals were to be achieved: (1) limb preference for each animal and (2) the animals had to learn to reach for the pellets via the slit opening. To achieve this, the pellet was placed directly in front of the opening at shaping day 1 and then the distance was gradually increased (the tempo of distance increase was adjusted per animals depending on the animal’s performance to allow sufficient time to learn) until a distance of 1.5 cm was achieved. During these sessions the limb preference was also determined by scoring and analyzing which of the two paws was used at 80% of a (successful) reach. Two shaping days were considered enough for identifying the preferred paw. In the subsequent shaping sessions pellets were placed contralateral to the preferred limb. To stimulate the animals’ motivation to reach for the sugar pellets, food was removed from their home cages maximum 1 hour before the shaping or testing sessions. As food deprivation was not an option due to the body weight loss in the SCA17 rats from the age 4.5 months old, this short food removal before the testing sessions was considered a mild stimulation for the animals to reach for the sugar pellets and did not cause any further body weight loss to the transgenic rats. Using this approach, all animals were keen on reaching for the pellets. Animal that repetitively used their tongue instead of their paws and were further trained by increasing the distance to 2 cm from the openings. At the end of the five shaping days all animals had reached a sufficient level of performing the test and were able to go to the next phase (a minimum of 20 reach attempts during one session was used as criterion). The next phase of the test was the testing sessions. In this phase each animal underwent a testing session for a total to 10 days. During these sessions, the animals received 30 pellets in total. Each pellet as considered a trial. The first 10 pellets were considered as a ‘warm-up’ and were therefore not scored and analysed. The next 20 pellets were considered the testing trials. During these trials the pellets were placed alternatively in one of the two sides of the box and in
a pseudo-random pattern between the two slits of each side of the box. This created a predicted order regarding the side of the pellet which helped the animals to move from one side of the chamber to the other and adjust their body posture after each reaching trial. All sessions were recorded using 2 infrared or night vision cameras and the MediaRecorder® software and vides were scored using The Observer v. XT 12 software. The first-attempt success rate was calculated for each rat and each session. A reach was considered first-attempt successful when the animal had managed to grab the pellet with the first attempt, placed it into his mouth and consumed it without dropping it on the floor. All other attempts were considered as failures and were not scored and analysed. A success rate per day per animal was calculated by dividing the number of successful reaches by the total number of 20 trials.

The habituation and shaping sessions were performed only at the 3 months old age point. Subsequently at each age point, a total of 10 training sessions were performed. Re-habituation and/or reshaping were not considered necessary, as from the very first session in the chamber at the ages of 6 and 9 months old the animals showed immediate response to the expectations of the test and performed reaching attempts.

### Table 2  Experimental schedule for fine motor control test

<table>
<thead>
<tr>
<th>Time point tested/Weeks</th>
<th>3 months old</th>
<th>6 months old</th>
<th>9 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 1</strong></td>
<td>Day 1-2 Habituation pellets in home cage</td>
<td>Day 1-2-3-4-5: Testing sessions</td>
<td>Day 1-2-3-4-5: Testing sessions</td>
</tr>
<tr>
<td></td>
<td>Day 3-4-5 Habituation in chamber and pellets</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Week 2</strong></td>
<td>Day 1-2-3-4-5: Shaping sessions</td>
<td>Day 1-2-3-4-5: Testing sessions</td>
<td>Day 1-2-3-4-5: Testing sessions</td>
</tr>
<tr>
<td><strong>Week 3 &amp; 4</strong></td>
<td>Day 1-2-3-4-5: Testing sessions</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Analysis

All data were analysed using IBM SPSS v.21 and v.22 statistical package (IBM, Corp, Chicago, IL) and graphs were made using GraphPad Prism v6.0 (GraphPad software, San Diego California, USA). Two-way repeated measures ANOVA (RMA) was used to analyze all parameters with age used as a within-subject factor and genotype as between-subject factor respectively. Bonferroni post-hoc test was used to investigate further any significant genotype x age effects if indicated by a significant group and/or age effect. A p value of < 0.05 was considered statistically significant. For the pellet reaching test, a RMA test was performed for each age point separately with testing days as within-subject factor and genotype remained a between-subject factor. For looking at
possible effects of the body weight on body weigh-sensitive CatWalk parameters, a partial correlation was performed between body weight and the relevant parameters, by using genotype as a covariate.

During the CatWalk experiment two control rats and one SCA17 rat kept turning back despite all efforts to obtain the sufficient number of runs in the CatWalk apparatus and were therefore excluded from the analysis. These dropouts did not have an effect on the power of the data. These rats were excluded during all three age points but performed satisfactory in the rest two tests (grip strength and beam walk) where they were normally included in the analysis.

RESULTS

**Cohort 1: Gross motor control**

**CatWalk test**

**Static parameters**

Based on the selection made during our pilots (Kyriakou et al., 2016), only a limited number of the CatWalk parameters were analysed which were shown to be relevant to our model. The regularity index (% of regular step patterns) did not show any differences between wildtype and SCA17 rats. (data not shown).

The front and hind leg spread respectively was calculated over the different runs at the three different testing ages. **Figure 1. A1 & A2** show the statistically significant decrease in the spread of the hind paws when walking at all three ages tested (genotype effect: (a) front paws: $F_{(1,18)}= 0.03$, $p= 0.96$; (b) hind paws: $F_{(1,18)}= 11.3$, $p<0.01$). For both parameters both groups were found to have larger front-paw and smaller hind-paw spread, respectively, by the final age of testing (age effects: (a) front paws: $F_{(2,36)}= 57.89$, $p<0.001$; (b) hind paws: $F_{(2,36)}= 28.18$, $p<0.001$). An interaction effect was present only for the front paws ($F_{(2,36)}= 6.83$, $p<0.01$), with further post-hoc analysis revealing a significant increase of the base of support (BoS) of the SCA17 rats at the age of 9 months ($p<0.05$). The width of the paw prints was also significantly smaller in the SCA17 rats compared to the wildtype counterparts in all three ages tested (**Figure 1. B1 & B2**) (genotype effect: (a) front paws: $F_{(1,18)}= 8.75$, $p<0.01$; (b) hind paws: $F_{(1,18)}= 8.14$, $p<0.01$), which was also changed with age (statistical trend only for the front paws, age effect: (a) front paws: $F_{(2,36)}= 2.81$, $p=0.07$; (b) hind paws: $F_{(2,36)}= 3.8$, $p<0.05$). A significantly shorter stride length of the hind paws of the SCA17 rats served as an indication of the distance between successive placements of the same paw (genotype effect: (a) front paws: $F_{(1,18)}= 2.88$, $p=0.1$; (b) hind paws: $F_{(1,18)}= 5.37$, $p<0.05$), which showed also age and interaction effects (age effect: hind paws: $F_{(2,36)}= 96.5$, $p<0.001$; interaction effect: $F_{(2,36)}= 6.63$, $p<0.01$) (**Figure 1. C1 & C2**). Stride length in the front paws was clearly affected by age (age
Motor characterization of the SCA17 rats

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effect: front paws: $F_{(2,36)} = 92.9, p<0.001$), and post-hoc analysis indicated a significant decrease for the SCA17 rats at the age of 9 months ($p<0.05$).

Significant differences in body weight between the two groups, can influence the results of parameters such as print width and max contact area which are directly linked to pressure and subsequently body weight. In this study, a significantly lower body weight of the SCA17 rats at the ages of 6 and 9 months was observed (data not shown). To ensure that this did not have a significant influence on the aforementioned parameters, a correlation analysis was performed for the age points 6 and 9 months. A significant correlation was noted between print width in the front paws and body weight at both 6 ($r^2=0.58, p<0.01$) and 9 months ($r^2=0.56, p<0.05$). For the hind paws, a significant negative correlation was observed only at the age of 6 months ($r^2=-0.55, p<0.05$). Correlations between max contact area and body weight are described in the supplementary material. A further analysis of static parameters relating to max contact area revealed a significant decrease from 3 to 9 months old for the front paws and decrease for the hind paws, respectively. These data are described and discussed in the supplementary material.

**Dynamic parameters**

Figure 2. A1 & 2. A2 illustrate the temporal relationship between placement of the lateral pair of paws within a step cycle, used as a measure of inter-paw coordination. An impairment is shown by the significant decrease in the phase dispersion for both diagonal pairs of paws in the SCA17 rats. This indicates a disturbed inter-paw coordination as the placement of the diagonal hind paws relative to the front paws were placed significantly earlier in the SCA17 rats compared with the control group (genotype effect: diagonal pairs, RF(ANCHOR) → LH(TARGET) $F_{(1,18)} = 14.5, p<0.001$; LF(ANCHOR) → RH(TARGET) $F_{(1,18)} = 11.6, p<0.01$). Diagonal pair paw placements were also affected by age but no interaction effect was observed (age effect: RF(ANCHOR) → LH(TARGET) $F_{(2,36)} = 7.46, p<0.01$; LF(ANCHOR) → RH(TARGET) $F_{(2,36)} = 7.4, p<0.01$; interaction: RF(ANCHOR) → LH(TARGET) $F_{(2,36)} = 23, p>0.05$; LF(ANCHOR) → RH(TARGET) $F_{(2,36)} = 1.8, p>0.05$). Post-hoc analysis exhibited that SCA17 rats had a significant impairment compared to the wildtype group at the age of 3 and 6 months old. The age of 9 months was not reaching a statistical significance most probably due to large within group variation. The speed at which the paw loses contact with the glass plate described by the stand index parameters, was significantly increased only for the front paws of the SCA17 rats (genotype effect: (a) front paws: $F_{(1,18)} = 10.9, p<0.01$; (b) hind paws: $F_{(1,18)} = 1.26, p>0.05$). Stand index was also in general decreased by age (age effect: (a) front paws: $F_{(2,36)} = 20.96, p<0.001$; (b) hind paws: $F_{(2,36)} = 30.42, p<0.001$) (Figure 2. B1 & 2. B2). Figure 2. C1 & 2. C2 shows the speed of the paw during swing, calculated by dividing the stride length by the swing duration. Swing speed of the front paws was significantly decreased for the SCA17 rats and a statistical trend was shown for the hind paws, respectively (genotype effect: (a) front paws: $F_{(1,18)} = 5.95$, $p<0.05$; (b) hind paws: $F_{(1,18)} = 3.04, p>0.05$).
Figure 1  Static parameters of the CatWalk test

(A1 & A2) Age development of the distance between the center points of the two fore and hind paws, respectively. (B1 & B2) Age development of the width (in vertical direction) of the complete fore and hind print expression. (C1 & C2) Age development of distance between successive placements of the same paw of the fore and hind paws. Data are expressed as means ± S.E.M. Two-way RMA ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
Figure 2 Dynamic parameters of the CatWalk test

(A1 & A2) Age development of the temporal relationship between placement of the lateral pair of paws within a step cycle for the two diagonal paw pairs, respectively. (B1 & B2) Age development of the speed at which the paw loses contact with the glass plate for the front and hind paws. (C1 & C2) Age development of the speed of the paws during swing for the front and hind paws. Data are expressed as means ± S.E.M. Two-way RMA ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
Swing speed was also significantly affected over time by an increase from 3 to 6 months, and a slight decrease from 6 to 9 months of age (age effect: (a) front paws: $F_{(2,36)}= 42.93$, $p<0.001$; (b) hind paws: $F_{(2,36)}= 37.18$, $p<0.001$). For further analysis of dynamic parameters such as *swing duration* please see the supplementary material.

**Grip strength test**

*Figure 3A* describes the average of the three highest values scored during the grip strength trials. Grip strength showed a statistically significant genotype, age and interaction effect, indicating that the SCA17 rats had a significantly lower fore grip compared to the control group and that grip strength was differently influenced by age in the two genotypes (genotype effect: $F_{(1,18)}= 55.7$, $p<0.001$; age effect: $F_{(2,36)}= 7.32$, $p<0.01$; interaction effect: $F_{(2,36)}= 15.92$, $p<0.001$). Post-hoc analysis revealed that SCA17 rats had significantly decreases in grip strength at all three age points tested (3 and 9 months: $p<0.001$; 6 months: $p<0.05$). *Figure 3B* shows the animals’ performance normalized to the individual body weight of each rat, in order to correct for any possible confounding factors due to the well-known weight differences between the two genotypes from the age of ~4.5 months old. When corrected for the body weight, the performance of the rats showed a worsening between the 3 and the 9 months old as reflected by the age effect ($F_{(2,36)}= 41.93$, $p<0.001$). A genotype effect was no longer present. Additionally, the interaction effect was also significant ($F_{(1,18)}= 5.32$, $p<0.010$) and subsequently post-hoc analysis revealed a significant decreased grip strength for the SCA17 rats at the age of 9 months ($p<0.001$).

**Beam walk test**

*Figure 3C* represents the average number of foot slips (including both front and hind paw foot slips), which did not show any significant genotype ($F_{(1,22)}= 2.45$, $p>0.05$) or interaction effects ($F_{(2,44)}= 0.35$, $p>0.05$). The animals’ performance did get worse with age, as indicated by the increased average number of foot slips between the ages of 3 and 9 month and reflected by the significant age effect ($F_{(2,44)}= 7.62$, $p<0.001$). *Figure 3D* shows a rat performing a cross attempt on the beam walk apparatus.

**Cohort 2: Fine motor control**

**Pellet Reaching test**

*Figure 4A* represents the mean success rate of reaching trials in the pellet reaching test. Performance was significantly decreased in the SCA17 rats compared to the wildtype counterparts (genotype effect: $F_{(1,18)}= 24.63$, $p<0.001$). Age did not seem to play a significant role in this test. However, there was a significant genotype-age interaction effect (age effect: $F_{(2,36)}= 2.9$, $p=0.067$; interaction effect: $F_{(2,36)}= 4.73$, $p<0.05$). Post-hoc
analysis showed that in all three ages tested there was a significant decrease of performance in the transgenic rats. (3 & 9 months: p<0.001; 6 months: p<0.05). Figure 4B shows a photo of a rat during the pellet reaching test at the shaping phase displaying olfactory engagement with the sugar pellet and preparing his body posture for initiation of a reach attempt. Repeated measures ANOVA analysis for each age point separately in order to assess the learning curve and performance during the whole testing period.
Figure 4 Pellet reaching task

(A) Mean success rate in the pellet reaching test across the three age points tested. (B) Photo displaying a rat during a shaping session of the pellet reaching test engaging in the test with the olfactory stimulus of the sugar pellet. (C) Success rate across the ten testing days at 3 months age. (D) Success rate across the ten testing days at 6 months age. (E) Success rate across the ten testing days at 9 months age. Data are expressed as means ± S.E.M. Two-way RMA ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
are depicted in Figure 4C, 4D and 4E, respectively. At all three age points, a significant genotype and days effect was observed, and only at the age of 9 months a significant interaction effect was seen (3 months: genotype effect: F(1,18)=14.33, p<0.01; days effect: F(9,162)=2.73, p<0.01; interaction effect: F(9,162)=1.75, p=0.08; 6 months: genotype effect: F(1,18)= 4.82, p<0.05; days effect: F(9,162)=3.1, p<0.01; interaction effect: F(9,162)=1.85, p=0.063; 9 months: genotype effect: F(1,18)= 28.9, p<0.001; days effect: F(9,162)= 3.84, p<0.001; interaction effect: F(9,162)= 2.06, p<0.05).

DISCUSSION

In this study, we focused on the development of motor deficits in the SCA17 rats by testing the animals at three different ages (3, 6 and 9 months), in an effort to outline a more comprehensive picture of the SCA17’s repertoire of motor function. SCA17 is a neurodegenerative disease best known for its effect on motor control and therefore confirming these deficits in an animal model of this disease is essential. In previous studies the SCA17 transgenic rats showed a severe neurological phenotype including ataxia and impairment of postural reflexes (Kelp et al., 2013). Here, we used a battery of motor tests with a combination of automated and traditional behavioural paradigms, allowing us to investigate in depth gross motor function, fine motor control, coordination and muscle strength. These findings will increase our knowledge of the motor phenotype of the SCA17 rats confirming specific deficits in different aspects of motor function.

Concerning gross motor control, SCA17 rats presented several significant impairments. Balance deficits were seen in the SCA17 rats most prominently at the age of 9 months by an increased base of support (BoS) in the front paws and a decrease in the hind paws. Distance between hind paws was significantly lower in all three ages tested. This might be attributed to the kyphotic (hunched) posture that the SCA rats displayed at the last months of their lives. That said, it can be argued that CatWalk is a sensitive and appropriate tool for detecting kyphotic or general skeletal differences between animals. A further thorough skeletal examination on the SCA17 rats in the future, at different ages can explain this further. It could also be related to the inflexible movement of the hind paws with a “dragged” gait style, which is compliant with reports in other SCA animal models (Chou et al., 2008; Guyenet et al., 2010). A wider BoS is characteristic of ataxic gait, working as a compensatory mechanism to maintain the balance. This mimics the ataxic gait of cerebellar patients who are reported to exhibit a wider BoS as a rescue mechanism to maintain their balance (Stolze et al., 2002). Interestingly, the differences in the BoS of the hind legs was present from the age of 3 months already, indicating that subtle differences exist already at an early stage before the full manifestation of the ataxic phenotype. Thus, BoS is an interesting indicative parameter to be used in future treatment studies.
Balance and coordination deficits were also confirmed by the lower *print width* (vertical direction of the complete paw print) in the SCA17 rats compared to the control group. A lower pressure was applied on both front and hind paws in the SCA17 rats reaching a statistical difference for the front paws and a trend for the hind paws at the age of 9 months. This suggests an imbalance due to muscle impairments (Vandeputte et al., 2010) which was not discovered in the beam walk test, clearly demonstrating the advantage of using automated gait analysis for the study of motor deficits. Reduced *print width* reflects reduced paw contact with the floor. Thus, the neuropathology caused by SCA17 may result in an impaired placement of the paw on the glass plate, due to an altered use of the plantar surface of the paw (Deumens et al., 2007) and is in line with effects seen in HD mice (Besusso et al., 2013). However, in literature an interdependency has been stated between the body weight and the *contact area*, *print area* and *print width* (Koopmans et al., 2007; Parkkinen et al., 2013a). In this study a significant difference was seen in the body weight of the two genotypes at 6 and 9 months (data not shown). Therefore, considering the significant positive correlation between the front paws print width and the body weight of the rats at the age of 9 months, a possible confounding effect on the *print width* results cannot be excluded and thus the results should be interpreted with caution.

*Stride length* measures the distance from the previous position of a hindlimb to the current position of the forelimb on the same side (i.e. the distance between successive placements of the same paw). A shorter *stride length* was observed in the SCA17 rats for both front and hind legs at the 9 months age point. This difference is typically indicative of disturbed gait and have been described in different animal models such as parkinsonian rats (Westin et al., 2012), BACHD rats (Huntington’s disease) (Abada et al., 2013), Rett syndrome mouse model (Robinson et al., 2012) and olivocerebellar ataxia rat model (Wecker et al., 2013).

Parameters significantly influenced by time are *phase dispersions*, *stand index* and *swing speed*. We found SCA17 rats to have an impaired inter-paw coordination as shown by the significantly decreased *phase dispersions* for both diagonal pairs of paws. Although post-hoc analysis only confirmed a significant genotype effect for 3 and 6 months of age, and no significance at 9 months of age, this parameter is considered an important marker for gait coordination dysfunction. This effect implies a faster placement of the fore paws in relation to the diagonal hind paws. The absence of statistical significance at 9 months is probably caused by a large within group variation and due to normal aging process, which also affect the gait of wildtype rats. These results are in line with findings in ataxic animal models and patients with neurologically impaired gait (Ebersbach et al., 1999; Vinueza Veloz et al., 2014). Another pronounced difference seen between the two genotypes is the significantly increased *stand index* in the SCA17 rats, which represents the speed at which the paw loses contact with the glass plate. This increase was mainly seen in the front paws, suggesting that the SCA17 rats moved slowly compared to the
wildtype littermates. The stand index has often been mentioned in evaluations of models of brain injury (Mountney et al., 2013) and of neurological diseases such as Huntington’s disease (Vandeputte et al., 2010) and amyotrophic lateral sclerosis (Mead et al., 2013; Vergouts et al., 2015). However, the results varied by model, and the evaluation of gait disturbances through changes in the stand index requires further study. Additionally, swing speed was generally lower in the front paws of the SCA17 rats without any significant age effects. This finding was expected, as the swing duration was similar between groups (see supplementary material), but the distance the paw moved was decreased, hence the speed had to be reduced as well. Thus, for each step a SCA17 rat took, its body was moved a shorter distance during a longer time span in comparison to a wildtype rat. This phenomenon has been also observed in the uncoordinated gait of Parkinson’s disease rat model (Westin et al., 2012). Taken together, shorter stride lengths, decreased swing speed, increased BoS are indicative of shuffling gait as observed in spinocerebellar ataxia patients (Bonnet et al., 2012).

With respect to muscle function, the SCA17 rats showed a significantly decreased fore limb grip strength, already from the age of 3 months. However, after correction for the body weight, these differences were confirmed only for the 9 months age point, indicating a reduced muscle function, independent of body weight. This explains that despite the different body composition (smaller in size and reduced body weight) of the SCA17 rats their muscle endurance remained intact until the last stages of disease. This is in line with observations in different types of SCA patients where it has been shown that patients are able to exert increased grip forces but fail to anticipate inertial load fluctuations (Rost et al., 2005). Since no load fluctuations were applied to the SCA17 rats but they were tested in a rather ‘passive’ form of grip strength measurement, this could explain why no differences were seen until the age of 9 months. Additionally, not all SCA17 patients have been reported to show muscle weakness and for those who have, muscle weakness was manifested when full disease symptomatology was displayed (Bruni et al., 2004; Rolfs et al., 2003). It must be noted that an age-related decrease in performance was noted in both genotypes, which might be either due to repeated testing or simply attributed to aging or a combination of those two factors (Xue et al., 2016).

The beam walk test did not show any significant differences between the two genotypes. Although SCA patients have been reported to have balance-related abnormalities (Ashizawa and Xia, 2016; Van de Warrenburg et al., 2005), this could not be replicated in the SCA17 rats in the beam walk test. Even though the relevance of the cerebellum for balance control in gait has been proven (Ilg et al., 2007), the use of this version of the beam walk test (i.e. the ledged tapered) was retrospectively considered not suitable or not sensitive enough for capturing any balance impairments. However, this may also be explained by taking into account the results of the gait analysis, which showed that the SCA17 rats had a reduced hind paw base width at 3 months, with a trend towards the same decrease at 9 months. Therefore, the reduced base width might mean that the rats
are less likely to slip, as the hindlimbs are further away from the edge of the beam (Trueman et al., 2017). Another possible explanation is that as already known, the SCA17 rats are markedly smaller in size and thinner than the wildtype rats already from the age of ~4.5 months old. That means that despite the challenge of the progressively narrowing beam this was wide enough for the small-sized body of the SCA17 rats to cross with slipping only one or two times at the very end part of the beam. On the contrary, the wildtype rats kept gaining weight until the end making it more challenging to keep their balance on the last narrowest part of the beam with a bigger-sized body. We presume that a repeated testing effect as well as the aging process also in the wildtype rats have played an important role in these results. Since no negative reinforcement was used in case the rats slipped and used the lowest platform, this may have influenced the animals’ performance by not making a great effort to stay on the highest platform. Due to the nature of the test, a correction of the body size and skeletal differences in the two genotypes could not be made, making this test less sensitive in detecting balance impairments. As the analysis of this test is based on recordings of crossing trials, body-size or skeletal data are not automatically captured. Therefore, for future experiments, one could consider measuring these parameters for all animals tested in the beam walk separately in order to be taken into account with the data analysis. Overall, several limitations of the beam walk test in this study have made these results not fully conclusive and/or representative of the balance-related phenotype of the SCA17 rats. On the other hand, balance impairments could be seen from the CatWalk results. This confirms that CatWalk is considered more sensitive than the beam walk test in detecting balance deficits since on CatWalk the animals are allowed to walk freely without having to put any effort in keeping their balance on a beam. Additionally, the CatWalk is less influenced by confounding factors such as differences in body size, or in the skeleton.

Regarding fine motor control, the SCA17 rats showed an impaired phenotype already from the age of 3 months old onwards, represented by the decrease in the mean success rate in the pellet reaching test. This is similar to SCA17 patients, but also patients with cerebellar degeneration, who are significantly impaired in fine motor skills such as hand transport and shaping, as well as general grasping (Ashizawa and Xia, 2016; Brandauer et al., 2008). Success rate in reaching attempts appeared to be significantly decreased in the SCA17 rats when challenged with the pellet reaching task at all three ages tested. These results are conform observations in rat models of stroke and brain injury (Ramanathan et al., 2006; Whishaw et al., 2008) but also models of neurological dysfunction (e.g. Parkinson’s disease) (Klein and Dunnett, 2012; Vergara-Aragon et al., 2003). To our knowledge this is the first animal model from all the spinocerebellar ataxias’ group that has been tested in this pellet reaching test and has shown such robust effects.

The strength of the current study focusing on motor profiling of the SCA17 rats is the wide range of behavioural tests, bringing together novel automated and traditional setups and paradigms under the same testing conditions, enabling behavioural monitoring
across different age points. However, it should be noted that we performed multiple behavioural tests with cohort 1 and repeated longitudinal testing in both cohorts at three different age points. Therefore, an influence of repeated testing on the results of the study cannot be entirely excluded. Nevertheless, the longitudinal testing offers also important advantages such as the monitoring of the same animals over time as having the animals serving as their own control. This is not of minor importance, considering the wide variety of symptomatology reported in the SCA17 patients.

In sum, in our study, we revealed specific motoric impairments in the SCA17 rats, which might be related to neuronal loss, particularly in the cerebellum as reported earlier (Kelp et al., 2013). The SCA17 rats’ impairment seen in a previous study in the beam walk test (Kelp et al., 2013), were not replicated by the ledged beam walk in the current study. Clear deficits were seen in fine and gross motor control as well as in grip strength which might be associated with reduced muscle mass. Above all, this study revealed novel readouts that can be used in future preclinical therapeutic studies for assessing the potency of candidate drugs on several motor related parameters.

**Conclusion**

Taken together, our results indicate that the SCA17 rat model displays a clear impaired motor phenotype over different ages in traditional and automated tests. Interestingly, the SCA17 rats showed impaired fine motor skills as well as inter-paw coordination from an early age already. The parameters that showed the most pronounced differences were related to fluidity of movement and support. In translation to the patients’ situation, these results are in line with impaired prehension kinematics and grasping forces and characteristic ataxic gait seen in early and/or late-symptomatic patients. Overall, our results mimic the general motor deficiencies reported for SCA17 patients, indicating that the SCA17 rat model could be suitable for future therapeutic studies.

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**Conflict of interest**

The authors E. Kyriakou and Dr. J.E. van der Harst were working for the EU funded “PhenoRat” project of which Noldus Information Technology, the manufacturer of
the PhenoTyper device and EthoVision XT 9 software, was a partner. At the time of the studies, J.E. van der Harst was employed as part-time scientific project advisor for PhenoRat employed by Noldus Information Technology, and E. Kyriakou was employed by Noldus Information Technology as part of the PhenoRat project.
SUPPLEMENTARY MATERIAL

Materials and Methods
In addition to the parameters presented in the main article, CatWalk parameters relating to max contact area (i.e. the maximum area of a paw that comes into contact with the glass plate) and the swing duration (i.e. the duration in seconds of no contact of a paw with the glass plate) were also analysed from the CatWalk experiment. Furthermore, the length of the body (longitudinally from the front paws to the hind paws) and from the nose until the base of the tail were measured at the end of the locomotor study at sacrifice.

Analysis
Analysis was performed as described in the main article. Body length was analysed using a Student t-test.

Results
Figure S1. A & B show the statistically significant decrease in the body length when this measured both from the front to the hind paws and from the nose to the base of the tail. (p<0.001 in both cases). Furthermore, max contact area remained unchanged between the two genotypes for both front and hind paws (genotype effect: (a) front: F(1,18)=3.2, p>0.05; (b) hind paws: F(1,18)=1.77, p>0.05) but was in general increased for the front paws and decreased for the hind paws respectively by age (age effect: (a) front paws: F(2,36)=4.0, p<0.01, (b) hind paws: F(2,36)=4.05, p<0.01). No interaction effect was observed ((a) front paws: F(2,36)=2.34, p>0.05, (b) hind paws: F(2,36)=1.01, p>0.05) (Figure S2. A1 & S2. A2). Swing duration was also intact for both front and hind paws (genotype effect: (a) front paws: F(1,18)=2.99, p>0.05, (b) hind paws: F(1,18)=0.15, p>0.05), showing only an age-related decrease in the hind paws (age effect: (a) front paws: F(2,36)=18.53, p=0.054, (b) hind paws: F(2,36)=10.46, p<0.001). No interaction effects were seen ((a) front paws: F(2,36)=0.083, p>0.05, (b) hind paws: F(2,36)=3.59, p>0.05) (Figure S2. B1 & S2. B2). As already mentioned in the main results section, to ensure that the significantly lower body weight of the SCA17 rats at the ages of 6 and 9 months did not significantly influence the results on max contact area, a correlation analysis was performed. This revealed a significant positive correlation for the max contact area in both front and hind paws only at 6 months old (front: r²=0.54, p<0.05; hind: r²=0.56, p<0.05).

Discussion
In literature, a decrease in the max contact area has been reported for several animal models such as in Parkinson’s disease rats and mice and stroke mice (Glasl et al., 2012; Hetze et al., 2012; Vlamings et al., 2007). However, in this study, max contact area remained intact in the SCA17 rats, a phenomenon also seen in cerebral ischemic rats (Parkkinen...
et al., 2013b). However, a strong age effect was observed throughout the three age points tested, which might mean that body weight changes from 3 to 9 months had influenced the max contact area. Concerning the possible effect of the body weight differences between the two genotypes at the ages of 6 and 9 months, a significant positive correlation was found for both front and hind paws only at the age of 6 months. Therefore, the difference observed for the front paws at 6 months old, should be interpreted with caution. Additionally, swing duration remained intact which is not in line with increases reported in spinocerebellar ataxia patients (Buckley et al., 2018) but similar effects have been observed in parkinsonian rats (Westin et al., 2012).

Figure S1  Length of the body at the age of 9 months

(A) Mean length of the body longitudinally from the front paws to the hind paws at the age of 9 months. (B) Mean length of the body from the nose to the base of the tail at the age of 9 months. Data are expressed as means ± S.E.M. Student’s t-test results are displayed in each graph. Statistical significance is indicated with asterisk(s) (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
Figure S2 Max contact area and swing duration of the CatWalk test

(A1 & A2) Age development of the maximum area of a paw that comes into contact with the glass plate for the front and hind paws respectively. (B1 & B2) Age development of the duration in seconds of no contact of a paw with the glass plate for the front and hind paws. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
Anxiety and risk assessment-related traits in a rat model of Spinocerebellar ataxia type 17

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ABSTRACT

Anxiety as a common feature of several neurodegenerative/polyglutamine diseases is an important aspect for the face validity of an animal model for Spinocerebellar Ataxia type 17 (SCA17). Risk assessment and anxiety-like traits were characterized in 3-6-9 months old rats of a transgenic model for SCA17 using the standard behavioural test elevated plus maze. In addition, c-Fos immunostainings in the basolateral amygdala evaluated neuronal activation in correlation to the behavioural responses. The most prominent behavioural effect was a higher level of risk assessment in the transgenic rats. In addition, an increase in anxiety-related behaviour in these rats was found. Although the EPM caused no overall effect on c-Fos expression, a negative correlation with the anxiety-like behavioural response was observed. Our results suggest that the SCA17 rat model displays an anxious phenotype already at 3 months of age resembling the generalized anxiety in early symptomatic SCA17 patients, thus confirming the validity of this rat model.
INTRODUCTION

The unraveling and treatment of neurodegenerative diseases is a constant challenge for the scientific community. One substantial group of the neurodegenerative diseases group involves the polyglutamine diseases which consist of Huntington’s, seven types of spinocerebellar ataxia, dentatorubropallidolysian atrophy (DRPLA), and Spinobulbar muscular atrophy. Spinocerebellar ataxia type 17 is one of the rarest forms of SCA caused by the abnormal expansion of the polyglutamine tract in the TATA box binding protein. The normal PolyQ stretch ranges between 29 and 42 glutamines while the abnormal expansion of 49-63 repeats leads to the onset of SCA17 in humans. Clinical features related to this disease, like many other neurodegenerative diseases, manifest a wide spectrum. In 2004, a human clinical study covered a 5-generation family study which recorded that from its 230 members, 16 members were affected by the disease. Thirteen of the 16 patients had undergone behavioural testing and it was observed that 54% of the affected patients suffered from anxiety as one of the disease symptoms (Bruni et al., 2004). In addition, in 2003, Rolfs and colleagues studied 4 families with autosomal dominant SCA17, and onset of disease appeared as early as 8 years of age and as late as 43 years of age. All patients suffered from at least one form of ataxia which included gait or limb ataxia, dysarthria, dysmetria and dysphagia, while psychiatric symptoms included schizophrenia, dementia, paranoia, depression and/or anxiety (Rolfs et al., 2003).

Due to the deleterious effects on patients’ lives, the importance of studying the psychiatric disorders along with the motor symptoms at both clinical and preclinical level is clearly important. Therefore, including psychiatric symptoms in the modeling of the disease with animals is crucial for the validity of preclinical studies. However, one has to keep in mind that the complexity of such symptoms can make it challenging to study such symptoms in a fully translational way in animal models. In this study we focused on anxiety as one of the non-motoric symptoms seen in SCA17 patients, and using the standard behavioural test elevated plus maze we concentrated on replicating the anxiety phenotype in an animal model for SCA17. As discussed in literature, rats are a more suitable model than mice for certain behavioural aspects such as cognition, anxiety, impulsivity and other neuropsychiatric behaviours (Ellenbroek and Youn, 2016). Therefore, for our study we use a recently generated SCA17 transgenic rat model that carries the full-length human TBP-cDNA with an abnormal CAG/CAA repeat of 64 codons. Primary characterization of the SCA17 rats showed a severe neurological phenotype, loss of body weight, early death (Kelp et al. 2013). However, the levels of anxiety-like behaviours across different developmental stages of the disease and their maintenance at the symptomatic stage are still unknown, which is important in order to identify whether anxiety can be related to the actual disease development. As in the Kelp and colleagues study there were indications of an anxiety phenotype only at the age of 4 months and taking into account the human situation, we decided to go further with the validation of this rat
model and investigate the development of anxiety in one earlier and two later age points. The ages are chosen in relation to the progression and manifestation of the motor symptoms as these have been described in literature: 3 months (pre-symptomatic), 6 months (early symptomatic) and 9 months (late stage of the disease). Thus, we aim at assessing the anxiety-like phenotype as a whole by using the well-established elevated plus maze (EPM) and open field (OF) tests. One other aspect of our design is the use of automated measurements which allows us to expand our analysis and check whether the anxiety-like phenotype can be influenced by the impaired motor capacities of these animals. Our analysis includes the traditional anxiety-related behaviour measured in the EPM (time spent in the open arms) but also the risk assessment behaviour (head dips in the open arms). The reasoning behind including the risk assessment is in order to extend our knowledge about the anxiety phenotype in this model and detect potentially subtle differences already in the pre-symptomatic stage. Notably, risk assessment is an important element when testing anxiety on the EPM, as it is part of the defensive behaviours in rodents which may mimic the symptoms of generalized anxiety in humans, and its analysis is believed to enhance the sensitivity of the task to detect subtle differences in anxiety (Wall, 2001). Since anxiety states and anxiety-related behaviours appear to be regulated by a distributed and highly interconnected system of forebrain structures including the basolateral amygdaloid complex (Hale et al., 2006), we correlate the behavioural response to the neuronal response in the basolateral amygdala, which has been described as the brain center for anxiety (Etkin and Wager, 2007). As very little is known about the functionality of the brain circuitry of anxiety in this SCA17 rat model, we chose the basolateral amygdala as our first target expecting the biggest changes (if any) to be presented there among all brain areas. To confirm the anxiety-related phenotype in the SCA17 rats we followed a separate cohort of animals longitudinally over the 3, 6 and 9 months and analysed their responses in the open field. In the open field our analysis included quantification of the locomotor activity over the three age points, but also the velocity with which the animals move in the arena and time spent in the central area as an index of anxiety in the SCA17 rats (Simon et al., 1994).

METHODS

Animals
Three different age groups of maze-naive male rats of both genotypes were used (3 months: SCA17 n=12 and WT n=10; 6 months: SCA17 n=11 and WT n=10; 9 months: SCA17 n=10 and WT n=10). For the open field test, the two genotype groups were tested longitudinally at all 3 age points (3, 6, and 9 months, SCA17 n=15; WT n=13). Animals were
housed socially under conditions in reversed day light cycle and water and food were available ad libitum. Testing was performed during the animals’ active (dark) phase. All experiments were performed after approval of the Ethical Committee for Animal Experiments of the Radboud University Nijmegen Medical Center for compliance to ethical standards and use of laboratory animals according to EU-guidelines.

**Elevated Plus Maze (EPM)**
This conventional test is used to evaluate anxiety-like behaviour in mice and rats based on the strong aversion of these animals to open areas and at the same time their high motivation to explore, resulting in an approach-avoidance conflict. The test was performed under a general room light intensity of 10 lux. At the start of the test the rat was placed in the center of the EPM facing one of the open arms. Subsequently, behavioural parameters were recorded and calculated by the EthoVision XT9 tracking system for a total trial duration of 300s.

The behavioural parameters recorded were: total distance moved, velocity, frequency of entries in open and closed arms, cumulative duration in open and closed arms, total frequency of arm visits, frequency of head dips in open arms (relatively short intensions to explore (“risk assessment”) as indicated by movement 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), cumulative duration of head dips in open arms, ratio of open/closed arm entries and relative open and closed arms visits.

**Immunohistochemistry**
Male SCA17 and wildtype littermates at 3, 6 and 9 months of age were transcardially perfused 90 min after the exposure to the EPM, through the left ventricle with 4% paraformaldehyde (PFA) (3 months: SCA17 n=8 and WT n=8; 6 months: SCA17 n=7 and WT n=7; 9 months: SCA17 n=8 and WT n=8). The brain was fixated overnight with 4%PFA, and equilibrated for 2 days in 30% sucrose solution. Coronal sections of 40 μm were then cut using a sliding microtome. Brain sections of the basolateral amygdala were blocked to prevent nonspecific binding using a 1% BSA solution and incubated with primary antibody against rabbit c-fos (1:100) (Santa Cruz), and chicken NeuN (1:1000) (Millipore). Secondary antibodies used included anti-rabbit (1:200) (Jackson ImmunoResearch Laboratories, Inc.), anti-chicken (Jackson ImmunoResearch Laboratories, Inc.). Images (for examples see supplementary figure 6) were then analyzed and compared using ImageJ software (NIH) version 1.50e as a tool for counting neurons number and c-fos positive (+) cells.

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3 All animals were socially housed by 3 or 2 in Makrolon-IVS cages (Techniplast, Italy) until 6 days prior to the EPM testing, when the animals were housed individually in the PhenoTyper 4500 (Noldus IT, Wageningen, The Netherlands) for homecage monitoring (not included in this manuscript) with food and water provided ad libitum. The uneven number of animals per genotype and age group is due to the variation in the litter sizes.
Open field
This assay has been generally used over the past decades to measure general locomotor activity levels and anxiety in rodents and is mainly based on rodents’ motivation to explore novel environments and hide from potential risks, thereby causing initial thigmotaxis, gradually (initial approach-avoidance conflict) evolving into more free exploration of the arena, including the center. The test was performed under red-light conditions and the apparatus used was a special-designed enlarged PhenoTyper 9000 with dimensions 90 x 90 cm. Behavioural parameters were recorded and calculated by the EthoVision XT9 tracking system for a trial duration of 300s. The behavioural parameters analysed were: total distance moved, velocity, cumulative duration and frequency of visits/crossings of the center (center was calculated as the inner 60 x 60 cm square area). Apart from the distance moved which is a classical parameter indicative of the exploratory activity of the animals in an open field, the time spent in the center has also been very often used for capturing anxiety-related behavioural patterns (Bailey and Crawley, 2009). Literature on the setup and general experimental conditions of an open field test show a broad variety between not only the shape of the arena (circular or square) but also the size of it (for review see Prut and Belzung 2003). However, the general principle of the tendency to explore the environment versus the avoidance of ‘open space’ can be generalized by analyzing the time spent in the ‘center’, which is not the exact center-spot, but is mostly defined as a certain area with several square centimeters. In line with this, and with the concept of thigmotaxis, in our analysis the arena is divided into 2 zones: periphery (30 cm, 1/3 of the total arena), based on the fact that it can fit an average distance of one body-length of an adult rat, and thus the rat can turn close to the wall without leaving the periphery-zone, and the center (60 cm, 2/3 of the total arena), representing the ‘open area’. This way the animal can be either in one or in the other zone, and thus, the time spent in the center is inversely related to the time spent in the periphery. Therefore, we only analyze and present the time spent in the center to draw our conclusions on the anxious phenotype of this animal model. Despite the diversity on the setups and conditions in which the open-field concept is used, the analysis including ours is similar being based on whether the animal dares to leave the safety of the walls and move to the center of the arena.

Statistical analysis
Data were analyzed using SPSS version 20. All results are shown as mean ± S.E.M. and are considered significant with a p value < 0.05. Two-way ANOVA was used as a statistical test comparing interaction between age and genotype for all behavioural parameters with significance p < 0.05 (*), p < 0.01 (**), and p <0.001(***)) with a Bonferroni post-hoc test for genotype effect per age category. For the open field data Repeated Measures ANOVA was performed to compare the interaction between the genotypes and ages for all behavioural parameters analysed, followed by a Bonferroni post-hoc test for genotype effects per
Anxiety profile of the SCA17 rats

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age category. Pearson’s r was used to examine correlations between c-Fos expression and behaviour of the SCA17 and the wildtype groups during the EPM exposure. All three age-points were pooled together for both genotype groups for the correlation analysis.

RESULTS

Elevated Plus Maze

Anxiety in the EPM is mostly characterised by less time spent in the open arms compared to the control group. However, risk assessment, as indicated by increased head dip frequency, also contributes significantly to assessing a more complete anxiety-like phenotype.

During a 5-min exposure to the EPM, SCA17 rats spent significantly less time in the open arms compared to their wildtype littermates. 2-way ANOVA revealed a significant difference in genotype ($F(1, 57) = 7.26, p<0.01$) with higher levels of time spent in the open arms for the control group. Also, an age effect was confirmed ($F(2, 57) = 8.26, p<0.001$), with the observation of an increase from 3 to 6 months and then a decline at the age of 9 for both genotypes. However, there was no genotype x age interaction ($F(2, 57) = 2.46, p>0.05$) (Figure 1A).

In addition, it was shown that the frequency of head dips to the open arms as an ethological parameter of risk assessment of the novel anxiogenic stimulus of the open arms was significantly increased for the SCA17 rats compared to the wildtypes ($F(1, 57) = 16.92, p<0.001$). No age effect was detected ($F(2, 57) = 0.08, p>0.05$) nor a genotype x age interaction ($F(2, 57) = 0.52, p>0.05$) (Figure 1B).

For the parameters frequency in the open arms, ratio open/closed arm visits, relative open and close arm visits and cumulative duration of head dipping, no differences reached statistical significance (data not shown). For other parameters such as the velocity ($F(2, 57) = 11.9, p<0.001$) (Figure S1B), distance moved ($F(2, 57) = 6.4, p<0.05$) (Figure S1B), frequency in the closed arms ($F(2, 57) = 5.66, p<0.01$) only a significant age effect was observed independent of genotype. Time spent in the closed arms showed a strong trend for genotype ($F(2, 57) = 3.6, p=0.06$) with the SCA17 rats spending more time that the control group and a significant age effect ($F(2, 57) = 4.5, p<0.05$) but no interaction was noted. Heatmaps of the overall activity per genotype and age-point group show the tracks of the animals during the 300s trial (Figure S3).

c-Fos neuronal activation in the BLA after EPM exposure

The levels of c-Fos expression in the basolateral amygdala of the SCA17 and wildtype animals were similar (genotype: $F(1, 40) = 0.24, p>0.05$; genotype x age: $F(2, 40) = 1.67, p>0.05$), but there was an age effect ($F(2, 40) = 3.71, p<0.05$). Similarly, the absolute number of neurons measured by the NeuN marker in the basolateral amygdala were not different.
between ages or genotypes and no interaction was observed (data not shown). This indicates that only the neuronal activity is altered in relation to anxiety as the total number of neurons remained the same across ages and genotypes. When correlating the time spent in the open arms and the frequency of head dipping to the number of c-Fos positive cells in the basolateral amygdala, we found a trend towards a positive correlation between the time spent in the open arms and the neuronal activation for the SCA17 genotype ($r= 0.42$, $p= 0.056$). However, there was no correlation for the control group ($r= 0.25$, $p>0.05$). Similarly, but on the opposite direction, the correlation between the frequency of head dipping and c-Fos activation was noted to be significantly negative for the SCA17 rats ($r= -0.43$, $p<0.05$) and again no correlation was observed for the control group ($r= 0.09$, $p>0.05$). Time spent in open arms and head dip frequency showed an increased anxiety in the SCA17 rats compared to the control group at the age of 6 months and at all three ages studied, respectively. However, both were inversely correlated with the c-Fos response of neurons in the basolateral amygdala. No significance was achieved when we correlated each age and genotype group separately with the number of the c-Fos positive cells (data not shown).

**Figure 1** Anxiety and risk assessment responses in the SCA17 rats compared to the WT at the ages of 3, 6 and 9 months

(A) Cumulative time spent in the open arms showed a significant genotype ($p<0.01$) and age effect ($p<0.001$); (B) Head dip frequency in the open arms with the body situated in the closed arms. An increase for the SCA17 rats compared to the WT ($F_{(1, 57)} = 16.92, p<0.001$) was confirmed. Data represent mean ±S.E.M.; with statistically significant differences in comparison with the control group (2-way ANOVA) *$p<0.05$; **$p<0.01$; ***$p<0.001$. 

between ages or genotypes and no interaction was observed (data not shown). This indicates that only the neuronal activity is altered in relation to anxiety as the total number of neurons remained the same across ages and genotypes. When correlating the time spent in the open arms and the frequency of head dipping to the number of c-Fos positive cells in the basolateral amygdala, we found a trend towards a positive correlation between the time spent in the open arms and the neuronal activation for the SCA17 genotype ($r= 0.42$, $p= 0.056$). However, there was no correlation for the control group ($r= 0.25$, $p>0.05$). Similarly, but on the opposite direction, the correlation between the frequency of head dipping and c-Fos activation was noted to be significantly negative for the SCA17 rats ($r= -0.43$, $p<0.05$) and again no correlation was observed for the control group ($r= 0.09$, $p>0.05$). Time spent in open arms and head dip frequency showed an increased anxiety in the SCA17 rats compared to the control group at the age of 6 months and at all three ages studied, respectively. However, both were inversely correlated with the c-Fos response of neurons in the basolateral amygdala. No significance was achieved when we correlated each age and genotype group separately with the number of the c-Fos positive cells (data not shown).
Figure 2 Correlations between anxiety and risk assessment and c-Fos neuronal activation in the basolateral amygdala in response to the EPM.

(A) Correlation between the head dip frequency and the c-Fos + cells counted in the BLA (black line) and correlation between the time spent in the open arms and the c-Fos + cells in the BLA (grey line) for the SCA17 rats; (B) Correlation between the head dip frequency and the c-Fos + cells counted in the BLA (black line) and correlation between the time spent in the open arms and the c-Fos + cells in the BLA (grey line) for the WT rats. (Pearson’s correlation analysis, *p<0.05; **p<0.01; ***p<0.001).
**Open field**

Anxiety in the open field is mostly characterised by less time spent in the center compared to the control group. The total distance travelled is indicative of the locomotor activity between the two groups, and can also provide insight in the anxiety-like profile.

During 5-min exposure to the open field, SCA17 rats spent significantly less time in the center compared to their wiltype counterparts. The total distance travelled did not show any genotype or genotype x age interaction effect ($F_{(1,26)}=0.12; p>0.05$ and $F_{(2,52)}=2.17; p>0.05$), but only an age effect ($F_{(2,52)}=4.2; p=0.02$) was observed, resulting in a decrease between the 3 and 9 months of age groups (**Figure 3A**). Regarding the time spent in the center, repeated measured ANOVA revealed a significant genotype effect, with the SCA17 rats spending significantly less time in the center of the arena ($F_{(1,26)}=5.26; p=0.03$) and an age-effect, where 3-month old group spending more time in the center than the 9-month old group ($F_{(0.4,52)}=3.7; p=0.047$) but no genotype x age interaction ($F_{(1,4.52)}=26.85; p>0.05$) (**Figure 3B**). Frequency of visiting the center was not different between ages ($F_{(2,52)}=1.4; p>0.05$) or genotypes ($F_{(1,26)}=56.9; p>0.05$), nor an age x genotype interaction effect was present ($F_{(2,52)}=1.42; p>0.05$) (**Figure S2A**). Velocity showed an age effect ($F_{(2,52)}=6.07; p<0.01$) but neither a genotype-effect ($F_{(1,26)}=0.049; p>0.05$) nor a genotype x age interaction ($F_{(2,52)}=1.14; p>0.05$) (**Figure S2B**).

**Figure 3** Anxiety and locomotor activity in the SCA17 rats compared to the WT at the ages of 3, 6 and 9 months

**(A)** Cumulative distance travelled during 5-min in the open field arena showed only an age effect ($p<0.05$) but neither a genotype nor a genotype x age interaction ($p>0.05$). **(B)** Cumulative time spent in the center of the open field arena. A decrease for the SCA17 rats compared to the WT ($p<0.05$) was confirmed. Age effect also reached significance ($p<0.05$) but no genotype x age interaction was noted ($p>0.05$). Data represent mean ±S.E.M.; with statistically significant differences in comparison with the control group (Repeated measures ANOVA) set at: *$p<0.05$; **$p<0.01$; ***$p<0.001$. 

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**Genotype:** ns  
**Age:** *  
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**Age:** *  
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![Figure 3A](image1.png)  
**SCA17**  
**Wildtype**

![Figure 3B](image2.png)
To illustrate a general movement pattern/preference for certain areas in the openfield, heatmaps are presented per genotype and age-point group to show where the animals spent most of their time during 5 minutes in the open field, (Figure S4).

**DISCUSSION AND CONCLUSIONS**

In this study, we focused on assessing the development of the anxiety-like phenotype over the lifespan of the SCA17 rat model (at 3, 6 and 9 months old). Our data demonstrated a high level of risk assessment behaviour for the SCA17 transgenic rats and increased anxiety-related behaviour, with the former being more prominent than the latter, across the 3 different developmental stages of the disease. These effects were not caused by locomotor differences or (dis)abilities between the transgenic and wildtype rats on the EPM, since velocity and distance moved did not show genotype-effects. Increased risk assessment behaviour, as reflected by an increased frequency of head dips was detected already from the age of 3 months old, recapitulating the human situation where psychiatric-like symptoms are being detected before the motoric impairments reported for the SCA17 rats (locomotor impairments, loss of weight, incoordination etc.- see Kelp et al. 2013; Kyriakou et al. 2015) appear. In addition, increased anxiety-related behaviour, as reflected by the decreased time spent in the open arms, was observed in the SCA17 rats at the age of 6 months. The fact that only the time spent in the open arms reached significance, whereas related parameters such as frequency of open arm visits, and duration and frequency of closed arm visits did not, indicates that the genotype-effect on this traditional anxiety-test is rather subtle. In addition, it indicates that both genotypes had an equal intention to explore but the SCA17 rats tend to go back to their enclosure sooner proving an anxiety-like phenotype.

In the same direction, when the rats were introduced into the open-field, the SCA17 transgenic rats inclined to stay mainly in the peripheral zone, as represented by the time spent in the center-zone, which is translated as an increased index of anxiety (Bailey and Crawley, 2009) compared to the control group that spent more time in the center-zone. The control-group also approached the center sooner (latency to enter center-zone - data not shown). To check for possible locomotor differences affecting the data we measured the total distance moved but no significant differences between the SCA17 and wildtype group were observed throughout the 3 ages tested. This data also suggests that the exploratory activity is also not per se influenced by the genotype so both groups have the same exploratory abilities. Increased anxiety-like behaviour as described by the stronger preference to stay in the periphery of the arena rather than exploring the center (as we presented by the time spent in the center) was prominent already from the age of 3 months and remained steadily high throughout the next 2 testing ages (6 and 9 months). Here we show for the first time that the anxiety-like phenotype can
already be present in a pre-symptomatic stage of the disease already, while in the past the increase of periphery-to-center ambulation in an automated-cage environment (PhenoMaster system, TSE) was detected only after the 7th month of age (Kelp et al., 2013). The lack of information on the time spent either in the center or in the periphery in that case, makes the comparison between these results and our findings difficult. However, a possible explanation for the fact that we detect an anxiety-like phenotype already at the age of 3 months can be the size of the two different open field-settings. In our study we have an arena of 90 x 90 cm which provides the animals with sufficient space to express a relatively natural behavioural response to an open area, whereas the automated cages used in the study of Kelp are quite small (standard type III) for the animals to show their full locomotor activity/exploration and approach-avoidance of the center. To our knowledge, no other study involving the phenotyping of transgenic SCA17 rodent models has looked into anxiety-related behaviour of their models (Chang et al., 2011; Friedman et al., 2007; Huang et al., 2015, 2011; Yang et al., 2014).

Thus the present results not only confirm the presence of psychiatric symptoms along the locomotor impairments during the progression of the disease but also add to previously reported findings for the SCA17 rats, where anxiety has been shown at the age of 4 months (Kelp et al., 2013). To our knowledge our study is the first one to show the appearance of an anxious phenotype at the early age of 3 months and to confirm that anxiety is part of the disease progression in a rodent model of SCA17. In literature, maze-naive animals are reported to have high levels of risk assessment towards the open arms, and explore these areas less extensively than other parts of the maze while neuro-physiological measurements sensitive/related to anxiety after the test have been shown to be elevated (Rodgers et al., 1999). Therefore, the employment of the ethological measurement of risk assessment in this study provides the insight that SCA17 pathology is associated with the assessment of risks related to potentially dangerous situations, allowing the animal to optimize the most adaptive strategy (Anseloni and Brandão, 1997; Rodgers et al., 1997). In the same context, our results support the notion that scoring beyond the classical behaviours provides a more comprehensive analysis that adds value to the EPM behavioural patterns. This was shown by the differences in risk assessment behaviour already at the age of 3 months old, whereas time spent in open arms revealed an anxious phenotype only in one of the three age points investigated. Interestingly, in literature risk assessment behaviour has been reported to be immensely valuable in identifying anxiolytic-like actions of drugs not detected by the conventional scoring parameters (Cole and Rodgers, 1994; Griebel et al., 1997; Setem et al., 1999).

Negative correlation of c-fos expression with the anxious-like phenotype (represented by an increased time spent in the open arms, and increased frequency of head-dipping in the open arms) in the SCA17 rats might be caused by reduced ability to activate the neurons as a result of the TBP mutation. That is, the presence of a TATAA sequence in the c-Fos promoter serves as a binding site for the TBP (Tansey et al., 1994) in order to
facilitate the transcription of the gene. Hence, the altered structure of the mutant TBP may result in a decreased transcription of the c-Fos gene and in turn to lower protein levels in the SCA17 rats. On the other hand, this genotype effect is likely to be similar for all SCA17 rats, regardless of the level of anxiety. The negative correlation found between the anxious-like phenotype and c-fos expression in SCA17 rats, implies that the correlation does have a functional meaning. Furthermore, the age effect of the positive c-Fos cells is in accordance with other findings in literature where old animals have showed a lower induction of c-Fos after engaging in learning and memory tasks (Boguszewski and Zagrodzka, 2005; Nagahara and Handa, 1997). The negative correlation we found between the c-Fos neuronal activation in the basolateral amygdala after the EPM exposure and the anxiety-related and risk-assessment behaviour expressed during the EPM is puzzling considering that most literature findings state an increase in c-Fos expression in the amygdala accompanied with anxiety behaviour in rats triggered by EPM testing (Campeau and Watson, 1997; Hinks et al., 1996; Singewald et al., 2003). It has been speculated that cognitive differences may be involved in risk assessment behaviour, as an individual’s perception of a threatening situation can determine his emotional reaction to it (File et al., 1994; Rodgers et al., 1999). Furthermore, neuronal activation in the amygdala (as well as other areas of the limbic system) has been suggested to be expressed not only as regulation of immediate responses to stress, but also as integration of fear and memory components of exploration of a novel environment (Hinks et al., 1996). That said, a mismatch between the behavioural index of anxiety/stress and the immediate early gene expression in SCA17 rats might imply that the transgenic rats are not able to integrate such information. The absence of a correlation between the behavioural parameters of anxiety and c-Fos activation in the wildtype animals might be attributed to the fact that the EPM experience can be considered as moderately anxiogenic for wildtype animals (Hinks et al., 1996). This may explain why there was a low amygdaloid response in wildtype rats compared to the more sensitive or anxiety-susceptible SCA17 rats. Importantly, a limiting factor to be taken into account for future studies is the absence of baseline measurements of c-Fos in the two genotypes not exposed to any stimulus. Such measurements would give more conclusive information for the effect of EPM on neuronal activation in both SCA17 and wildtype rats compared to the baseline levels. All the significant correlations reported here were found when all three age groups of each genotype exposed to the elevated plus-maze (i.e., SCA17 3, 6, and 9 months and WT 3, 6 and 9 months together, respectively) were included in the analysis, but not when each genotype-age group was analyzed separately. We speculate that the need to collapse across plus-maze groups to find the significant correlations may be due to the fact that a larger sample size is needed to provide sufficient variability, particularly on the behavioral variable, before a correlation of any strength could be revealed. On the other hand, it is of critical importance to note that the effects we are looking at are rather subtle, as there were no differences in the absolute numbers of the c-Fos positive cells.
Taken together, our results indicate that the SCA17 rat model displays an anxious-like phenotype already at 3 months of age, before the exhibition of any locomotor symptoms. In addition, the genotype effect on risk-assessment and thigmotactic behaviours continues across all three age points tested, which indicates that psychiatric symptoms may develop before and along the locomotor deficits in the SCA17 rat model. In translation to patients’ situation, our results are in line with findings of mood changes including stress and anxiety in early- and late-symptomatic patients. However, it is difficult to say the same for the pre-symptomatic phase considering that very little or no information about the psychiatric status of the patients is usually known before the manifestation of the first motor symptoms. Overall, our results mimic the generalized anxiety as seen in case reports (Butler et al., 2005), making it a suitable model for further therapeutic studies.

**Acknowledgements**

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**Conflict of interest**

The authors E. Kyriakou and Dr. J.E. van der Harst are working for the EU funded “PhenoRat” project of which Noldus Information Technology is an industrial partner. Dr. J.E. van der Harst is part-time scientific project advisor for “PhenoRat” employed by Noldus Information Technology.
SUPPLEMENTARY MATERIAL

Figure S1  Anxiety and locomotor activity in the SCA17 rats compared to the WT at the ages of 3, 6 and 9 months in the elevated plus maze.

(A)  Cumulative distance travelled on the elevated plus maze apparatus during the 5-min trials showed no significant genotype or genotype x age interaction but a significant age effect (p<0.05).
(B)  Velocity of moving through the elevated plus maze during the 5-min trials showed only an age effect (p<0.001) but no genotype or genotype x age interaction effects were noted. Data represent mean ±S.E.M.; with statistically significant differences in comparison with the control group (2-way ANOVA) *p < 0.05; **p < 0.01; ***p < 0.001.

Figure S2  Anxiety and locomotor activity in the SCA17 rats compared to the WT at the ages of 3, 6 and 9 months in the open field

(A)  Frequency of visits in the center showed no genotype, age or genotype x age interaction differences.
(B)  Velocity moving within the open field arena showed an age effect (p<0.001) but no genotype or age x genotype differences. Data represent mean ±S.E.M.; with statistically significant differences in comparison with the control group (Repeated measures ANOVA) *p < 0.05; **p < 0.01; ***p < 0.001.
Figure S3  Heatmaps generated by EthoVision XT9 to create a representative image of the time that animals spent at certain locations on the elevated plus maze during 300s. Data are represented as group means.
Figure S4  Heatmaps generated by EthoVision XT9 to create a representative image of the time that animals spent at certain locations in the open field arena during 300s. Data are represented as group means.
Figure S5  Double staining in the BLA of the amygdala in WT and SCA17 rats (3 months).

(A, C) Immunohistochemistry with antibody NeuN (green) against positive (+) neurons in the BLA of WT and SCA17 rats respectively. (B, D) Immunohistochemistry with antibody c-fos (red) against positive (+) active neurons in the BLA of WT and SCA17 rats respectively. Scale bars: A-H, 100 μm

Figure S6  Double staining in the BLA of the amygdala in WT and SCA17 rats (6 months)

(A, C) Immunohistochemistry with antibody NeuN (green) against positive (+) neurons in the BLA of WT and SCA17 rats respectively. (B, D) Immunohistochemistry with antibody c-fos (red) against positive (+) active neurons in the BLA of WT and SCA17 rats respectively. Scale bars: A-H, 100 μm
Figure S7 Double staining in the BLA of the amygdala in WT and SCA17 rats (9 months)

(A, C) Immunohistochemistry with antibody NeuN (green) against positive (+) neurons in the BLA of WT and SCA17 rats respectively. (B, D) Immunohistochemistry with antibody c-fos (red) against positive (+) active neurons in the BLA of WT and SCA17 rats respectively. Scale bars: A-H, 100 μm
Home-cage anxiety levels in a transgenic rat model for Spinocerebellar Ataxia type 17 measured by an approach-avoidance task: the light spot test

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HIGHLIGHTS

- Light spot used for measuring anxiety in the home cage for the first time in rats.
- Light spot caused an initial increase in activity and time spent in the shelter.
- Light spot test evoked avoidance responses in rats.
- Light spot effects in SCA17 rats at 6 and 9 months.

ABSTRACT

Background: Measuring anxiety in a reliable manner is essential for behavioural phenotyping of rodent models such as the rat model for Spinocerebellar ataxia type 17 (SCA17) where anxiety is reported in patients. An automated tool for assessing anxiety within the home cage can minimize human intervention, stress of handling, transportation and novelty.

New method: We applied the anxiety test “light spot” (LS) (white led directed at the food-hopper) to our transgenic SCA17 rat model in the PhenoTyper 4500® to extend the knowledge of this automated tool for behavioural phenotyping and to verify an anxiety-like phenotype at three different disease stages for use in future therapeutic studies.

Results: Locomotor activity was increased in SCA17 rats at 6 and 9 months during the first 15 minutes of the LS, potentially reflecting increased risk assessment. Both genotypes responded to the test with lower duration in the LS zone and higher time spent inside the shelter compared to baseline.

Comparison with existing methods: We present the first data of a rat model subjected to the LS. The LS can be considered more biologically relevant than a traditional test as it measures anxiety in a familiar situation.

Conclusions: The LS successfully evoked avoidance and shelter-seeking in rats. SCA17 rats showed a stronger approach-avoidance conflict reflected by increased activity in the area outside the LS. This home cage test, continuously monitoring pre- and post-effects, provides the opportunity for in-depth analysis, making it a potentially useful tool for detecting subtle or complex anxiety-related traits in rodents.

Keywords: home cage behaviour; approach-avoidance test; light spot; anxiety; SCA17; animal model
INTRODUCTION

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). However, despite this physiological and evolutionary aspect of anxiety which serves as a natural adaptive reaction, anxiety can also become pathological and interfere with the ability to cope with the stressful events encountered (Steimer, 2002). For these reasons, anxiety is considered an important component of neurological phenotyping of animal models in behavioural neuroscience (Yvonne K. Urbach et al., 2010). Different behavioural tests can be used to measure several ethologically relevant aspects such as approach-avoidance, defense, stress, panic etc. depending on the protocol and set-up used (File and Seth, 2003; Pfeiffer et al., 2013). A disruption in any of the aspects of the anxiety spectrum can be indicative of psychopathology. Rodents, and especially rats, are highly suitable animals for preclinical studies as their brain complexity and their vast repertoire of behaviour and natural tendency to explore but also avoid risks make them suitable for studying anxiety (Abbott, 2004).

When evaluating anxiety in laboratory animals, unnatural and highly controlled structured environments are used. The apparatuses used, vary broadly from mazes to open area boxes and arenas (Bailey and Crawley, 2009). Over the last years, along with the advancements of behavioural neuroscience, there is a notion and urge towards high-throughput behavioural screening (Anderson and Perona, 2014; McGonigle and Ruggeri, 2014; Tecott and Nestler, 2004). Automation instead of excessive animal handling is highly needed. Some of the latest trends and needs in behavioural neuroscience are (1) limiting human intervention as much as possible (2) mimicking the natural environment of the animal to the maximum and (3) implementing the tests neuroscientists need to perform into the home cage environment.

As rats are nocturnal animals sensitive to light, they experience bright lightning as a noxious/negative stimulus (Burn, 2008). When possible, a rat will actively choose for the lowest light intensity available in its surroundings (Blom et al., 1992). Many classical anxiety behavioural tests are based on the rodents’ natural aversion to the bright light such as highly illuminated versions of open field and mazes and light-dark boxes. In this study we used an anxiety test implemented in the automated home cage PhenoTyper 4500 (Noldus IT, Wageningen, NL) hereafter mentioned as ‘light spot test’ (LS). The LS 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 dark (i.e. active) phase. Shining a light at the feeding area, which the animals visit in order to eat, is a negative stimulus that induces an approach-avoidance conflict (Cosquer et al., 2005; Godsil and Fanselow, 2004). One would
expect that anxious animals will tend to spend less time in the LS zone and seek security in the shelter that is available in the automated home cages. The LS test is designed in such a way that it creates an approach-avoidance conflict in the subject which is based on two important factors: (1) the location where the light is being presented (the feeding area, i.e. in front of the food hopper) and (2) the time when the test is being executed (i.e. 15 min after the start of the dark (active) phase). Previous activity patterns analysis throughout the dark-light cycle of rodents have shown that animals are most active during the first hour(s) right after the start of the dark phase and visit frequently the feeding zone.

Approach-avoidance conflict paradigms have been routinely used to investigate anxiety-related behaviours in psychology and also in rodents (Brown, 1948). Unlike other tests used in this context (for review see (Corr, 2013)), in the case of the LS test there is no acute reward-punishment concept. No punishment is given if the animal disregards the bright-light and visits the feeder zone and no special or extra food reward is provided except for the access to food in case the animal dares to visit the feeding area. There is only an internal dilemma between the animal’s motivation to eat and its natural response to avoid the light. This type of tests is considered valuable for studying anxiety (Aupperle et al., 2012; Shin and Liberzon, 2010) since it is based on the natural response of the animals to the stimulus preventing any influence from stress, novelty of transfer to an artificial environment as well as any manipulation by punishment and reward which can potentially influence the results. The LS test provides a mild anxiogenic stimulus, to which the response can be closely monitored for a longer period of time and can also be analysed in detail by means of a high sampling-rate in the automated home cage. It is therefore considered able to detect subtle anxiety-related effects. Thus far, this test has been validated for mice, showing that C57BL/6L mice had a strong behavioural response to the LS test which was significantly decreased when administering anxiolytic drug (Aarts et al., 2015). More specifically, the LS caused a decrease in the time spent outside the shelter in mice, which Aarts and colleagues were able to significantly decrease after the administration of diazepam. This is the first evidence that the LS can be a valuable tool for screening therapeutic agents and compounds. In the current study we show for the first time the LS test implemented in the rat automated home cage PhenoTyper 4500 for screening the anxiety levels in a transgenic rat model for a neurodegenerative disease.

Avoidance has been described as an important part of anxiety (Steimer, 2011), and anxiety is one of the symptoms reported in human patients with Spinocerebellar Ataxia type 17 (SCA17) (Bruni et al., 2004; Rolfs et al., 2003). In our previous findings by using classical behavioural tests such as the elevated plus maze and the open field, our transgenic SCA17 rat model showed an anxiety-like phenotype when tested at the ages of 3, 6 and 9 months old (Kyriakou et al., 2017). In order to investigate the disease development over time in relation to anxiety, we tested SCA17 and wildtype rats at 3 different ages before and during the stage when the motor impairments of the animals
become evident. These motor impairments have been described as ataxic gait, balance impairment, loss of postural reflexes and coordination disturbances by using the ataxia scoring (including the ledge test, hind limb clasping and gait assessment (with scoring 0-3), rotarod, beam walk and CatWalk. Weight loss starts at 4 months, the first motor symptoms start at the age of 5 months, and the life span of these rats is shortened to approximately 10 months of age (Kelp et al., 2013; Kyriakou et al., 2016). Given this information, we chose 3 different ages for our test: 3 months (3mo; pre-symptomatic), 6 months (6mo; symptomatic) and 9 months (9mo; late-symptomatic) of age which were the same ages used in our previous study.

In this study we aim to answer the following questions: (1) what are the effects of the LS on the behaviour of rats at different ages, (2) do the transgenic SCA17 rats show increased approach-avoidance responses compared to the wildtype rats (3) does the LS verify and/or extend our findings compared to classical tests and can we provide more information that contribute to showing the usefulness of the LS as a tool for measuring anxiety.

METHODS

Animals
Experimental animals were obtained by breeding heterozygous male SCA17 rats with wildtype female Sprague Dawley rats. After weaning transgenic rats were identified by using a transgene-specific PCR protocol as described earlier (Kelp et al., 2013). Three different age groups of naïve male rats of both genotypes were used (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). All animals were socially housed (3 or 2 per cage) in Makrolon-IVS cages (Techniplast, Italy) prior to the 7-day individual housing period in the PhenoTyper 4500 (Noldus IT, Wageningen, The Netherlands). In both housing conditions food and water was provided *ad libitum* and reversed day-light conditions (dark: 08:00-20:00) were used in the housing and experimental room with dim red-lights used throughout the dark-cycle. All experiments were performed in compliance to the EU guidelines for the use of animals for scientific purposes and evaluate and approved by a local Animal Ethics Committee.

LS test
Rats were housed individually in PhenoTyper 4500 cages with dimensions 45 X 45 X 55 cm (L/W/H) for a total duration of 7 days. The first 5 days were considered habituation days in which the rats were allowed to acclimatize to the new home cage environment (*Figure 1A*). After this 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, 15 min after the start of the dark phase (i.e. at 08:15) a focused white led light beam with an intensity of 500 lux was projected on the feeding area for a duration of three hours (Figure 1B). This induces an approach-avoidance dilemma for the animal: the choice between approaching the feeder zone during a period when normally the animals visit frequently the food hopper in order to get food, and avoiding the aversive bright light, and thus to voluntarily choose not to eat. During the LS period the animal has the opportunity to display a large variety of voluntary responses: from avoiding the LS zone, seeking shelter, approaching and exploring the area around the LS zone, to ignoring the light and entering the feeding area. The reason why this specific time of the day was chosen is due to the activity patterns of rodents from literature and our own data (unpublished) which have shown that already half an hour before the light-dark transition but especially right at the beginning of the dark phase and for the next 1-2 h the animals are the most active regarding food consumption (Borbély and Neuhaus, 1978; De Visser, 2008; De Visser et al., 2006).

Figure 1  Schematic representation of the home cage environment and the direction of the “LS test” in the cage (PhenoTyper 4500®)

The cage is equipped with a food-hopper, 2 water-spouts and a black shelter with 2 entrances/ openings on the front and on the side. (A) Home cage environment on days 1-5. (B) LS shining directly through the feeding area for 3h on day 6.
ANALYSIS

Behavioural analysis
From the 3-hour LS, we analysed the first hour of the response of the animals to the LS measurements were taken between 08:15-09:15 on day 5 (baseline) and the same time on day 6, when the LS was on. We focused our analysis on the first hour as the period with the strongest initial anxiety-related reactions. Further analysis of the 2nd and 3rd hour is presented in the Supplementary material (Figure S1-3). All measurements were taken using three-point detection to make sure visits in the respective zones were calculated when the whole body of the animal was inside the zone. This was done since frequent nose-tail swaps occurred in the version of EthoVision used in this study and therefore the registration of specific body parts, for instance representing head dipping in the feeder zone could not be accurately measured. Due to the length of our observations manual corrections were hardly feasible and it was chosen to focus on full-body entrance of the pre-defined zones (feeder, shelter).

To investigate the approach-avoidance response, the parameters mentioned below were chosen for analysis and were translated as follows: (1) time spent and frequency in the feeder zone (LS): indicative of approach, (2) time spent and frequency in the shelter: indicative of avoidance behaviour and (3) distance travelled: indicative of an approach-avoidance conflict and potentially representative of risk assessment behaviour.

Statistical analysis
Data analysis was performed at 3 different levels:
I. 3-way mixed ANOVA with the condition (baseline or LS) and the four 15-min time bins as within-subjects parameters and genotype as between-subject parameter for each age separately
II. 2-way mixed ANOVA focusing only on the first 15 min of the condition (baseline or LS) as within-subjects parameter and genotype as between-subject parameter for each age separately
III. Factorial mixed repeated measures ANOVA (thereafter mentioned “mixed RMA”) in order to fully compare the development of the response to the LS throughout the four 15-min time bins of the first hour and the different conditions (baseline, LS) (time bins and condition as within-subjects parameters) between the two genotypes (SCA17, wildtype) and three ages (3, 6 and 9 months old) (genotype and age as between subjects parameters).

Data were analysed using SPSS version 21. All results are shown as mean ± S.E.M. and are considered significant with a p value < 0.05. Statistical significance is indicated as p<0.05 (*), p<0.01 (**), and p<0.001(***). with a Bonferroni post-hoc test applied when necessary. In figure 3 normality was not achieved for a few time bins however due to the robustness of the ANOVA against heterogeneity of variance data are still presented as analysed.
RESULTS

1. 3-way ANOVA

   Analysis with condition (baseline or LS) and the four 15-min time bins as within-subjects parameters and genotype as between-subject parameter for each age separately.
   a) Distance travelled

   At all separately analysed ages, no 3-way interactions were observed (p>0.05) (Figure 2). For all 3 ages ANOVA revealed a significant condition x time bins interaction (3mo: F(3,54)=10.38, p<0.001, 6mo: F(3,63)=4.25, p<0.01, 9mo: F(3,60)=5.99, p<0.001). Considering the simple main effects, a time bin effect was significant for 3 (F(3,54)=9.61, p<0.001)

![Figure 2](image-url)

**Figure 2** Distance travelled during the baseline (black lines) and LS (yellow dashed lines) divided into four 15-min time bins for both genotypes

The LS test was performed at 3 different ages: (A) 3, (B) 6 and (C) 9 months old (3 months: SCA17 n=12 and WT n=9, 6 months: SCA17 n=12 and WT n=11, 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text
The light spot test in the SCA17 rats

(Figure 2A) and 6 months old ($F_{(3,63)}=4.05, p<0.05$) (Figure 2B) and a condition effect for 9 months ($F_{(1,20)}=4.93, p<0.05$) (Figure 2C). Post-hoc Bonferroni within-group comparisons per time bin between the two conditions showed a significant LS effect in the distance travelled during the first 15-min time bin compared to the baseline conditions, at both 3 and 6 months old ($p<0.001$ and $p<0.05$ respectively) (Figure 2A & B).

Same comparisons between the two conditions revealed a significant decrease in activity during the second and third 15-min time bin at 9 months ($p<0.01$ in both cases) (Figure 2C). Further pairwise comparisons focusing on the first time bin will be discussed at section II.

b) Time spent and frequency of visits to the feeder zone

At all ages analysed, no 3-way or 2-way interactions were observed ($p>0.05$) (Figure 3). For all 3 ages ANOVA revealed a significant condition simple main effect (3mo: $F_{(1,18)}=22.77, p<0.001$ (Figure 3A), 6mo: $F_{(1,21)}=10.88$ (Figure 3B), $p<0.01$, 9mo: $F_{(1,20)}=5.06, p<0.05$ (Figure 3C)). Post-hoc Bonferroni within-group comparisons between conditions showed that LS caused a significant decrease in the time spent in the feeder zone compared to the baseline measurements at all 3 ages analysed ($p<0.05$ in all three ages).

ANOVA for the frequency of visits in the feeder zone (data not shown) revealed a condition effect for all three ages (3mo: $F_{(1,18)}=8.29, p<0.05$, 6mo: $F_{(1,21)}=9.39, p<0.01$, 9mo: $F_{(1,20)}=7.33, p<0.05$). Furthermore a genotype effect was confirmed for the age of 6 months only ($F_{(1,21)}=1.96, p<0.05$). Post-hoc Bonferroni within-group comparisons between conditions showed that during the LS the rats visited the feeder zone significantly less often compared to the baseline at all 3 ages ($p<0.001$ in all three cases).

Further comparisons per genotype showed that during the baseline conditions the SCA17 rats visit significantly more frequently the feeder zone at the age of 6 months ($p<0.05$) (data not shown).

c) Time spent and frequency of visits to the shelter

At all separately analysed ages, no 3-way interactions were observed ($p>0.05$) (Figure 4).

For the ages of 3 and 9 months ANOVA revealed a significant condition x time bins interaction (3mo: $F_{(3,54)}=6.12, p<0.001$ (Figure 4A), 6mo: $F_{(3,63)}=1.8, p=ns$ (Fig. 4B), 9mo: $F_{(3,60)}=5.6, p<0.01$ (Figure 4C)). Considering the simple main effects, a condition effect was significant for 6 ($F_{(1,20)}=5.58, p<0.05$) (Figure 4B) and 9 months old ($F_{(1,20)}=4.93, p<0.05$) (Figure 4C). Post-hoc Bonferroni within-group comparison between conditions showed that the LS resulted in a significant increase in the time spent inside the shelter compared to the baseline for both at 6, and 9 months old groups ($p<0.01$ in both cases). A simple time bin significant effect for the ages of 3 ($p<0.05$), 6 ($p<0.05$) and 9 months old ($p<0.001$) was noted. Post-hoc Bonferroni analysis showed specifically a significant increase of the time spent in the shelter during both the second and third 15-min time bin compared to baseline for all three ages tested, irrespective of genotype (Figure 4A, B & C).
Frequency of visits in the shelter (data not shown) was influenced by the LS as shown by the 2-way condition x time bins effect for 3 and 6 months old (3mo: F(3,54)=8.31, p<0.01, 6mo: F(3,63)=5.73, p<0.05) as well as simple main condition (3mo: F(1,18)=8.36, p<0.05, 6mo: F(1,20)=10.65, p<0.01) and time bins effect (3mo: F(3,54)=7.49, p<0.001, 6mo: F(3,63)=6.53, p<0.01). For 9 months only a trend for a condition effect (F(1,20)=4.27, p=0.052) was noted. Post-hoc Bonferroni within-group comparisons per time bin between the two condition showed the LS resulted in increased frequency of visits in the shelter during the first and second 15-min time bin in comparison with the baseline at the 3 months old (p<0.05) and respectively during the first and fourth 15-min time bin at the 6 months old (p<0.05) (data not shown).

**Figure 3** Time spent in the feeder zone during the baseline (black lines) and LS (yellow dashed lines) divided into four 15-min time bins for both genotypes

The test was performed at 3 different ages: (A) 3, (B) 6 and (C) 9 months old (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
II. 15-min analysis

Two-way mixed ANOVA focusing on the first 15-min time bin of the condition (baseline or LS) as within-subjects parameters and genotype as between-subject parameter for each age separately.

As indicated by the previous 3-way analysis, the strongest responses to the bright light seem to happen during the first 15-min time bin. In addition, to be able to compare these results to results of standard short-lasting behavioural tests such as the elevated plus maze or the open field, in this section we will zoom into the effects taking place during the first 15-min of the LS test. The data of distance travelled during the first time bin is also visualised in bar graphs (Figure 5A).

Figure 4  Time spent inside the shelter during the baseline (black lines) and LS (yellow dashed lines) divided into four 15-min time bins for both genotypes at the ages of (A) 3, (B) 6 and (C) 9 months old (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
Figure 5 (A) Distance travelled, (B) time spent in the feeder zone and (C) time spent inside the shelter during the baseline (black and grey bars) and LS (yellow dotted and striped bars) during the first 15 min of the 1 hour LS and respective baseline (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M and statistical significance indicated as p<0.05 (*), p<0.01 (**), and p<0.001 (***).
a) Distance travelled
At all three ages analysed, no 2-way interactions were observed (p>0.05) (Figure 5A). However, ANOVA revealed a significant condition effect for the ages of 3 and 6 months (3mo: $F_{(1,19)}=19.56$, p<0.001, 6mo: $F_{(1,20)}=5.43$, p<0.05) (Figure 5A) and a significant genotype effect ($F_{(1,20)}=5.34$, p<0.05) for the age of 6 months as well as a statistical trend the age of 9 months ($F_{(1,20)}=4.02$, p=0.059) (Figure 5A). Bonferroni within-group comparisons between conditions revealed that LS caused a significant increase in the distance travelled during the first 15 min at both 3 and 6 months of age compared to baseline irrespective of genotype (p<0.01). Further comparisons per genotype between the two different conditions showed that the SCA17 rats travelled a higher distance compared to the wildtypes during the first time bin at the ages of 6 and 9 months during the LS (both p<0.05) (Figure 5A).

b) Time spent and frequency of visits to the feeder zone
At all separately analysed ages, no 2-way interactions were observed (p>0.05) (Figure 5B). In addition, no simple main effects reached significance for any of the 3 ages (p>0.05) (Figure 5B). Frequency of visits in the feeder zone reached no 2-way significant interactions as well as no simple main effects (p>0.05, data not shown).

c) Time spent and frequency of visits to the shelter
At all three ages analysed, no 2-way interactions were observed (p>0.05) (Figure 5C) but only a significant condition effect for the age of 3 months (3mo: $F_{(1,19)}=8.07$, p<0.05) (Figure 5C). Bonferroni within-group comparisons between conditions revealed that the LS resulted in a decrease in the time spent inside the shelter compared to the baseline for the age of 3 months (Figure 5C).

Frequency of visits in the shelter showed a LS effect for the ages of 3 and 6 months (3mo: $F_{(1,19)}=10.68$, p<0.01, 6mo: $F_{(1,20)}=8.44$, p<0.01) irrespective of genotype (data not shown). Bonferroni within-group comparisons between conditions confirmed a significant LS effect with an increase in the frequency of visits in the shelter in comparison with the baseline at the ages of 3 and 6 months (p<0.01 in both cases).

III. Factorial analysis
*Factorial mixed RMA with condition (baseline or LS) and the four 15-min time bins as within-subjects parameters and genotype and the three ages as between-subject parameters.*

Due to the complexity of this design and for the sake of brevity, only statistically different effects will be described. Factorial analysis is a useful analysis as it combines the whole data-set of all three experiments and takes into consideration both the different conditions (baseline and LS) and the four time bins as well as the genotype and age. However, the number of animals per group in our study should probably be larger to
support sufficient power for such a complex analysis. Thus, this analysis may only reveal very strong effects and may not confirm the more subtle effects that were reported above in the less complex analyses.

a) Distance travelled
During the first hour factorial mixed RMA indicated no 4-way interaction. Two-way interactions for condition x time bins (F(3,177)=18.85; p<0.001); age x time bins (F(6,177)=5.36; p<0.001) and age x condition (F(6,177)=3.52; p<0.05) were statistically significant (Figure 2). Simple main effects were significant for the time bins (F(3,177)=9.42; p<0.001) (Figure 2). Bonferroni within-group comparisons per time bin between the two conditions revealed that the LS significantly increased the distance travelled during the first time bin (p<0.01), followed by a significant decrease during the second and third time bin compared to the baseline (p<0.05 in both cases). In addition, within-group pairwise comparison per condition between the three different ages revealed a significant decreased distance travelled between the 3- and 9-months old animals, independent of genotype (p<0.05) (Figure 2A & C).

b) Time spent and frequency of visits to the feeder zone
During the first hour factorial mixed RMA indicated no 4-way interaction. Two-way interactions for condition x time bins (F(3,177)=3.25; p<0.05); age x time bins (F(6,177)=2.43; p<0.05) and age x condition (F(2,59)=11.13; p<0.001) were significant (Figure 3). Simple main effects were significantly different for condition (F(1,59)=39.71; p<0.001) and age (F(2,59)=11.96; p<0.001) (Figure 3). Bonferroni within-group comparisons between conditions revealed a significant LS effect expressed as a decrease in the time spent in the feeder zone compared to the baseline (p<0.001) (independent of genotypes and ages). Further comparisons per condition between the different ages showed a significant decrease between 3 and 6 and between 3- and 9-months old age groups (Figure 3A, B & C) during both baseline (p<0.05) and LS conditions respectively (p<0.01) (independent of genotypes). Conversely, within-group comparisons per age groups between the different conditions showed a significant decrease of the time spent in the feeder zone was during the LS only for the 3 months old age-group compared to the baseline measurements (p<0.05) (Figure 3A). In addition, time bin comparisons revealed that the LS caused a significantly decreased time spent in the feeder zone for all four 15-min time bins compared to the baseline (p<0.05) (Figure 3).

Frequency of visits in the feeder zone showed a condition x time bins interaction (F(3,177)=2.81; p<0.05) and a condition simple main effect (F(1,59)=23.96; p<0.001) as was the case for the duration. Bonferroni within-group comparisons between conditions confirmed a significant decrease in number of visits to the feeder zone when the LS was on compared to the baseline (p<0.05). Respectively, within-group comparisons per time bins between the two conditions revealed a significant decrease in number of visits to
the feeder zone during LS compared to the baseline during the second, third and fourth 15-min time bin (p<0.05 in all three cases - data not shown).

c) Time spent and frequency of visits to the shelter
RMA on the four 15-min time bins of the first hour of the LS and the corresponding baseline measurements 24h before did not confirm any interaction between the four parameters (F(6,177)=0.48; p>0.05) (Figure 4). ANOVA revealed a significant 2-way interaction between condition x time bins (F(3,177)=11.21; p<0.001) and a condition simple main effect (F(1,59)=12.67; p<0.01) (Figure 4). Bonferroni within-group comparisons between conditions demonstrated a significant increase of the time spent in the shelter when the LS was on compared to the baseline measurements (p<0.05). In addition, within-group comparisons per time bins between the two conditions show that this significant decrease during the LS happened during the second and third 15-min time bin (p<0.05 in both cases). Such an observation provides evidence that the avoidance response comes later, specifically during the second- and third-time bin in this case (Figure 4).

Frequency of visits in the shelter showed a condition x time bins interaction (F(3,177)=10.01; p<0.01) and a condition simple main effect (F(1,59)=22.06; p<0.001) as was the case for the duration in the shelter. Bonferroni within-group comparisons between conditions revealed a significant increased visit in shelter visits when the LS was on (p<0.01). In addition, within-group comparisons per time bins between the two conditions showed that this increase was significant during the first and second 15-min time bin during the LS (p<0.05 in both cases - data not shown).

DISCUSSION

In the present study, we used an anxiety test implemented in an automated home cage environment, aiming at investigating: (1) what are the effects of the LS at the 3 different ages tested, (2) how does the SCA17 rat model react to the anxiogenic stimulus compared to their wildtype counterparts and (3) if the LS can confirm the anxiety-like phenotype in the SCA17 rats. Like every new test, the LS test has both advantages as well as potential disadvantages, which we will outline and discuss below.

Behavioural phenotype of the SCA17 model subjected to the LS test
In order to fully capture potential genotype effects concerning anxiety-related traits and to eliminate any influence of repeated testing, naïve wildtype and SCA17 rats were used in all three experiments. In our previous findings, the SCA17 rats showed a higher level of risk assessment already at the age of 3 months and a strong anxiety-related response in the elevated plus maze at the age of 6 months, as well as in the open field at all three ages.
tested (3, 6 and 9 months old) (Kyriakou et al., 2017). In addition, our group has described an increased periphery-to-center ambulatory activity at the age of 7 months (Kelp et al., 2013). Based on these findings, in this study we aimed to investigate whether, compared to the control group, the SCA17 rats show a stronger, or more subtle anxious phenotype when subject to a mild aversive stimulus in their home cage (LS) and verify potential development over time of anxiety-traits in this model. Importantly, the additional value of the present approach in relation to previous findings, is the benefits that the home cage-based experiments offer by making a clear distinction between nonspecific (e.g. exposed in an open/elevated arena/maze for a short time under limited non-standardized circumstances or influenced by human handling and/or transportation) (Stiedl et al., 2014) and specific stressors (e.g. anxiogenic stimulus presented in the homecage). In the present study, the homecage setup provided the benefit of capturing the animals’ behaviour after a long habituation (minimizing the novelty nonspecific stressor factor) as well as an extended analysis to a longer period of time which included both baseline and subsequent to LS measurements. Such useful characteristics were missing from previous studies.

No strict comparison between the mouse LS study and our experiments can be made for a number of reasons. First, in the mouse study anxiolytic drugs were administered in order to investigate if they can reverse the effects caused by the LS. Furthermore, in the mouse study no control group was used, since they did not use transgenic models and the animals were used as their own controls comparing ‘no drug’ with drug-effects on behaviour. Additionally, in our study the time spent inside the shelter did not yield clear results. As the LS test puts relatively low pressure on the animals since it is conducted in the homecage, it may not yield very robust effects on one single parameter and may therefore urge for more in-depth analysis of subtle effects (with potentially higher translational value). As stated by Spruijt and colleagues, we also share the opinion that despite all the technological advancements, novel automated tests are frequently still treated with suspicion (Spruijt et al., 2014). For this reason, we chose to characterize the anxiety profile of the SCA17 rat model by using and comparing both classical as well as novel automated approaches.

a) Distance travelled
In order to eliminate any possibility of confounding effects due to the ataxia related phenotype of the SCA17 rats, we measured the distance travelled in all three ages. Overall, during baseline no genotype differences were found, confirming that despite their ataxia, under normal conditions the SCA17 rats are still able to move around the cage at the same level of activity as the control group. The presence of LS in the cage induced a significant increase in the distance travelled compared to baseline proving that the LS was noticed by the animals (irrespective of genotype), resulting in a change in their normal behaviour. During the LS, 9 months old rats travelled less distance compared
to rats of 3 months old (independent of the genotype). That indicates that the LS had a different effect on the different ages of the animals tested. Such an observation makes the LS an interesting tool for aging studies as it appears sensitive enough in evoking different responses throughout different ages. At the same time, that may be useful information to take into account for future studies as the choice of the age of the animals may potentially play an important role in the results obtained.

To be able to compare these findings with our previous findings with classical short lasting behavioural tests we focused also separately on the first 15 min of the LS. When zooming into the first time bin when the strongest reaction occurred we found that the SCA17 rats travel a higher distance during the LS at the ages of 6 and 9 months, which may be indicative of an approach-avoidance conflict. This can be translated as a higher risk assessment behaviour compared to the wildtypes, which is in line with our previous findings from classical tests where a risk assessment behaviour was prominent in the SCA17 rats (Kyriakou et al., 2017). The risk assessment profile we have observed with the classical tests involved increased head dipping in the open arms during the elevated plus maze and avoidance of the central area in the open field from already the age of 3 months and throughout the 6 and 9 months old testings as well. We believe that the increased mobility during the LS, may be a sign of evaluating the potential threat from the sudden change in the light conditions in their home cage, together with the conflict to approach the feeder zone. We cannot exclude that this increase is also related to general arousal, however, the lack of increased time spent or frequency of visits in the LS zone makes such an interpretation less likely, since the animals clearly avoided this zone while increasing their activity. However, as arousal has also been reported in literature as part of the anxiety response (Cryan and Holmes, 2005; Steimer, 2002; Weiss et al., 1998), the findings suggest that the SCA17 rats show signs of higher anxiety-related responses compared to their wildtype counterparts at the ages of 6 and 9 months. Such findings provide supporting evidence that the LS test can be a valid tool for measuring anxiety in the home cage.

The distance moved was calculated for the remaining area (excluding the LS zone (~325 cm²) and the in shelter zone (~ 225 cm²)), which is only about ~1475 cm². This remaining space in length is a bit more than one body length of a rat. Dividing the remaining area into more zones leaves a relatively small area which may not be the most representative of the activity and approach-avoidance response to the LS, since the animals cannot really avoid the bright light otherwise then seeking shelter. Therefore, the analysis of more zones can cause high variation in the data, since the animals are tracked by the center of gravity. In addition, the growth of the animals during this longitudinal study from 3 up to 9 months, can make zone entries variable over the course of time. Especially the growth curve of SCA17 rats is suppressed compared to WT and ends at lower body weight and length (data not shown) than WT rats, complicating the interpretation of zone entries if small zones are defined. Last, every animal may assess
the “new situation” differently (from either closer or further away). In either case, approach-avoidance will be translated in moving around in the remaining zone, and not by sitting still. Therefore, we considered the movement in the relatively small remaining cage floor area as indicative of approach-avoidance conflict.

b) **Time spent and frequency of visits to the feeder zone**
We found that both genotypes responded to the LS resulting in a decrease in the time spent in the LS zone, making it clear that the LS was able to evoke aversive responses as expected. However, due to the strength of the avoidance responses no difference in these responses between the two genotypes was found. Analysis of the average bout length of feeder zone visits (i.e. duration divided by frequency, reflecting the mean duration of each visit – data not shown) did not have any added value to the interpretation of the results.

Effects of the LS were rather strong, as shown by the extremely low time spent in the feeder zone which may have led to a floor effect. Once again, age played an important role as 6- and 9-months old rats spent less time in the feeder zone under both conditions compared to the youngest age group of 3 months. These age differences are of great value as it suggests that the animals have different feeding needs and activity patterns over time regardless of any disease or condition. Again, the age effects observed suggests as mentioned before that the LS is able to induce different effects at different ages, making it a potentially useful tool for aging research. The lack of any genotype effects may be attributed to the extremity of the responses to the LS which makes it difficult to identify any potential genotype effects. The same applies to the comparison between the LS and the classical behavioural tests. Due to the strong avoidance reactions, no direct comparison can be made as during both the elevated plus maze and the open field, most animals had at least visited the open arms and canter respectively during the first minutes showing a clear genotype effect in their responses (with the SCA17 rats being more anxious than the wildtype). In addition, due to the frequent nose-tail swaps that occurred in the version of EthoVision used in this study, head dipping in the feeder zone could not be accurately measured. This complicates the comparison between the two methods. Thus, the time spent in the LS zone might not be the most representative parameter for analysis when it comes to detecting subtle differences between experimental groups. Our findings regarding the frequency of the visits to the feeder zone were no different from those of the duration adding more evidence on the value of the effects.

Notably, under baseline conditions the SCA17 rats spent more time in the feeder zone at the age of 6 and 9 months, although this was not statistically significant. Such observation is in line with previous findings that although the SCA17 rats weigh significantly less than their wildtype counterparts already from the age of 5 months and thereafter, the food consumption in relation to their body weight was higher compared to the
control group (Kelp et al., 2013). The absolute amounts of food consumed during either the baseline or the LS were however not measured during this study to prevent disturbance of the natural behavioural pattern of the animals before and during the test.

c) Time spent and frequency of visits to the shelter

Time spent inside the shelter was analysed and presented which also showed a LS effect. However, this appeared not to be the most robust parameter. The significant increase of the time spent inside the shelter during the LS indicates the influence of the test on the animals’ behaviour, confirming the effectiveness of the LS to evoke avoidance responses. Similar to the feeder zone data, the average bout duration in the shelter was not described as no additional effects were observed. Nevertheless, for other studies with different effect size, bout durations can be of interest and hence their analysis is recommended.

In line with the strong decrease in time spent in the feeder, both genotypes responded with a strong increase in time spent in the shelter when the LS was on. Interestingly, unlike the other two parameters analysed, the absence of any age-related differences on avoidance, suggests that maybe in this test the avoidance responses are not dependent on age. Such an observation may be useful information for future aging studies and the analysis and interpretation of the outcomes. The consistency of the condition x time bin interaction effect noted in all three parameters analysed, emphasizes the strength of the long-lasting analysis of the LS compared to the short-lasting standard tests. In the case of the time spent in the shelter, the longer analysis (i.e. further than the first 15 min) revealed that true avoidance responses come at a later stage, specifically during the second, and third time bin, rather showing a more acute response such as the increased distance travelled. The effect on frequency of visits to the shelter was in line with the duration effects.

Regarding the effectiveness of the LS as an anxiety test, the findings of the time spent in the shelter suggest that by analyzing the later time bins and not focusing entirely on the acute responses, can reveal potential differences in avoidance. This is important when comparing the LS with the classical behavioural tests as in the latter the analysis is mostly limited to the first 15 min or even less. Such limitation may result in lacking insight in the development of the avoidance response over a longer period of time, as it may be a more dynamic process compared to initial risk assessment.

Evaluating more critically the classical tests one can say approach and avoidance responses are strictly mutually exclusive meaning that scientists usually focus on the approach (for example of the open arms or the center zone) considering that when animals do not approach these areas they automatically avoid them, although this may not be a conscious choice of the animal. However, in the case of the LS when the animal does not approach the feeder zone, it has other possibilities than hiding in the shelter. That said, during the LS the approach-avoidance responses can be more clearly
distinguished as the choice of the animal (it can either avoid the LS by not entering this zone, or show a stronger avoidance-response by hiding in the shelter). Further analysis of the strength and weaknesses of the current method will be described in the sections below.

**Strengths & Weaknesses of the LS test to measure home cage anxiety**

**Strengths**

Moving towards automation in behavioural neuroscience has been an attractive topic of discussion over the past years (Richardson, 2015; Schaefer and Claridge-Chang, 2012). Several options for home cage monitoring are commercially available for both mice and rats. As a matter of interest, quite often in literature the PhenoTyper home cages but also many other automated home cages are only used for extracting measurements such as general locomotor/ambulatory activity, velocity, food and water consumption monitoring etc. It is not often the case to see specific tests such as anxiety tests to be implemented within such automated cages. In our opinion this would not only make better use of large and sophisticated laboratory set-ups, but also add ethological value to the implemented tests (compared to the standard tests used until now). Therefore, the use of an automated home cage environment instead of a so-called ‘standard’ and classical novel maze or open field is probably of greater importance from a behavioural point of view (Spruijt and DeVisser, 2006). Having useful and biologically relevant screening tools that allows the thorough quantification of anxiety phenotypes is quite essential, especially when respective ethological techniques are integrated (Rodgers et al., 1997).

Unlike other more demanding tests with higher discomfort level and disturbance of natural activity patterns of the animal, the LS test has the benefit that it is based on the rodents’ natural aversion for brightly lit areas and the assurance that a covered shelter is offered (Bourin and Hascoët, 2003). Therefore, the implementation of 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 behaviour. Quantification of anxiety levels for the purpose of behavioural characterization of an animal model often contains the application of a battery of tests in order to capture the whole phenotype (Steimer, 2011). However, repeated testing and inherent handling and disturbance, can cause a high level of discomfort which is mostly unsuitable since it can interfere with the results and cause a negative impact on animal welfare (Burn, 2008). For these reasons, the LS test could be added into the ‘unconditioned behaviour’ category of tasks (Cole and Rodgers, 1994; Ennaceur, 2014) as no prior manipulations such as food restriction or deprivation or any kind of training is needed to stimulate the animal’s participation. Such a classification adds power and ethological validity to the LS test as these tests are less confounded by specific manipulations such as any learning-memory, thirst or hunger impedance which in turn can make the behavioural screening
The light spot test in the SCA17 rats is quite thorough and more biologically relevant (Campos et al., 2013). The lack of a need for prior training and food or water deprivation are also in favor of the use of the LS in the same way as described for the “light-dark box” (LDB) (Hascoët et al., 2001): 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 a ‘light-dark box’ experiment. In the case of the ‘light-dark’ box, the animal has also the freedom to choose at which of the two sides it will stay without being forced to get exposed into the bright light (Ennaceur, 2014). However, in the case of the LS the animal is being confronted with its natural instinct of feeding itself whereas in the ‘light-dark box’ there is the instinct of exploration of the novel box (Hascoët and Bourin, 1998). In addition, in the ‘light-dark box’ the animals are directly placed in the light compartment whereas the place of the animal at the beginning of the LS is not pre-defined. Interestingly, in the LS test there is also the element of curiosity for exploring the new situation presented in the home cage and assessing the potential threat.

One should note that the location of the LS as in this study over the food hopper zone could confound the results in studies that use drugs or animal models which affect feeding behaviour. In such cases, LS can be moved to a different location. However, it should be kept in mind that the imposed dilemma between the exposure to, and avoidance of the bright light and motivation to approach the food hopper would no longer exist. One way to address this would be to present the LS in the shelter, also a highly preferred zone. This is practically feasible in the PhenoTyper cages (see Maroteaux et al., 2012 used it in mice in a learning paradigm). However, this approach may disturb the natural behaviour of the animals when the normally safe shelter and will be associated with a negative stimulus. As a consequence, the animals cannot seek shelter, which is an important benefit of automated tests in the PhenoTyper (i.e. testing animals in a semi-natural familiar environment with food, shelter etc. that offers the opportunity to respond in a natural way).

Despite its limited applications so far, the LS holds a great advantage in relation to all other classical behavioural. By definition, all classical tests are claimed to be based on conflicting drives, although this has been also highly questionable (Ennaceur, 2014). In contrast to classical tests (Lohr et al., 2007; Zvolensky et al., 2000) LS’s choices do have a positive and negative stimulus interaction, including a real approach-avoidance conflict.

It should be emphasized that in comparison to the classical short-lasting behavioural tests, the LS has the advantage of having baseline measurements of the same animals allowing within-group analysis. Additionally, the test 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 and for in depth analysis tools to optimally use the detailed data acquired.
Weaknesses

The LS test is a rather recent test which to our knowledge has only been reported once for mice thus far (Aarts et al., 2015). 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 likewise be influenced by those characteristics and therefore further research by using several different mouse and rat strains may be needed, although the option to use a within-group baseline offers a solid basis for interpretation of the data of separate experiments. Furthermore, because in the LS test the escape option (i.e. stay in the shelter) is possible and easily accessible, it may lead to a (relatively) fast abortion of any other anxiety-like response (Torres, 1961), although the motivation to eat will most likely provide a temporal dynamics in the approach-avoidance response.

Albeit Aarts and colleagues claimed to have proven the pharmacological validity of the LS test, it is believed that the old-school opinion of “pharmacological validation” being a pre-requisite for proving the value of any new behavioural test of anxiety” (Gray, 1982) is rather outdated and should be reconsidered. Instead, weight should be given to the ethological and construct validity of the respective test (Hånell and Marklund, 2014; Lister, 1990; Rodgers et al., 1997).

Despite the benefits given from the baseline measurements of the same animals, this may also be a weakness due to the large variation within the population in the responses of each animal. In the same line of reasoning, the large variation, especially within the control group, can be attributed to the fact that after such a long habituation, when the stimulus is presented, the animals may have a slightly different behavioural activity pattern and some of the animals may not even be fully awake by the time the stimulus is presented. Analyzing only 1 hour out of the 24 hours of a day gives insight in the large variation in the natural behavioural pattern of a group of animals, although this was considered in the choice of the timing of the LS. Furthermore, an additional analysis such as that of the head dipping (i.e. shortly entering the exposed zone with the nose and/or head) or the body length as an index of stretch-attend posture (Hager et al., 2014) would provide more insight in the risk assessment responses of the animals. For such analysis, we recommend for future studies video material to be obtained for the duration of the LS, the lack of which is a limitation in this study. However, due to the limitations of the version of the EthoVision software used in this study such measurements could not be reliably extracted. The same applies to measuring the actual food consumed which at this point is not possible to be measured without disturbing the animals.

Conclusion

In this study we provide supporting evidence for the relevance of a recently introduced methodology for measuring anxiety in rodents in a home cage environment as an addition to, or replacement for the classical approaches that are systematically used. These ‘old school’ tests keep being applied without too much criticism due to the
scepticism and suspicion towards any novel test (Spruijt et al., 2014) as many times pointed out by research in the field of neurobiology and pharmacology (Bouwknecht and Paylor, 2008; Ennaceur, 2014; Rodgers, 1997).

In conclusion, in this study we confirmed that the LS test implemented in a home cage environment (PhenoTyper 4500):

1. has successfully induced avoidance and shelter seeking in rats as well as age-related effects (such as distance travelled, approach response and time spent in the feeder zone)
2. has evoked a stronger approach-avoidance conflict in the SCA17 rats compared to the control group reflected by the increased distance moved during the first 15 min of the LS at the ages of 6 and 9 months potentially indicating higher risk assessment which is in line with previous findings using traditional tests
3. is a promising test for measuring and evaluating avoidance and responses to negative stimuli
4. allows continuously monitoring of pre- and post-effects and provides the opportunity for in-depth analysis. These properties make it a potentially useful tool which despite the mild nature of the stimulus, the setting and the lack of punitive character can detect subtle or complex anxiety-related traits in rodents.

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Conflict of interest
The authors E. Kyriakou and Dr. J.E. van der Harst are working for the EU funded “PhenoRat” project of which Noldus Information Technology, the manufacturer of the PhenoTyper device and EthoVision XT 9 software. At the time of the studies, J.E. van der Harst was employed as part-time scientific project advisor for PhenoRat employed by Noldus Information Technology and E. Kyriakou was employed by Noldus Information Technology as part of the PhenoRat project. We thank Celina Tomczak for genotyping of SCA17 rats.
I. Results throughout the whole 3 hour duration of the LS and respective baseline

As described in the materials and methods, the duration of the LS was 3 h. For the sake of focusing on the most prominent and robust reactions we have analysed and presented the first hour. Here we describe the results throughout the whole 3 h period of the LS in comparison to the same 3 h of the day before (baseline). We analyze the three age points separately with 3-way RMA.

Figure S1 Distance travelled at the age of 3 (A1), 6 (A2) and 9 (A3) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
a) Distance travelled
During the first hour, factorial mixed RMA indicated no 4-way interaction. Two-way interactions for condition x time bins were statistically significant for the ages of 3 mo, (F(11,198)=4.63, p<0.001); 6 mo (F(11,231)=1.97, p<0.05); and 9 mo respectively (F(11,220)=2.28, p<0.05) (Figure S1A1-3). Simple main effects were significant for the condition only for the ages of 6 and 9 months old (3 mo: F(1,18)=1.26, p>0.05; 6 mo: F(1,21)=6.17, p<0.05 9 mo: F(1,20)=7.18, p<0.05). Bonferroni within-group comparisons per time bin between the two conditions revealed that at the age of 3 mo the LS caused a significant increase in the distance travelled during the first time bin (p<0.01), followed by a significant decrease during the eighth and ninth time bin compared to the baseline (p<0.05 in both cases). In the 6mo experiment the LS caused a significant increase in the distance moved during the first time bin, followed by a significant decrease during the tenth time bin compared to the baseline (p<0.05). Last, during the 9 mo experiment the LS evoked a significant increase during the second, third, and seventh time bin (p<0.05 in all cases). In addition, pairwise comparisons between the two conditions for the three ages separately revealed a significant decrease in the distance moved during the LS in comparison to the baseline measurements for the ages of 6 and 9 months old (p<0.01 in both cases).

b) Time spent in the feeder zone
During the first hour factorial mixed RMA indicated no 4-way interaction. Two-way interactions for condition x time bins were statistically significant only for the age of 3 mo, (F(11,198)=2.08, p<0.05) (6 mo (F(11,231)=1.02, p>0.05); and 9 mo respectively (F(11,220)=1.16, p>0.05) (Figure S2B1-3). Simple main effects were significantly different for condition for all three ages tested (3 mo: F (1,18)=53.96, p<0.001; 6 mo: F (1,21)=16.15, p<0.001 9 mo: F(1,20)=12.62, p<0.01). Additionally, a significant genotype effect was noted for the age of 9 mo only (F(0,20)=10.95, p<0.01). Bonferroni within-group comparisons per time bin between the two conditions revealed that at the age of 3 mo the LS caused a significant decrease in the time spent in the LS zone during the 2nd, and 3rd and throughout the 5th and 11th time bin (p<0.05 in all cases). Further comparisons between the two conditions showed a significant decrease in the time spent in the feeder zone in all three ages tested compared to the baseline activity (3mo: p<0.001; 6 and 9 mo: p<0.01 in both cases). Last, at the age of 9 mo the SCA17 rats spent significantly more time in the feeder zone compared to the wildtype counterparts (irrespective of condition) (p<0.01). Further comparisons per condition between the two genotypes revealed that difference concerned mainly the baseline conditions during which the SCA17 rats spent significantly more time in the feeder compared to the control group (p<0.01).

c) Time spent in the shelter
During the first hour, factorial mixed RMA indicated no 4-way interaction. Two-way interactions for condition x time bins were statistically significant only for the age of 3
mo, (F(1,199)=3.03; p<0.05) (6 mo (F(1,23)=0.92; p>0.05); and 9 mo respectively (F(1,220)=1.83; p=0.05)) (Figure S3C1-3). Simple main effects were significantly different for condition for all three ages tested (3 mo: F(1,18)=6.32, p<0.05; 6 mo: F(1,21)=10.88, p<0.01 9 mo: F(1,20)=14.55, p<0.01). No simple main effects were noted. Bonferroni within-group comparisons per time bin between the two conditions revealed that at the age of 3 mo the LS caused a significant decrease in the time spent in the shelter during the 1st time bin and consequently a significant increase compared to baseline during the 5th, 9th and 11th time bin (p<0.05 in all cases). Regarding the statistical trend for the age of 9 months, further comparisons per time bin between the two conditions revealed a significant increase in the time spent in the shelter compared to the baseline conditions.

Figure S2 Time spent in the feeder zone at the age of 3 (B1), 6 (B2) and 9 (B3) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
during the 2\textsuperscript{nd}, 3\textsuperscript{rd}, 6\textsuperscript{th} and 7\textsuperscript{th} time bin (\(p<0.05\) in all cases). Further comparisons between the two conditions showed a significant increase in the time spent in the shelter in all three ages testes compared to the baseline activity (\(p<0.05\) in all three cases).

II. Results 1 hour after LS and respective baseline

Here we describe the results throughout the first hour after the LS exposure in comparison to the same 1 h 24 hours before used as baseline. For the sake of simplicity, we used the first method of analysis as described in the main body of this manuscript, analyzing the three age points separately with 3-way RMA.

![Graphs showing time spent in the shelter during baseline and LS exposure](image)

**Figure S3** Time spent in the shelter at the age of 3 (C\(_1\)), 6 (C\(_2\)) and 9 (C\(_3\)) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 \(n=12\) and WT \(n=9\); 6 months: SCA17 \(n=12\) and WT \(n=11\); 9 months: SCA17 \(n=12\) and WT \(n=10\)). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
a) Distance travelled

During the first hour, factorial mixed RMA indicated no 4-way, 3-way or 2-way interactions. In addition, RMA revealed no genotype differences. No other simple main effects such as between the two conditions or between the time bins was found ($p>0.05$ in all cases). This shown that all effects noted throughout the 3 h of the LS duration, faded out right after the termination of the LS providing supporting evidence that the animals returned to their normal rhythm without permanent change in their behaviour (Figure S4A1-3).

![Graphs showing distance travelled at the age of 3 (A1), 6 (A2) and 9 (A3) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.](image-url)
b) Time spent in the feeder zone

During the first hour, factorial mixed RMA indicated no 4-way, 3-way or 2-way interactions. In addition, RMA revealed no genotype differences. No other simple main effects such as between the two conditions or between the time bins was found (p>0.05 in all cases). This shown that all effects noted throughout the 3 h of the LS duration, faded out right after the termination of the LS providing supporting evidence that the animals returned to their normal rhythm without permanent change in their behaviour (Figure S5B1-3).

Figure S5  Time spent in the feeder zone at the age of 3 (B1), 6 (B2) and 9 (B3) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
c) **Time spent in the shelter**

During the first hour, factorial mixed RMA indicated no 4-way, 3-way or 2-way interactions. In addition, RMA revealed no genotype differences. No other simple main effects such as between the two conditions or between the time bins was found (p>0.05 in all cases). This shown that all effects noted throughout the 3 h of the LS duration, faded out right after the termination of the LS providing supporting evidence that the animals returned to their normal rhythm without permanent change in their behaviour (Figure S6C1-3).

![Figure S6](image)

*Figure S6*  Time spent in the shelter at the age of 3 (C1), 6 (C2) and 9 (C3) months old during the baseline (black lines) and LS (yellow dashed lines) during all three hours divided into 12 15-min time bins for both genotypes (3 months: SCA17 n=12 and WT n=9; 6 months: SCA17 n=12 and WT n=11; 9 months: SCA17 n=12 and WT n=10). Each point represents the mean ±S.E.M. Due to complexity of the graphs, significance is described only in the text.
The light spot test in the SCA17 rats
A longitudinal investigation of social interaction in a transgenic rat model of Spinocerebellar ataxia type 17

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ABSTRACT

Background: Spinocerebellar Ataxia type 17 (SCA17) is a neurodegenerative disease characterized by ataxia, dementia, and other psychiatric symptoms, including reports of altered social behaviour. The SCA17 transgenic rat model is a promising model which reproduces several of the patients' symptoms, including motor and anxiety-like phenotype, but nothing is so far known about its social phenotype.

Objective: To explore social behaviour in the transgenic SCA17 rat model, using the social interaction test in a spacious arena.

Methods: Social interaction parameters were assessed longitudinally in SCA17 transgenic and wildtype rats across the ages of 3, 6 and 9 months, using the large (90x90 cm) PhenoTyper® 9000 arena. All animals were tested with their familiar cagemate after a short isolation prior to the social interaction test. At 9 months, an extra social interaction test was performed with unfamiliar pairs of the same genotype to investigate social novelty at the last stage of the disease development.

Results: 9 months old SCA17 rats displayed a decrease in both the frequency of interactions and the total time spent on approaching the familiar and unfamiliar test-partner. This suggests difficulties initiating social interactions with their conspecifics, which may be indicative of social anxiety, a feature also reported in SCA17 patients. However, despite this prominent decrease in the total distance moved and velocity in the SCA17 rats, no social withdrawal was observed as the total time of social interaction remained intact by the longer mean duration of each social interaction bout. Thus, the motivation to engage in social interaction remains intact. It cannot be excluded that the overall lower activity level of the SCA rats at this age (shown by a significant lower distance moved and velocity) plays a role here. Unfamiliarity between pairs, as tested at the age of 9 months, resulted in unchanged total time spent interacting compared to the wildtype, while the mean duration of social interaction bouts was increased in the SCA17 rats, similarly to the test with the familiar partner.

Conclusions: The results showed alterations in social behaviour at 9 months in the SCA17 rats, which was enlarged by testing with novel partner. Most differences were related to initiation of social contact with a familiar or an unfamiliar animal. The results could be related to the lower activity level in the SCA17, but could also be caused by a lower level of (social) stimulus-processing, and needs to be further investigated. The alterations observed in several aspects of social interaction seem to hold promise for the SCA17 transgenic rats as a model to provide a scala of possible future readouts for treatment studies.
INTRODUCTION

Social interaction is an essential and adaptive component of the biology of numerous species and plays an important role in the survival of the individual, while social recognition is critical for the structure and stability of the networks and relationships that define societies (Kaidanovich-Beilin et al., 2011). Traditionally, neuroscience focused mainly on studying single subjects. However, since the great complexity of the rats' social interactions has been recognized 50 years ago by Barnett, 1976, researchers have been shown a great interest in social behaviour of animals. Recent technological advancements both in manipulating brain activity and automatic analysis of behavior, made investigating social interactions much more accessible, which partially explains the rapid growth of this field of research (Kondrakiewicz et al., 2019). At present, social interaction has been recognized as an important component of psychiatric research as well as neurological testing of animal models in behavioral neuroscience (Yvonne K. Urbach et al., 2010).

As part of the emotional screening of a model social behaviour is a very sensitive marker which can demonstrate when an animal experiences anxiety, stress or can relate to aspects such as play and sexual behavior (File and Seth, 2003). For this reason, parameters representative of social behaviour are being used as readout parameters to recognize these psychiatric conditions. Abnormal social behavior can be indicative of a psychiatric pathology (Peters et al., 2015) and can therefore inform us of the onset or progression of conditions such as schizophrenia (Wilson and Koenig, 2014), Huntington’s (Urbach et al., 2014), Alzheimer's disease (Lewejohann et al., 2009), or Rett syndrome (Veeraragavan et al., 2016). When studying social behaviour, it is of great importance to limit stress and possible aversive conditions to the animals as the expression of social behaviour is highly dependent on the animal’s emotional state (File and Seth, 2003).

Rats can express a wide range of social behaviours including affiliative, play, aggression, maternal and sexual behaviour. Studying the social interaction in preclinical studies has certainly been a challenge, mostly attributed to its dynamic nature involving at least two individuals, making it more difficult to quantify and requiring more complex testing environments, behavioural observational methods and techniques. Although nowadays the importance of social behaviour has been acknowledged in preclinical research, extra attention should be paid to both housing and experimental conditions in order to allow an optimal manifestation of the social behaviour in a study (Spruijt et al., 2014). That said, a more ethologically-based approach in preclinical studies is important in order to achieve a better understanding of the mechanisms underlying social behaviour (Peters et al., 2015). Adopting such an experimental approach that allows for natural and self-rewarding behaviours of laboratory animals has been a challenge which many have tried to tackle by developing systems for monitoring social interacting animals. These have been developed mostly for mice (de Chaumont et al., 2012; Giancardo et al., 2013; Kabra et al., 2013; Ohayon et al., 2013; Shemesh et al., 2014; Weissbrod et al., 2013) and to
a lesser extent for rats (Castelhano-Carlos et al., 2017), while the rat might provide a highly useful animal model for studying social interaction. Including social behaviour in preclinical animal research and rodent phenotyping can increase their predictive power and value for the transition to clinical trials and treatments for humans (Peters et al., 2015; Richardson, 2015).

Spinocerebellar ataxia type 17 (SCA17) is a family member of Autosomal Dominant Cerebellar Ataxias (ADCA) characterized by variable manifestations caused by expansion of CAA/CAG repeats that are translated to an expanded polyglutamine repeat in TATA-box binding protein (TBP) (Cui et al., 2017). Although CAG repeats expansion mutation has been described in several neurodegenerative diseases and was first described in Huntington’s disease (HD) (Riley and Orr, 2006; Zoghbi and Orr, 2000), in case of SCA17 the CAG expansion affects a ubiquitous transcription initiation factor which as a component of the transcriptional complex, activates the expression of most genes (Bauer and Nukina, 2009; Koide et al., 1999; van Roon-Mom et al., 2005). The clinical features of SCA17 patients can be broad and variable presenting mainly a spectrum of prominent symptoms in an age-dependent manner. Besides the most frequently seen symptoms such as ataxic gait, abnormal movements, parkinsonism and pyramidal signs, several non-motoric symptoms such as dementia, psychiatric disturbances and epilepsy have also been reported (Bruni et al., 2004; Rolfs et al., 2003; Stevanin and Brice, 2006). More specifically, psychiatric symptoms, such as aggression (Nielsen et al., 2012), paranoia (Fujigasaki et al., 2001), euphoria (Lin et al., 2007), depression (Herrema et al., 2014; Mariotti et al., 2007), psychosis and mood changes (Zühlke et al., 2001) are observed frequently in SCA17 patients. Behavior or personality changes as initial symptoms may indicate the presence of psychiatric disorders. In SCA17 patients, cognitive deficits in situations related to social behaviour have been described (Lasek, 2006). In addition, although it was originally believed that the cerebellum is involved only in motor functions, numerous neuropsychological studies have now implicated its role also in (social) cognition (Hoche et al., 2016; Sokolovsky et al., 2010). Case reports often refer to behavioural abnormalities, personality changes, social isolation, and marked deficits in understanding social cues observed in cerebellar patients (Pollack et al., 1995; Riva and Giorgi, 2000; Tavano et al., 2007). This clinical evidence could suggest involvement of polyQ expansions in social behaviour impairments.

The SCA17 transgenic rat model is carrying a full human cDNA fragment of the TBP gene with 64CAA/CAG repeats (TBPQ64) and was generated using construct containing a fragment of the murine prion promoter (Prp), a N-terminal myc tag, full-length human TBP cDNA, and a poly-A tail (Kelp et al., 2013). The SCA17 rats have shown until now a strong motor phenotype as well as emotional alterations: previous characterization studies in the SCA17 rats showed clasping behaviour starting at the age of 8 months, robust deficits in the ataxia score test and beam walk test already at 3.5 months of age, hyperactivity in early stages (at 3 months old) followed by reduced activity in the
Phenomaster at the age of 9 months (Kelp et al., 2013). Gait impairments in the CatWalk test have also been shown already from 3 months of age, as well as deficits in fine motor control shown in the pellet reaching task (chapter 2). From an early age (3 months), the SCA17 rats also display changes in emotionality as suggested by an increased anxiety-like phenotype in an elevated plus maze set-up and in an automated homecage implemented anxiety test. This resembles the generalized anxiety in early symptomatic SCA17 patients (Kyriakou et al., 2018, 2017). Aside from the relatively extensive investigation of the motor phenotype in the SCA17 rats, its psychiatric phenotype has only been partly investigated, indicating that there is need for further phenotyping of these aspects. In one study, where a similar transgenic SCA17 model was used containing 64 CAG repeats under the control of the rat Htt promoter (von Horsten et al., 2003), the authors claimed to have captured schizophrenia-like prodromal symptoms earlier than the exhibition of the motor disorder and dysregulated monoamine levels at baseline and in response to amphetamine (Amato et al., 2017).

In the current study, social behaviour was investigated in the SCA17 rats in order to evaluate potential deficits that recapitulate the psychiatric symptoms related to social behaviour seen in patients. The background strain of the SCA17 rats and the strain of the control group (Sprague-Dawley) provided an extra advantage to study the social behaviour of this transgenic rat model. That is, literature suggests that Sprague Dawley rats express higher levels of social behaviour than other strains such as Wistar rats (Manduca et al., 2014a, 2014b). It is known that there are numerous different variations of the social interaction test that can influence what is actually measured. For example, both the light level and the familiarity of the test arena can manipulate the levels of anxiety in the animals (File and Seth, 2003). Another variation factor of the social interaction test is the use of familiar or unfamiliar conspecifics. When familiar pairs are used, social interactions reflect the existence of specific social bonds between the two familiar partners. When unfamiliar pairs are used, social interactions measured rely upon nonspecific social attraction between and interest for conspecifics (Cirulli et al., 1996).

Furthermore, a reunion with a familiar partner after a 24h isolation results in a significant decrease in cortisol levels whereas the reunion with an unfamiliar conspecific cause high levels of cortisol (Carter et al., 1988). These observations suggest that in rodents, attachment underlies social relationships and that social interactions with unfamiliar peers are more likely to provoke more anxiety-like behaviours than the investigation of familiar peers.

In order to limit the anxiety-related aspects in this study, both measurements during the active phase using red light conditions, and thorough habituation in the test arena were used. Additionally, an enlarged instrumented homecage-like arena (PhenoTyper® 9000, Noldus Information Technology, The Netherlands) was used to allow for more natural behavior while monitoring the development and changes of social interaction over time in pairs of SCA17 rats and their wildtype littermates. Also, for the longitudinal...
study familiar pairs of rats were used for the social interaction measurements to mimic the (familiar) social setting where SCA-patients are reported to show alterations. To investigate the social behavior repertoire of the SCA17 rats, we performed detailed analyses of social behaviour and examined several different parameters, obtained from both automated analysis and manual observations. This was done in a longitudinal design, in order to monitor the development of potential changes over time. Based on the information available regarding the disease progression in the SCA17 rats, three testing ages were chosen: (1) 3 months old when no obvious motor phenotype has been developed yet; (2) 6 months old which is considered an early-symptomatic stage for the SCA17 rats and (3) 9 months old when the SCA phenotype has been fully developed. Last, after the end of the longitudinal measurements at the age of 9 months, an extra measurement was performed using same genotype but unfamiliar pairs in order to gain insight on whether familiarity affected the social interaction differently in the SCA17 rats compared to the wildtype pairs.

MATERIALS AND METHODS

Animals
For the social interaction test, a total of 40 rats (20 SCA17 and 20 wildtype rats, equivalent to 10 SCA17 and 10 wildtype testing pairs). Transgenic animals were obtained from an in-house colony preserved and maintained at Radboud UMC (Nijmegen, The Netherlands) by crossbreeding transgenic males with wildtype female rats (Charles River, Sulzfeld, Germany). Pups were weaned 21 days postnatal and were group housed per 2 with littermates of the same sex and genotype after confirming the genotype via PCR analysis with the use of DNA isolated from ear tissue sample collected during weaning (procedure described in detail by Kelp et al. (2013)). All transgenic animals used in this study were heterozygous and had a Sprague Dawley background. Body weights of all animals were measured on a weekly basis throughout the duration of the study in order to monitor at first the growth and subsequently the health status of the SCA17 rats as it is known that SCA17 rats lose a significant amount of weight at the age of 5 months old already. All animals were socially housed by 2 in Makrolon-IVS polycarbonate cages (Techniplast, Italy) expect during designated experimental procedures. For this study, male rats were selected and used. Female littermates of both genotypes were kept for other (pilot) experiments (see Lorbach et al. (2018), or not included in this thesis). At all times food and water was provided ad libitum and reversed day-light conditions (dark: 08:00-20:00) were used in the housing and experimental room with dim red-lights used throughout the dark-cycle. All experiments were performed in compliance to the EU-guidelines for the use of animals for scientific purposes and evaluated and approved by an external independent Ethics Committee.
Study Design
Experimental apparatus and software

It has been previously shown that particularly in the case of studying of the social behaviour, the size of the test environment is important in order to demonstrate social behaviour and social interaction in rats (Peters et al., 2016; Spruijt et al., 2014). More specifically, Spruijt et al., 2014 have argued that the effect of short individual housing can only become apparent in an enlarged arena. This effect is expressed by an increase in social interaction, shown by a decrease in the distance between the two rats. For this reason, a relatively big arena was used (PhenoTyper® 9000, Noldus Information Technology, The Netherlands). All experiments were performed during the dark phase under red light conditions, to reduce anxiety. The cage consisted of a 90 × 90 cm black floor, transparent Perspex 100cm high walls and a roof equipped with infrared emitting LED’s (peak range average of 950 nm). The videos were recorded from a top-view perspective with a standard PhenoTyper® top unit (image resolution 704 × 576, 25 fps) placed on the roof of the cage which contained an infrared-sensitive camera (CCD 1/3” Sony Super HAD CCD black/white) and IR-filter (type Kodak 87C). Two PhenoTypers® 9000 were connected to 1 computer and used simultaneously. No bedding and accessories were used during the study procedures. Video recordings were made on computers placed outside the experimental room using the MediaRecorder® v. 2.0 software (Noldus Information Technology). The videos were subsequently scored blindly to the rats’ genotype via Observer XT 12.5 (Noldus Information Technology). The dataset acquired in this study comprised 79 videos of a social interaction test in this enlarged instrumented arena with two rats. Each recording captured 15 min of interactions between different rat pairs. Figure 1 shows examples of the captured interactions in the PhenoTyper® 9000.

Social interaction test

A social interaction test consisted always of two rats with the same genotype, simultaneously placed into the PhenoTyper® 9000 arena. A total of 10 pairs per genotype were used in this study. All pairs were tested longitudinally at the ages of 3, 6 and 9 months old in order to monitor the progression of any possible deficits. At all three age points, the same testing schedule was followed as described in table 1 below. For the purposes of subject identification, the color back or red was randomly assigned to each individual within a pair. Marking was done using an indelible marker (Edding, Germany). Since red is not visible under infrared lighting conditions, the color red was chosen for control purposes of the effect of marking. All animals were marked at the rear half of their body up to 30 min prior to testing and were returned into their home cage until the start of the test. Testing order of pairs was randomized on all days. Only after the last test session at the age of 9 months and after a wash-out period of one week, the animals were tested in the social interaction test with the same protocol. The exception was that
the session of habituation in pairs was skipped and that the subjects were paired with an unfamiliar but same-genotype and weight-matched partner (see table 2).

**Familiar testing**

The protocol used in this study is adapted from the social interaction protocol described by Peters et al., 2015, which is based on social interaction with a familiar cage mate. In table 1, the day-by-day schedule of the social interaction testing is described. In more detail, at day 1, the animals were individually habituated in the PhenoTyper® 9000 arena for 30 minutes. On day 2, both cagemates were placed into the arena and given another 20-min habituation session. Day 3 was assigned as rest day, so animals remained in their home cage. On day 4 and approximately 24 hours prior to the start of the social interaction testing session, the animals were isolated from their cagemates and housed in a Makrolon type-III cage and provided with food and water *ad libitum*. During this separation period, animals could not see or touch each other but they could hear and smell each other as they were housed in the same housing room. On day 5, the animals were reunited in the PhenoTyper® 9000 and were allowed to interact freely for a session of 15 min (during the habituation sessions, a decline in movements in the arena was observed and was therefore decided to shorten the social interaction testing session-data not shown). The use of these two habituation sessions before the start of the test were considered beneficial in order to minimize the effect of repeated testing as much as possible, but also to reduce the novelty of the arena while not encouraging territorial behaviour. This way, any initial novelty-induced behaviour is expected to decline after repeated exposure to the test environment (Rossetti et al., 2016; Spruijt et al., 2014). Additionally, short isolation from peers before the performance of the social interaction test is a common handling to manipulate and stimulate/increase social behaviour in rats (Niesink and Van Ree, 1982). The same procedural protocol was followed at all three age points.

**Table 1** Social Interaction testing protocol for familiar pairs

<table>
<thead>
<tr>
<th>Age</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3 months</td>
<td>Single</td>
</tr>
<tr>
<td>old</td>
<td>habituation</td>
</tr>
<tr>
<td>6 months</td>
<td>Single</td>
</tr>
<tr>
<td>old</td>
<td>habituation</td>
</tr>
<tr>
<td>9 months</td>
<td>Single</td>
</tr>
<tr>
<td>old</td>
<td>habituation</td>
</tr>
</tbody>
</table>
Unfamiliar testing

One week after the social interaction test with familiar pairs, animals were tested one more time, with the only difference using unfamiliar pairs this time. No littermates and no cagemates were used during this testing. This additional measurement was performed to monitor any possible effects of exposure to an unfamiliar pair on social interaction.

Table 2 Social interaction testing protocol for unfamiliar pairs at 9 months

<table>
<thead>
<tr>
<th>Age</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 months old</td>
<td>Single habituation</td>
</tr>
<tr>
<td></td>
<td>Rest day</td>
</tr>
<tr>
<td></td>
<td>Separation – Individual housing</td>
</tr>
<tr>
<td></td>
<td>Unfamiliar Social Interaction test</td>
</tr>
</tbody>
</table>

Manual scoring

All video recording collected were scored manually by one observer blind to the genotype. Software Observer v. XT 12.5 (Noldus Information Technology, The Netherlands) was used to score the behaviours using a ½ playback speed of the video in order to achieve a precise coding of the behaviours. The ethogram of all behaviours scored and their definitions are explained in table 3. Both time spent and frequency of occurrence of all behaviours were analyzed. All social behaviours were scored as the behaviour of the pair and therefore no distinction was made between the role of the actor of the receiver in our data analysis. In other words, while scoring, only one animal of the pair was followed (i.e. continuous focal animal sampling was used).

Apart from the manual scoring of the social behaviours, all video recordings were processed afterwards with the video tracking software EthoVision v. XT 10 (Noldus Information Technology) using the detection settings ‘static subtraction’. With the use of EthoVision, parameters such as the total moved during the social interaction sessions as well as the velocity with which the animals moved in the arena were analysed. Those parameters were chosen in order to monitor the changes in mobility over time especially in the SCA17 rats since as described in the introduction the SCA17 transgenic rat model exhibits reduced activity at the age of 9 months.

Data analysis

All data were analysed using IBM SPSS v.25 statistical package (IBM, Corp, Chicago, IL) and graphs were made using GraphPad Prism v8.0 (GraphPad software, San Diego California, USA). Two-way ANOVA was used to analyze all parameters of the longitudinal study with age and genotype was used as between-subject factors. Total distance moved per pair was used a covariate to correct for the significant differences in the distance moved at the age of 9 months. Bonferroni post-hoc test was used to investigate further
any significant genotype x age effects indicated by the results. Multiple comparisons Tukey post-hoc test results were used for the different assigned levels when interaction effects were not significant. A p-value of < 0.05 was considered statistically significant. It is important to note that in this study, each pair was considered a statistical unit (i.e. with 20 rats per genotype, resulting in 10 pairs of animals per genotype). Right before the 9 months old testing, one SCA17 rat had to be euthanized as it had reached a humane endpoint. Subsequently this SCA17 pair was excluded from the statistical analysis. Only in the familiar testing one SCA17 pair was identified as an outlier (based on the standard deviation of the mean) and was therefore excluded from the statistical analysis. For the statistical analysis of the familiar and unfamiliar testing at the age of 9 months, a two-way ANOVA method was used, with genotype and familiarity used as between-subject factors and total distance moved of both animals of the pair as covariate. Finally, total distance moved and mean velocity were measured per individual and were analysed using the repeated measures ANOVA with age as the within-subject factor and genotype as the between-subjects factor.

Figure 1  (A) Example of the PhenoTyper® 9000 system as a homecage environment used for observation of group housed rats. (B) A top-view example photo of two adult male rats in the PhenoTyper® 9000 cage during the social interaction test after a 24h isolation period.
Box 1 explanations for the experimental design

**Familiar pairs**
- To increase the translational value of the results of this study since patients suffering from polyglutamine diseases have been reported to face great challenges socially within their family life (Margolis et al., 1999; Vamos et al., 2007).

**Same-genotype pairs**
- Social interaction requires the participation of at least two individuals and the behavioral output of a mixed genotype cannot be easily attributed to the genetic complement of one individual (Hahn and Schanz, 1996; Lijam et al., 1997). In our analysis we were able to distinguish social behaviours attributed to SCA17 due to the uniformity of both the genetic background of the pairs and the housing per genotype. This way, long-term observations could be made on individual genotypes.
- Same-genotype pairs were chosen to eliminate the potential effects of the large body weight differences between the two genotypes (Kelp et al., 2013) and the marked differences in locomotion and gait (see chapter 2), which if applicable in future studies, should be carefully taken into consideration when designing the experiments.
- In the case of mixed-genotype pairs, scoring of behaviours should be performed per rat separately to be able to extract biologically relevant results attributed to the SCA17 genotype. In this study, due to feasibility, all behaviours were scored per pair without discriminating from which rat the behaviour was originated.

**Role of familiarity in social interaction**
- Other than attachment, interactions can also reflect a general social attraction between same-age conspecifics, which doesn't necessarily require previous familiarity. In literature it has been reported that the behavioural repertoire of rats is markedly affected by familiarity (Cirulli et al., 1996). For this reason, we considered it relevant to investigate whether SCA17 influences the motivating role of partner novelty in a familiar environment (i.e. less anxiogenic situation) on social behaviour compared to the control situation.
- The use of the social interaction test in a familiar (longitudinally) and unfamiliar (one-time testing at 9 months) context in addition to performing the social interaction test in a more ethologically relevant manner, allowed the investigation of any potential SCA17-related changes in different specific aspects of social behaviour. This increases our knowledge beyond the well-known motor phenotype of the SCA17 rats, by providing new information on specific changes in different aspects of social interaction.

**Limitations and future considerations**
- Each breeding cycle provides only a specific number of transgenic males. Thus, not all different study designs were at this point feasible. The parameters of interest we present here can serve as a basis for performing power analyses for future experiments and subsequently use these parameters for appropriate analysis of social interaction.
- For this study, the same-genotype longitudinal familiar testing and one-time unfamiliar testing was considered an appropriate start for investigating the social profile of the transgenic SCA17 rat model. Ideally, if feasible, further social interaction experiments using longitudinal unfamiliar same-genotype pairs and mixed-genotype familiar and unfamiliar pairs would provide interesting information to further characterize the social phenotype of SCA17 rats.
Table 3 Ethogram – modified after Peters, (2018). All behaviours in the social interaction (SI) test were scored as described below

<table>
<thead>
<tr>
<th>Behavioural Category</th>
<th>Behavioural element</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-contact social interaction</td>
<td>Allogrooming</td>
<td>The grooming animal has one or both front paws on the other animal and pulls at its fur. The head of the grooming animal often makes nodding-like movements.</td>
</tr>
<tr>
<td></td>
<td>Nape attack</td>
<td>One animal approaches or attacks the neck area of the other with its front part of the body, (i.e., its paws and head). This movement can be a head-turn, a leap, or a translational motion.</td>
</tr>
<tr>
<td></td>
<td>Pinning</td>
<td>The attacker then pins the other animal to the ground by pushing it down with its forepaws or its whole body. The attacked animal may turn on its back. It actively tries to keep the other on its back.</td>
</tr>
<tr>
<td></td>
<td>Social nose contact</td>
<td>One individual establishes contact or near contact with its nose to another’s body parts. Both animals remain mainly stationary during this interaction.</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>An animal actively interacts with the other animal, but the interaction is not defined. Examples for not defined interactions are: boxing, kicking, crawling over/under each other or wrestling.</td>
</tr>
<tr>
<td>Non-contact social interaction</td>
<td>Approaching</td>
<td>A try to get into proximity of the other animal. The two animals are not in proximity yet and one animal moves in a direct way towards the other until (near) contact is established.</td>
</tr>
<tr>
<td></td>
<td>Following</td>
<td>A following animal attempts to maintain a close distance to another animal while the latter is moving within a tail length distance. During chasing, the two animals run after each other in close contact and their path often describes an S- or 8-like shape.</td>
</tr>
<tr>
<td></td>
<td>Moving away</td>
<td>One animal is actively moving away from its partner after being in close proximity. Its movement is directed away from the other animal, i.e., their distance increases. Often considered as negative social interaction behaviour, seen as an action of actively stopping social interaction or of not having any more social interest.</td>
</tr>
</tbody>
</table>
RESULTS

Social interaction at ages of 3, 6 and 9 months with familiar pairs
When compared to their wildtype littermates, transgenic animals spent more time on interacting per interaction bout at 9 months (p<0.05) but no significant genotype, age or interaction effects were noted (genotype: F(1,49) = 2.61, p>0.05; age: F(2,49) = 2.33, p>0.05; interaction: F(2,49) = 1.9, p>0.05) (Figure 2A). Conversely, animals of both genotypes showed an overall significant decrease of time spent interacting socially between 3 and 9 months of age (age effect: F(2,49) = 7.65, p<0.001) but no further genotype or interaction effects were confirmed (genotype: F(1,49) = 0.16, p>0.05; interaction: F(2,49) = 0.97, p>0.05) (Figure 2B). Additionally, in order to monitor the animals’ general activity during the social interaction sessions, total distance moved and velocity were analysed. At the three different ages tested, animals showed a decrease in the total distance moved and velocity with age and a significant interaction effect (a) distance moved: age effect: F(2,72) = 15.39, p<0.001; interaction: F(2,72) = 6.42, p<0.01; (b) velocity: age effect: F(2,72) = 14.05, p<0.001; interaction: F(2,72) = 3.77, p<0.05) but no genotype effect was observed (genotype: (a) distance moved: F(1,36) = 0.07, P>0.05; (b) velocity: F(1,36) = 0.78, p>0.05). Post-hoc analysis revealed that the SCA17 rats moved significantly less and with a lower velocity than the control group at the age of 9 months. (p<0.05 in both cases) (Figures 2E & 2F). When looking more into detail into the type of social interaction that occurred at all three ages, animals showed a significant decrease across time in non-contact social behaviours whereas in-contact behaviours remained unchanged (age effect: non-contact S.I.: F(2,49) = 7.86, p<0.001; in-contact S.I.: F(2,49) = 1.42, p>0.05) with no significant interaction effects in both parameters (non-contact S.I.: F(2,49) = 1.82, p>0.05; in-contact S.I.: F(2,49) = 0.57, p>0.05). Post-hoc analysis revealed a statistical trend for a decrease only in the non-contact social interaction behaviours in the SCA17 rats compared to the control group at the age of 9 months (p= 0.06) (genotype effect: non-contact S.I.: F(1,49) = 1.19, p>0.05; in-contact S.I.: F(1,49) = 0.68, p>0.05) (Figures 2C & 2D).

Table 3 Continued

<table>
<thead>
<tr>
<th>Behavioural Category</th>
<th>Behavioural element</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-social contact</td>
<td>Solitary behaviour</td>
<td>The animals are not actively interacting or perform individual actions such as self-grooming, rearing or exploration with at least one body length distance between the two animals.</td>
</tr>
</tbody>
</table>
When looking at all behaviours separately, animals of both genotypes spent significantly more time on allogrooming between the 3 and 9 months of age (age effect: $F_{(2, 49)} = 3.42$, $p<0.05$) with no significant interaction ($F_{(2, 49)} = 1.8$, $p>0.05$) and genotype effect ($F_{(1, 49)} = 0.004$, $p>0.05$) (Figure 4A). Conversely, all animals spent significantly less time on nape attacking across the three ages we tested (age effect: $F_{(2, 49)} = 8.9$, $p<0.001$) but showed no other effect (genotype: $F_{(1, 49)} = 0.2$, $p>0.05$; interaction: $F_{(2, 49)} = 1.1$, $p>0.05$) (Figure 4B). Moreover, the SCA17 rats showed in general more pinning behaviour than the wildtype rats (genotype effect: $F_{(2, 49)} = 6.33$, $p<0.05$) showing no further significant effects (age: $F_{(1, 49)} = 1.36$, $p>0.05$; interaction: $F_{(2, 49)} = 0.06$, $p>0.05$) (Figure 4C). Social nose contact showed no age, genotype or interaction effects (genotype: $F_{(1, 49)} = 1.43$, $p>0.05$; age: $F_{(2, 49)} = 0.29$, $p>0.05$; interaction: $F_{(2, 49)} = 0.17$, $p>0.05$) (Figure 4D). Both approaching and following behaviours showed a significant age effect while post-hoc analysis revealed a decrease of approaching in the SCA17 rats at the age of 9 months ($p<0.05$) (approaching: genotype: $F_{(1, 49)} = 3.1$, $p>0.05$; age: $F_{(2, 49)} = 4.6$, $p<0.05$; interaction: $F_{(2, 49)} = 1.85$, $p>0.05$) (following: genotype: $F_{(1, 49)} = 0.1$, $p>0.05$; age: $F_{(2, 49)} = 4.2$, $p<0.05$; interaction: $F_{(2, 49)} = 0.63$, $p>0.05$) (Figure 4E & 4F). Moving away behaviour was significantly decreased in both groups of animals between 3 and 9 months old ($F_{(2, 49)} = 5.1$, $p<0.05$) and post-hoc analysis indicated a significant reduction in the time spent moving away from the partner of 9 months old SCA17 rats compared to the wildtype rats ($p<0.05$). No further effects were shown in the moving away parameter (genotype: $F_{(1, 49)} = 3.1$, $p>0.05$; interaction: $F_{(1, 49)} = 0.91$, $p>0.05$) (Figure 4G). Finally, an increase in solitary behaviour was observed with age ($F_{(2, 49)} = 7.1$, $p<0.001$) but no other effects were noted (genotype: $F_{(1, 49)} = 0.47$, $p>0.05$; interaction: $F_{(2, 32)} = 1.11$, $p>0.05$) (Figure 4H).

### Social interaction at 9 months of age with unfamiliar pairs

When comparing social interaction at 9 months between familiar and unfamiliar pairs, a significant increase in the total time spent socializing in the unfamiliar pairs rats compared to the familiar situation became apparent ($F_{(1, 32)} = 4.81$, $p<0.05$) but with no further interaction or genotype effect (genotype: $F_{(1, 32)} = 0.21$, $p>0.05$; interaction: $F_{(1, 32)} = 0.69$, $p>0.05$) (Figure 3B). Looking at the mean duration of the interaction bouts, a significant genotype effect was observed ($F_{(1, 32)} = 13.9$, $p<0.001$). Post-hoc analyses indicated an increase in the mean duration of the interaction bouts in the SCA17 rats compared to the control group in both familiarity situations ($p<0.05$ and $p<0.01$ respectively). No further effects were observed (familiarity: $F_{(1, 32)} = 1.52$, $p>0.05$; interaction: $F_{(1, 32)} = 2.57$, $p>0.05$) (Figure 3A). Further analysis of the in contact social behaviours in both familiarity situations revealed a significant increase in the SCA17 rats compared to the control group ($F_{(1, 32)} = 7.38$, $p<0.05$) and a significant familiarity effect ($F_{(1, 32)} = 15.04$, $p<0.001$) but no interaction effect ($F_{(1, 32)} = 0.74$, $p>0.05$) (Figure 3C). Post-hoc analysis showed a significant increase of the in-contact social behaviours in the SCA17 rats in the unfamiliar situation compared to the wildtype pairs ($p<0.05$). Total duration of the
non-contact social behaviours were decreased in the SCA17 rats when interacting with both familiar and unfamiliar partners (post-hoc analyses between familiar: $p=0.06$ and unfamiliar: $p<0.01$ pairs; genotype effect: $F_{(1,32)}=11.95$, $p<0.01$). No familiarity ($F_{(1,32)}=1.68$, $p>0.05$) or interaction effect was observed ($F_{(1,32)}=1.56$, $p>0.05$) (Figure 3D).

Allogrooming was significantly increased in the SCA17 rats at the age of 9 months compared to the control group. Post-hoc analysis confirmed that this increase was statistically greater between the unfamiliar pairs ($p<0.001$) (genotype: $F_{(1,32)}=15.52$, $p<0.001$; familiarity: $F_{(1,32)}=0.44$, $p>0.05$; interaction: $F_{(1,32)}=6.04$, $p<0.05$) (Figure 5A). Nape attack and pinning showed no significant effects (nape attack: genotype: $F_{(1,32)}=2.61$, $p>0.05$; familiarity: $F_{(1,32)}=2.33$, $p>0.05$; interaction: $F_{(1,32)}=0.84$, $p>0.05$) (pinning: genotype: $F_{(1,32)}=2.7$, $p>0.05$; familiarity: $F_{(1,32)}=0.049$, $p>0.05$; interaction: $F_{(1,32)}=0.12$, $p>0.05$) (Figure 5B & 5C). Social nose contact showed a significant familiarity effect, with a statistical increase between unfamiliar rats while no other differences were seen (genotype: $F_{(1,32)}=0.18$, $p>0.05$; familiarity: $F_{(1,32)}=8.21$, $p<0.01$; interaction: $F_{(1,32)}=0.01$, $p>0.05$) (Figure 5D). Approaching behaviour was affected by genotype and familiarity, as the SCA17 rats showed significantly less time approaching their partner ($F_{(1,32)}=7.45$, $p<0.01$), and familiar pairs spent significantly more time approaching in comparison with the unfamiliar situation ($F_{(1,32)}=5.21$, $p<0.05$). No interaction effect was noted ($F_{(1,32)}=0.8$, $p>0.05$) (Figure 5E). Both following and moving away behaviours showed a decrease in the SCA17 rats (following: $F_{(1,32)}=5.67$, $p<0.05$; moving away: $F_{(1,32)}=3.6$, $p=0.06$). Familiarity showed a significant effect only in the case of moving away (following: $F_{(1,32)}=0.25$, $p>0.05$; moving away: $F_{(1,32)}=55.15$, $p<0.001$). For both parameters no interaction effects were seen (following: $F_{(1,32)}=0.82$, $p>0.05$; moving away: $F_{(1,32)}=1.79$, $p>0.05$) (Figure 5F & 5G). Post-hoc analysis showed a significant decrease in following in the SCA17 rats during the unfamiliar testing ($p<0.05$) and in moving away during both familiarity situations (in both familiar and unfamiliar: $p<0.05$).
Figure 2  Social interaction (S.I.) test results at ages of 3, 6 and 9 months of age between familiar pairs (cagemates, same genotype)

See table 3 for explanation of the different elements of the social interaction analysis. (A) Mean duration of social interaction bouts across the three age points tested. (B) Total time spent interacting socially during the 15-min session across the three age points tested. (C) Total time of non-contact S.I. (D) Total time of in-contact S.I. (E) Distance moved (F) Velocity.
social interaction behaviours requiring no physical contact of the two animals across all three age points tested. (D) Total time of social interaction behaviours requiring physical contact across all three ages tested. (E) Total distance and (F) mean velocity of the pairs moved during the social interaction sessions across all three age points tested, as measured by an automated tracking system (EthoVision XT 11). Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; #= 0.05<p<0.1; ns=not significant).

Figure 3 Social interaction (S.I.) test at 9 months of age between unfamiliar pairs (same genotypes)

(A) Mean duration of social interaction bouts at 9 months in familiar and unfamiliar pairs. (B) Total time spent interacting socially during the 15-min session at 9 months in familiar and unfamiliar pairs. (C) Total time of social interaction behaviours requiring no physical contact of the two animals across at 9 months in familiar and unfamiliar pairs. (D) Total time of social interaction behaviours requiring physical contact at 9 months in familiar and unfamiliar pairs. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; #= 0.05<p<0.1; ns=not significant).
**Figure 4** Social interaction (S.I.) test at ages of 3, 6 and 9 months of age between familiar pairs

(A) Allogrooming  (B) Nape attack  (C) Pinning  (D) Social nose contact  (E) Approaching  (F) Following  (G) Moving away  (H) Solitary. Data are expressed as means ± S.E.M. Two-way RMA ANOVA results are displayed in each graph. Results from post hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***=p<0.001; ns=not significant).
Figure 5  Social interaction test at 9 months of age between unfamiliar pairs

(A) Allogrooming. (B) Nape attack. (C) Pinning. (D) Social nose contact. (E) Approaching. (F) Following. (G) Moving away. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ns=not significant).
DISCUSSION

The SCA17 transgenic rat model has been a promising model so far, however, its social phenotype has not yet been investigated. We explored social behaviour in the transgenic SCA17 rat model, using the social interaction test in a spacious arena. The social interaction test as it was performed here, aimed to investigate the motivation (social interest) for social interaction by testing in an environment that is large enough to display natural behavior, in the sense that animals could really move away or keep distance from each other. Furthermore, as stress factors seem to have an important effect on social interaction (Varlinskaya and Spear, 2008), extra attention was paid to eliminate such effects by habituation-sessions to the test-environment and the fact that the experimenter was familiar to the animals. Our results showed specific changes in social behaviour in the SCA17 rat model at 9 months of age. Differences that could be related to social anxiety, specifically shown by the lower frequency initiating a social interaction and the lower duration of approaching with a familiar and an unfamiliar animal were observed. However, the motivation for social interaction (social interest) was unchanged no social withdrawal was observed in the SCA17 rats. Unfamiliarity between pairs resulted in unchanged total time spent interacting, while the mean duration of social interaction bouts was increased in the SCA17 rats, similarly to the test-situation with familiar partners.

The social interaction test revealed no significant genotype differences in social behaviour, shown by the unchanged total social interaction time. Yet, a significant increase of the mean duration of the social interaction bouts and a statistical trend for a decrease in the frequency of occurrence of social interactions were observed in the SCA17 rats at the age of 9 months, in the test-situation with familiar partners. Furthermore, both total distance moved and velocity were shown to be significantly lower in the SCA17 rats during the 9 months age point. These results suggest that when the SCA17 phenotype and symptomatology is fully manifested, considerable alterations with initiating social interactions with their conspecifics are present. As it has been described, the decrease in the number of contacts may be associated with hypoactivity (Kaidanovich-Beilin et al., 2011), confirmed by the decreased distance moved and the trend for a lower frequency of social interactions in the SCA17 rats of 9 months old. However, this decrease may also be associated with depressive- and/or anxiety-like behavior, with the latter being evaluated and confirmed for the SCA17 rats by using both traditional (Kyriakou et al., 2017) and automated novel behavioural paradigms (Kyriakou et al., 2018). That said, it might eventually be the combination of these two factors that result in a fewer number of contacts of the SCA17 rats with their conspecifics. This is in line with reports that in SCA17 patients social phobia has frequently been observed (Lasek, 2006). It is worth mentioning that to prevent a strong confounding effect of the distance moved-related differences observed in the SCA17 rats at the age of 9 months on the social interaction measurements, distance moved was used as a covariate in our data analysis. Thus, it can

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be argued that the prominent difference in the total distance moved and velocity did not result in social withdrawal, as measured by the unchanged total social interaction time and solitary time of the SCA17 rats compared to the wildtype pairs. Our results showed no evidence of social withdrawal in the SCA17 rats at any of the ages we tested. This is of great interest, as social withdrawal is an important parameter used for measuring the negative symptoms of psychosis (Wilson and Koenig, 2014). Studies on SCA17 or other polyQ-related patients, report the presence of psychosis, often before developing full neurological symptoms (Rolfs et al., 2003; Tsuang et al., 1998). To this day, only one study has claimed schizophrenia-like symptoms in the SCA17 rats later in their life, expressed by sensorimotor gating deficits (Amato et al., 2017).

During the social interaction test at the age of 9 months, SCA17 rats spent less time in each other's proximity. This behavioural category reflects approaching, following and moving away. Only for approaching and moving away the differences reached statistical significance. On the contrary, behaviour that required physical contact showed no differences between the two genotypes. The in-contact behavioural category includes a combination of passive and active behaviours such as allogrooming, nape attack, pinning and social nose contact. With this category, the amount of time the two genotypes engaged in social play could also be evaluated (i.e., pouncing/nape attack and pinning). To that extent, play fighting is the most common form of play behavior in rats. It is initiated when one partner uses its snout to nuzzle the nape of the neck of the other animal and the partner, in turn, defends their nape from such attacks by rotating to its dorsal surface or evading the attacker (Himmler et al., 2016; Ku et al., 2016; Pellis et al., 1989). Total duration of nape attacks, a characteristic feature of rat play behavior, was not different in the SCA17 rats. However, it was as expected significantly influenced by age resulting into a decrease between 3 and 9 months of age. These results are in agreement with previous reports that play behaviour in the rat peaks during early adolescence and declines thereafter (Panksepp, 1981; Panksepp et al., 1984; Panksepp and Beatty, 1980; Vanderschuren et al., 1997; Varlinskaya et al., 1999; Varlinskaya and Spear, 2008). Another behavioural feature of play is pinning, which involves contact of the nape, a full rotation around the longitudinal axis of the recipient animal’s body, ending in a supine position with the other subject standing over it (Trezza et al., 2010). Pinning was in general increased in the SCA17 rats compared to the wildtypes. Both pinning and nape attack are representative of a more aggressive style of play behaviour which may reflect abnormal social behaviour. This might be in turn indicative of altered adult emotionality including impaired social behavior, enhanced aggression and violence (Veenema and Neumann, 2009). Aggressive episodes have been sporadically reported in SCA17 patients (Rolfs et al., 2003), which could relate to the increased pinning time. However, total pinning time was generally low and with a large group variation. Therefore, based only on this parameter, no clear conclusions can be drawn. Future studies can include measurements on aggressive characteristics of the social play behaviour in their analysis,
to investigate this aspect further. Conversely, following was significantly decreased over
the ages in both genotypes, which implies a decrease in propensity for a social interaction
over time. Social approach is known as a crucial behaviour for establishing relationships
among individuals (Panksepp et al., 2007). In line with this, classical theories on motivation
(Glickman and Schiff, 1967; Young, 1959) also propose that approach behaviours can be
highly rewarding experiences for an individual (Berridge and Robinson, 1998). In the
present study, we showed that SCA17 rats were significantly less prone than the control
group to approach and move away from their conspecifics only at the age of 9 months,
while following showed only a decrease with age from 3 to 9 months old. This lower
approaching in the SCA17 rats may suggest a social anxiety phenotype in the SCA17 rats,
or is caused by the lower activity level and compromised condition of the SCA17 rat
model. However, the total social interaction levels remained similar, suggesting that
social motivation (social interest) to interact remained intact in the SCA17 rats, despite,
the locomotion deficits of the SCA17 rats (chapter 2).

In literature, an unfamiliar testing situation is traditionally viewed as an anxiogenic
condition, and the decrease of social interactions under this condition has been used as
an animal model of anxiety (File, 1980; File and Hyde, 1978; File and Seth, 2003). In this
study, when exposed to an unfamiliar partner, SCA17 rats showed no difference in the
total time spent on social interaction. Additionally, the mean duration of social interaction
bouts was increased in the SCA17 rats when encountering an unfamiliar partner, as it was
also observed with familiar pairs. Conversely, in SCA17 rats, non-contact social behaviour
was decreased in the test with the unfamiliar partner, whereas the in-contact social
behaviour was significantly increased, compared to the wildtype control group. The
frequency of social interaction with the unfamiliar test-partner was significantly
decreased in the SCA17 rats at 9 months, enlarging the slight decrease observed with the
familiar test partner. Additionally, the significant decrease in the duration of both
approaching and following, in the test with the unfamiliar partner, provides more
information to support the hypothesized social anxiety profile of the SCA17 rats. In a
different way, the significantly increased allogrooming in the test with the unfamiliar
partner could also be an indicator of anxiety (either social anxiety or related to the
environmental stressors), since in literature, stressful situations in humans have been
reported to promote affiliative behavior (Beery and Kaufer, 2015; Taylor, 2006; Teichman,
1974; Zucker et al., 1968). Also in rodents, there has been evidence that allogrooming is
increased during a dyadic social interaction as a sign of empathy when one of the
partners is in distress (Li et al., 2014; Lu et al., 2018; Lü et al., 2017). In addition, grooming
is particularly sensitive to stress (Kalueff and Tuohimaa, 2005).

Concerning social novelty, our results showed no alterations in transgenic animals,
supported by the lack of genotype differences in the total social interaction time in the
test with the unfamiliar partner that was conducted at the age of 9 months. However,
the familiarity effect that was observed, proved that the exposure to an unfamiliar
partner increased the time spent interacting with the novel conspecific. Moreover, the introduction to a novel social partner did not result in increased social approach, but rather in a significant decrease in time spent approaching the new partner in the SCA17 rats compared to the wildtype pairs. Whether establishment of a dominance-relationship, and again the effect of the lower activity level and condition in SCA rats is involved here, is not clear. In the test with the familiar partner, when the pairs were already cage mates, it is expected that this hierarchy was probably already established in the home cage and had not to be re-established in the social interaction arena. This was supported by the absence of any sign of severe dominance or aggression during all social interaction sessions, between rats irrespective of genotype.

It is worth mentioning that a clear effect of short isolation on social behaviour was demonstrated by an overall increase in the total distance moved during the social interaction sessions in comparison with the habituation session (data not shown).

Taken together, our results indicate that despite the changes that the SCA7 rat model displays in different parameters of social interaction, there are, at the same time, no signs of lack of motivation for social interaction (social interest). However, specific changes suggest a certain level of social anxiety, mainly related to the hesitation initiating social contact (as shown by a lower frequency of social interaction and lower duration approaching) which matches the anxiety-like profile of the SCA17 rat model described in chapters 3 and 4. To our knowledge, beyond the described motor and anxiety-like phenotypes of the transgenic SCA17 rat model, this is the first study that explores other behavioural aspects of this model such as its social phenotype. Although the decreased activity and diminished condition of the SCA17 rats could play a role in the engagement of social interactions, this did not result in differences in the total social interaction. The observed bidirectional changes in social behavior may not be entirely unexpected given the larger burden of psychiatric illness in SCA17 patients compared to healthy controls, and the great diversity of the psychiatric and cognitive symptoms reported in the SCA17 patients (Rolfs et al., 2003), making it difficult for a rat model to recapitulate all the different psychiatric symptoms observed in the SCA17 patients.

**Conclusion**

This study aimed to get insight in potential changes in the characteristics of social behaviour in the SCA1 transgenic rat model. Specific alterations in social behaviour in the SCA17 rat model were revealed at 9 months of age. The results showed that most differences were related to a decreased initiation of social interaction with both a familiar or an unfamiliar animal, as shown by several contact- and non-contact (e.g. approach) behaviour related to social interest or social interaction. This could be indicative of social anxiety, but the equal total time spent on social interaction, and the longer duration of the separate social interaction bouts suggested that despite these alterations in social interaction, the motivation and interest for social interaction was unchanged in the
SCA17 rats. The alterations observed in several aspects of social interaction seem to hold promise for the SCA17 transgenic rats as a model to provide a scala of possible future readouts for treatment studies.

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Conflict of interest
The authors E. Kyriakou and Dr. J.E. van der Harst were project-members of the EU funded “PhenoRat” project (Marie Curie Initial Training Network (ITN) (FP7/2012; grant agreement No. 317259). Noldus Information Technology, the manufacturer of the PhenoTyper® device and EthoVision XT 9 software was consortium-member as Industrial partner of the PhenoRat project. At the time of the studies, J.E. van der Harst was employed as part-time scientific project advisor for PhenoRat employed by Noldus Information Technology and E. Kyriakou was employed by Noldus Information Technology as part of the PhenoRat project.
SUPPLEMENTARY MATERIAL

Materials and Methods
In addition to the parameters presented in the main text of this chapter, social interaction parameters relating to the frequency of social interactions were also analysed to provide insight in all elements of the social behavior and support interpretation of the data. This was done for both the data from the social interaction experiment in the familiar context at the ages of 3, 6 and 9 months, and the data of the test in the unfamiliar setting at the age of 9 months.

Analysis
Analysis was performed as described in the materials and methods section in the main chapter.

Results
The frequency at which animals engaged in social interactions (S.I.) (Figure S1A) shows no change over time within both groups and no change between genotypes over time (Repeated Measures ANOVA: genotype: F(1, 49) = 1.12, p>0.05; age: F(2, 49) = 1.5, p>0.05; interaction-effect (age x genotype): F(2, 49) = 1.51, p>0.05). Post-hoc analysis revealed that when compared to their wildtype littermates, transgenic animals showed a statistical trend towards significance for a decreased frequency of social interactions with their familiar conspecifics at the age of 9 months (0.05<p<0.1). Interestingly, the frequency of interactions with an unfamiliar test-partner, as tested at 9 months, was significantly lower in the SCA17 rats as compared to their wildtype littermates, suggesting that the unfamiliarity enlarged the genotype effect seen with the familiar test-partner (genotype: F(1, 32) = 5.86, p<0.05; familiarity: F(1, 32) = 1.74, p>0.05; interaction effect (genotype x familiarity of test-partner): F(1, 32) = 0.39, p>0.05) (Figure S1B).
Figure S1  Social interaction (S.I.) test (for definitions see Table 3) at ages of 3, 6 and 9 months of age between familiar pairs & Social interaction test at 9 months of age between unfamiliar pairs

(A) Frequency of social interactions across the three age points tested.  (B) Frequency of social interactions at 9 months in familiar and unfamiliar pairs. Data are expressed as means ± S.E.M. Two-way Repeated Measures ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; #= 0.05>p>0.1; ns=not significant).
Social interaction profile of the SCA17 rats
Protein aggregation, neuroinflammation and monoamine levels in the transgenic rat model of Spinocerebellar Ataxia type 17 (SCA17)

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ABSTRACT

Background: Spinocerebellar ataxia type 17 (SCA17) is an autosomal dominantly inherited neurodegenerative disorder caused by the expansion of a CAG repeat in the TBP gene resulting in an expanded polyglutamine track in the TBP protein. Patients show protein aggregates, inflammation and changes in monoamine neurotransmission. Although several mouse models have been reported which show some of the SCA17 features, a good animal model that fully recapitulates the features seen in patients’ is still needed.

Objective: The present study aimed at characterizing protein aggregation, neuroinflammation and monoamine levels in a transgenic SCA17 rat model carrying the full human TBP gene with 64 polyQ repeats.

Methods: Brain tissues from wildtype and transgenic animals were collected from different age points. Aggregate levels were measured using the filter trap assay. For quantification of TBP protein and neuroinflammation levels, brain lysates were analysed using a western blot assay. Monoamine levels were also investigated using high-performance liquid chromatography only at final disease stage.

Results: Molecular characterization of the SCA17 rat model exhibited strong aggregate accumulation, high levels of soluble mutant TBP, and reduced levels of endogenous TBP and neuroinflammation responses at early disease stages, especially in the cerebellum and less in the cortex. Moderate monoamine changes were also observed, mostly in the striatum and amygdala.

Conclusions: Our results revealed changes in age-dependent protein aggregation, intranuclear inclusions and monoamine alterations in the SCA17 rat model. Hereby, this study adds to the validity of this transgenic rat model, making this a suitable model for future therapeutic studies.
INTRODUCTION

Polyglutamine (polyQ) diseases are a group of neurodegenerative disorders caused by cytosine–adenine–guanine (CAG) trinucleotide repeat expansions, which encode subsequently a large polyQ tract that makes the disease proteins prone to misfold and aggregate (Orr and Zoghbi, 2007). Although the proteins responsible for each disorder of this family are not related to each other and differ in function and intracellular localization, polyQ diseases share several common pathological features apart from the known CAG elongation in causative genes (Margulis et al., 2013). It has been shown that the accumulation of polyQ proteins can impair and damage the mitochondria, chaperone, and ubiquitin proteasome system (Bennett et al., 2007; Chafekar and Duennwald, 2012; Solans et al., 2006). Interestingly, although the mutant proteins are expressed throughout the body, selective degeneration is observed in specific regions of the brain. This suggests that certain specific cellular conditions exist in vulnerable neurons that may cause the selective cytotoxicity by their gene products. The aggregations formed can in turn lead to degeneration of the neurons in different brain areas such as the cerebellum, the brainstem and the spinal track (Zoghbi and Orr, 1999). The amounts of mRNA and protein produced seem to be relatively unaffected in these conditions and no deletions or point mutations in most of their genes were reported. This suggests that these disorders do not result from a loss of gene function but rather from a gain of toxic function (Bauer and Nukina, 2009). Also, there is no clear relationship between expression pattern and site of pathology, except for SCA6, where the gene product is expressed predominantly in Purkinje cells (Ishikawa et al., 1999). Symptoms of these diseases usually appear at middle age and progressively worsen until death, over about 15-20 years, with longer repeat sizes being associated with an earlier age of disease onset (Persichetti et al., 1994). However, the threshold number of repeats that leads to pathology differs significantly between the different diseases from 20 for SCA6 to 52 for SCA3 (Margulis et al., 2013).

Although aggregation is one of the key features of polyQ diseases, its exact role in disease pathogenesis is controversial. Until today, it is still not entirely clear whether the presence of visible aggregates or the smaller intermediary species generated during the aggregation process are responsible for the toxicity of the expanded polyQ proteins (Bauer and Nukina, 2009). Nuclear accumulation of mutant proteins and inclusions have been identified as predominant in HD, SCA1, SCA3, SCA7, SCA17, DRPLA, and SBMA patients (Schöls et al., 2004), however, cytoplasmic inclusions have also been found in affected brain regions of HD and SCA2 patients (DiFiglia, 2002; Huynh et al., 2000). In addition, in the SCA family and other related polyQ neurodegenerative diseases neurological damage has frequently been paired with neuroinflammation and activation of glia cells surrounding damaged neurons, (especially astrocytes and microglia) (Bradford et al., 2010; Olejniczak et al., 2015; Orr and Zoghbi, 2007; Shao and Diamond, 2007).
Beyond aggregation, in terms of neurochemical changes, polyglutaminopathies have been associated with a disturbance of the metabolism of dopamine (DA) in the nuclei of the basal ganglia and striatum (caudate putamen) (Bernheimer et al., 1973; Huot et al., 2006; Lin et al., 2007). In SCA3 for example, a reduced dopamine (DA) serotonin (5-HT) turnover rate, and reduced 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentration in the CPu have been shown (Bichelmeier et al., 2007). Conversely, in HD no significant differences in the concentrations of DA and HVA in the have been observed. However, levels of serotonin or 5-hydroxyindoleacetic acid were elevated in most striatal subareas (Huot et al., 2006; Kish et al., 1987; Reynolds and Garrett, 1986).

Spinocerebellar ataxia type 17 (SCA17) is a rare and severe autosomal-dominant neurological disorder, with generally a late-onset, commonly observed during the second and third decade of life (Nakamura et al., 2001; Stevanin and Brice, 2006). The SCA17 clinical description consists of progressive gait and limb ataxia (95%), cognitive dysfunctions and dementia (~90%), and involuntary movements (extrapyramidal features) (~70%), including chorea, spasticity, parkinsonism and dystonia, as well as neuropsychiatric symptoms, pyramidal signs, and rigidity (Cellini et al., 2004; Toyoshima et al., 2004). SCA17 is caused by a CAG/CAA repeat expansion of 45 or more in the TBP gene (Koide et al., 1999). TBP is nuclear-localized and is the DNA-binding subunit of the RNA polymerase II transcription factor D (TFIID), which is essential for the expression of most protein-encoding genes, suggesting that polyQ expansion results in transcriptional dysregulation by affecting the interaction of TBP with its binding partners (Friedman et al., 2008, 2007). It has been shown that the mutant TBP displays reduced binding to TATA box DNA in vitro suggesting that the mutant TBP can induce neurotoxicity independent of its association with DNA (Friedman et al., 2008). Neuroradiological examinations in patients have shown a reduction in the availability of presynaptic dopamine transporters in the striatum as well as in glucose metabolism in the basal ganglia, whereas postsynaptic dopamine D2 receptor binding capacity was only slightly reduced (Günther et al., 2004). It is known that the formation of neuronal intranuclear inclusions (NIls) is a common hallmark of the CAG repeat diseases (Yamada et al., 2000). The molecular mechanisms responsible for the pathogenesis of the polyQ diseases including SCA17 have not yet been completely explained (Lupton et al., 2015; Xu et al., 2015). Although at the beginning it was thought that mutant polyQ aggregates were responsible for neurodegeneration seen in the disease, several studies later on have supported the disassociation between the inclusions and the neurodegeneration (Cummings et al., 1999; Klement et al., 1998; Saudou et al., 1998). The last few years data have been published indicating a protective role of the inclusions, most probably as a result of the sequestration of the mutant protein (Arrasate et al., 2004; Bowman et al., 2007). That said, despite the similarities in the pathogenesis between SCA17 and other polyQ diseases, it remains still unclear to what manner the polyQ track affects the functionality of the TBP protein.
For this reason, several different models, including cells (Reid et al., 2003; Schaffar et al., 2004), Drosophila (Hsu et al., 2014; Ren et al., 2011; Xu et al., 2015) and rodents (Friedman et al., 2007; Huang et al., 2011; Kelp et al., 2013; Lee et al., 2015), have been generated in order to shed more light into the pathoetiology of SCA17. In several of these models it has been shown that overexpression of full-length-mutant TBP and truncated-mutant TBP which lack the DNA-binding domains can cause formation of nuclear inclusions and can cause a severe neurological phenotype. This suggests that insoluble aggregates are causative factors and that the neurotoxicity of mutant TBP is independent of DNA binding (Hsu et al., 2014; Reid et al., 2003). In Drosophila SCA17 models that overexpressed polyQ-expanded TBP, neurotoxic aggregates were demonstrated, while the mutant TBP sequestered the wild-type TBP in the neuroblasts of the flies and the normal function of wild-type TBP was impaired. Additionally, Drosophila mutants with loss of Drosophila TBP confirmed that loss of TBP function caused age-associated neurodegeneration. This suggests that dysfunction of TBP may play a universal role in polyQ-induced neurodegeneration (Hsu et al., 2014). These findings together indicate that dysfunction of TBP may play a universal role in polyQ-induced neurodegeneration. Thus, to clarify the causes of SCA17 which will also help clarify the causes of other polyglutamine diseases such as Huntington’s, the study of the molecular underpinnings of SCA17 is important (Toyoshima and Takahashi, 2018).

In this study, a recently developed transgenic rat model for SCA17 was used in order to study the development of protein aggregation, neuroinflammation and monoamine levels of dopamine and serotonin in different brain areas and ages throughout the disease development. This rat model is to our knowledge the first transgenic rat model for any inherited spinocerebellar ataxia and carries a full human cDNA fragment of the TBP gene with 64CAA/CAG repeats (TBPQ64). First phenotyping efforts in TBPQ64 rats showed a severe neurological phenotype including ataxia, impairment of postural reflexes as indicated by significant ataxia scores already from the age of 5 months. This severe phenotype seen in the SCA17 rats was neuropathologically associated with neuronal loss, especially in the cerebellum. Clear degeneration of Purkinje, basket, and stellate cells, changes in the morphology of the dendrites, nuclear TBP-positive immunoreactivity, and axonal torpedos were seen using light and electron microscopy (Kelp et al., 2013). While some of these changes were successfully outlined in other existing mouse models for SCA17, evidence was provided that some crucial characteristics of SCA17 are better mirrored in TBPQ64 rats.

In order to investigate the disease development over time in relation to the aggregates accumulated in several different brain areas, in this study, we focused on methods that may reveal differences in this molecular trait related to SCA17 patients. Additionally, this study aimed also at analyzing the distribution of metabolic content with dopamine (DA) and serotonin (5-HT) as precursor, and the turnover rate of dopamine and serotonin in several brain areas in the SCA17 rats at the age of 9 months. To achieve
that, a combination of molecular techniques was used, for detection and quantification of protein aggregation, neuroinflammation and monoamine levels in the SCA17 rats.

MATERIALS AND METHODS

Ethical statement
The analyses described here were carried out at two different locations, using tissues collected from animals housed at both locations. In both cases, all experiments were approved by the local ethical committees (‘Dier Experimenten Commissie RU-DEC’ of the Radboud University Nijmegen Medical Center for the Netherlands and by the local ethics committee ‘Regierungspraesidium Tuebingen’ for the Tuebingen University for Germany) as well as according to ethical standards and use of laboratory animals according to EU-guidelines. Animals of both locations were handled in compliance with the legal requirements of Dutch legislation (Animal testing act, WOD) and the German animal welfare act.

Study 1: Quantification of mutant TBP aggregates, TBP protein levels, Purkinje cell, reactive gliosis and neuroinflammation markers

Animals and tissue collection
For the western blot (WT: n=5 and SCA17: n=5 per age point and tissue) and filter trap assays (WT: n=1 and SCA17: n=5 per age point) (University of Tuebingen, Germany), brains were collected from stock animals of the SCA17 colony preserved and maintained at Tuebingen University. Animals were bred as described above by paring heterozygous SCA17 males with wildtype females. Both wildtype and transgenic animals had a Sprague Dawley genetic background and the confirmation of the transgene was performed by PCR using ear biopsy tissue collected during weaning at postnatal day 21. All animals were provided food and water ad libitum and were housed in rooms with constant temperature (20 ± 2 °C) and humidity (55 ± 10%) with a regular light dark cycle at Tuebingen University (lights on/off at 06:00AM/06:00PM). Animals were socially housed per 3 up to 5 depending on their weight and age in Makrolon-IVS cages (Tecniplast, Italy) according to the guidelines of the Federation of European laboratory animal science associations. For the western blot and filter trap analyses, animals were sacrificed by CO2 inhalation. Brains were immediately dissected on ice and whole brain, or brain regions, were sampled. Tissue was shock-frozen in liquid nitrogen and stored at −80°C until further use.

Quantification of mutant TBP aggregates
The SCA17 transgenic animal model used in this study carries exogenous DNA incorporated into the animal genome, which results into the overexpression of the exogenous mutant
TBP. At the same time, these animals express also their own normal or endogenous TBP. However, often the integration of a transgene into the genome can disturb the expression of the endogenous gene (Dyck et al., 2003). For this reason, and to prove the validity of the model, the quantification of the expression of the exogenous mutant TBP was considered relevant in several different brain areas and at different age points. Furthermore, in order to investigate the effect of the overexpression of the mutant TBP on the endogenous TBP levels, the quantification of the endogenous TBP protein levels was measured at different brain areas at different age points. Filter trap assay (FTA) (also known also filter retardation assay) is a well-known method used for detection and quantification of aggregation of the stretched protein. With FTA, reaction products filtered through membrane that traps and retains large protein aggregates while small species including protein monomers pass through. Combined with immunodetection, this method can yield a sensitive estimation of protein aggregation. The analysis we performed included investigation of the aggregation levels in the cortex and cerebellum at the ages of 3, 6, and 10 months old brains of transgenic and control rats (SCA 17: n=5 and WT: n=1 per age point). Subsequently, for exploratory purposes, a comparison in the aggregation levels between the cerebellum, cortex and striatum was performed only in 10 months old brains of both genotypes.

For the detection of SDS-insoluble TBP aggregates, homogenates of tissue samples were first made by lysis with DPBS + 1% (v/v) Triton X-100 followed by sonication for 10s. Subsequently, protein concentration was measured using Bradford assay. Next, 12.5 μg of cerebellar, cortical or striatal homogenates were diluted in 100ml DPBS (Life Technologies) with 2% SDS and incubated for 5 min at room temperature. Denaturation was performed by warming the samples at 95°C for 5 min and let them cool down at room temperature to avoid precipitation. A nitrocellulose membrane (0.45 mm; Bio-Rad) was equilibrated in 0.1% SDS in DPBS and samples were filtered through this membrane using a Minifold® II Slot Blot System (Schleicher & Schuell). The membrane was then washed twice with DPBS and blocked with 5% SlimFast milk (Unilever) in Tris-buffered saline for 1h at room temperature. Retained SDS-insoluble TBP was detected using two different primary antibodies. In cerebellar tissues where the highest amount of aggregates was detected, both N-terminal and C-terminal antibodies were tested, in order to identify whether the abnormal polyQ stretch is located at the N-terminal or the C-terminal domain. The TBP-specific N-12 rabbit anti-TBP antibody was used at a concentration of 1:250. Secondary antibody anti-rabbit was diluted 1:10000. The polyQ-specific mouse 1C2 antibody (clone 5TF1-1C2; Millipore) was used at a concentration of 1:1000. Secondary antibody anti-mouse HRP (NXA931; GE Healthcare) was diluted 1:1000. Chemiluminescence signals were detected with the LI-COR ODYSSEY FC Imaging system and quantified using the ODYSSEY® Server software version 4.1 (both LI-COR Biosciences) (method also described here: Clemens et al., 2015).
Quantification of TBP Purkinje cell, reactive gliosis and neuroinflammation markers

Western blot analysis is a well-established method used for protein separation and identification. In this study, western blot was used to investigate the expression of endogenous soluble TBP in the cerebellum and cortex from the five transgenic and five control animals at the ages of 3, 6 and 10 months old. Additionally, exploratory analysis in the striatum of tissues from animals at 3 and 10 months of age was performed in order to quantify the transgenic and endogenous soluble TBP levels in the striatum (6 months age point was skipped for logistic reasons due to limited amount of tissues available). Comparative analysis of the levels of transgenic and endogenous soluble TBP in cerebellum, cortex and striatum was also performed at the ages of 3 and 10 months old. The distinction between the endogenous and transgenic soluble TBP, was based on the different size of these two proteins, showing different bands in the Western blots. Endogenous TBP was detected with a band at the size of ca. 38 kDa and transgenic TBP was detected with a band at the size of ca. 49 kDa. Aggregates of TBP can also be detected in the western blot, as due to their size, are getting trapped in the stacking part of the gel. Furthermore, comparative analysis within the transgenic cerebellar tissues was performed in order to monitor the levels of transgenic and endogenous soluble TBP in the cerebellum at 3, 6 and 10 months of age. Finally, we looked at Purkinje-cell loss and microglia and astrocyte activation in the cerebellum at the ages of 3, 6, and 10 months old tissues. Astrocyte activation was also investigated in the cortex at the ages of 3, 6 and 10 months old.

Immunoblotting was performed with lysates from all tissue samples mentioned. For this, tissues were thawed and homogenized on ice in DPBS-T buffer (DPBS with 1% Triton X-100) containing protease and phosphatase inhibitors (4% Complete® Protease Inhibitor Cocktail, Roche). Homogenates were diluted 1:10 in DPBS-T buffer, incubated for 25 min at 4°C and further centrifuged for 15 min at 16,100 G. The supernatant was collected and stored at −80°C containing 10% glycerol. At the time of immunoblotting, all samples were thawed on ice and the protein concentration was measured spectrophotometrically using Bradford reagent. Western blot analysis was performed according to standard procedures. Briefly, 30 μg of lysate protein were assayed by SDS-PAGE using purchased Bolt® 4-12% Bis-Tris gels (Life Technologies). Proteins were transferred on nitrocellulose membranes and probed overnight at 4°C with the respective primary antibody. One hour of incubation with a respective IRDye® antibody (goat anti-mouse or goat anti-rabbit, 800CW, 1:10000; LI-COR Biosciences) at room temperature followed. Chemiluminescence and fluorescence signals were detected with the LI-COR ODYSSEY FC Imaging system (LI-COR Biosciences). Protein levels were quantified by densitometry using Image Studio™ Lite software version. 4.0 (LI-COR Biosciences). The following primary antibodies were used in our western blots: (1) rabbit anti-TBP N-12 antibody was used at a concentration of 1:250; (2) mouse anti-polyglutamine 1C2 antibody (clone
Molecular changes in the SCA17 rats

5TF1-1C2; Millipore) was used at a concentration of 1:1000; (3) rabbit anti-Calbindin antibody working as a Purkinje neuron-specific marker (1:2000; Sigma, St. Louis, MO, USA); (4) rabbit anti-Iba1 working as a microglia-specific marker (1:2000) (Wako, Osaka, Japan); (5) rabbit anti-GFAP working as an astrocyte-specific marker (1:2000); (6) mouse anti-α-tubulin was used as a loading control (1:500; clone DM1A. CP06).

Analysis

In the filter trap analysis, a one-way ANOVA was performed with either age as between subjects parameter in case of analysis of one tissue at the ages of 3, 6 and 10 months old tissues, or with brain areas as between subjects parameter in the case of analysis of tissues collected from 10 months old animals comparing aggregates in cerebellum, cortex and striatum. To analyze the western blot data, (1) Student t-test was performed when comparing levels of transgenic or endogenous soluble TBP, calbindin or GFAP levels between wildtype and transgenic tissues at ages 3, 6 and 10 months old separately, (2) one-way ANOVA was performed with either age as between subjects parameter in case of analysis of one tissue at the ages of 3, 6 and 10 months old tissues, or with brain areas as between subjects parameter in the case of analysis of tissues collected from 10 months old animals comparing aggregates in cerebellum, cortex and striatum.

Study 2: Monoamine analysis

Animals and tissue collection

For the high-performance liquid chromatography (HPLC) measurements, brains were collected from 9 months old WT (n= 10) and SCA17 (n= 10) Sprague Dawley rats. Animals were previously exposed to a one-week isolated placement into the PhenoTyper 4500® cages at the ages of 3, 6 and 9 months old, as part of the phenotyping of the behavioural profile of the SCA17 rat model. These animals were supplied from an in-house SCA17 breeding colony at Radboud University Medical Center (Nijmegen, The Netherlands) by cross breeding transgenic rat males with wildtype females (provided from Charles River in Sulzfeld, Germany). All animals were provided food and water ad libitum and were housed in rooms with constant temperature (20 ± 2 °C) and humidity (55 ± 10%) with a reversed 12h light/dark cycle at Radboud (lights on/off at 08:00PM/08:00AM). Animals were socially housed per 3 up to 5 depending on their weight and age in Makrolon-IVS cages (Tecniplast, Italy) according to the guidelines of the Federation of European laboratory animal science associations.

Monoamine analysis

Neurochemical analyses were performed by High Pressure Liquid Chromatography (HPLC). HPLC is a method used commonly neurochemical analysis that allows to separate, identify, and quantify each component in a mixture, as it relies on pumps to pass a
pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample injected interacts in a different way with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column (Zapata et al., 2010). Here HPLC was used to determine the metabolic content and turnover rate in several brain areas of dopamine (DA) and serotonin (5-HT) in 9 months old SCA17 and wildtype male rats. The distribution of metabolites was investigated, with DA as precursor, being subsequently converted into epinephrine (E), norepinephrine (NE), 3,4-Dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylglycol (MHPG), 3-methoxytyramine (3-MT), normetanephrine (NM), homovanillic acid (HVA), were investigated. 5-HT and hydroxyindolacetic acid (5-HIAA) were also examined.

Sample collection and preparation
The dissected sample areas were obtained via punching, in a Cryostat with an object temperature of 11 °C and chamber temperature of 12 °C, s; from whole brains previously stored in -80 °C. The brains were put in -20 °C 24 hour prior to the punching. The regions of interest (200 μm thick slices) were punched with different needle widths (NW) and collected in Eppendorf tubes, with the exception of the cerebellum, which was taken as a whole. The Eppendorf tubes were weighed pre- and post-punching. The following brain regions were selected using a rat brain atlas (Paxinos and Watson, 2006): the medial prefrontal cortex (mPFC; NW: 2.00 mm; 8 sections), the primary motor cortex (M1; NW: 1.50 mm; 8 sections), the caudate putamen (CPu; NW: 2.00 mm; 10 sections), the basal lateral amygdale (BLA; NW: 1.00 mm; 8 sections), the central amygdala (CeC; NW: 1.00 mm; 8 sections), the raphe nucleus (DRN; NW: 0.75 mm; 8 sections), and the cerebellum (as a whole). The prepared punched samples were initially homogenized, at a constant speed and duration, together with 100 mL/ 3 mg wet tissue 0.2 M perchloric acid; and 1 μL/ 1 mg wet tissue of 1 ng/ 1 μL ISO in solution A (i.e. internal standard). Afterwards, the homogenized samples were kept on melting ice for 30 minutes to denature the proteins, and then centrifuged for 15min at 4°C on 17000 RPMs. Subsequently, the supernatant was removed and pH-adjusted with 3 μL 1.0 M sodium acetate per 25 μL 0.2 M perchloric acid, filtered and ultimately stored at -80 °C.

Data collection
For the analysis, an Eicom HTEC-500 – methanol graded HPLC, was used with an Eicompak SC-50DS (ID 3.0 x 150 mm) separation column. The pressure of the liquid was consistent between 7.9 – 8.1 MPa at a constant flow rate of 400 μL/min and an applied potential of + 750 mV – coupled with an Ag/AgCl indicator. Additionally, the working electrode graphite used, was the WE 3G (Gasket GS-25), including an Eicom EAS-20 Auto Injector. The data were processed via the Eicom EPC-300 Data Processor at a temperature of 25 °C and time constant of 3 seconds. The recorded data was then visualized in
PowerChrom v2.2.4 software, in which every sample was analyzed through the HPLC for 25 minutes post injection. Ahead of every “prepared sample” analysis of each day, the “standard samples” retention times had to be certified for each monoamine. The injected amount of “standard” sample, per monoamines, stands on 100 pg, resulting in a 25 μL injection volume (with a continuous movement and without any air interference) of standard solution 1 and 2; and a 5 μL (i.e. minimum injection volume) injection of 3-methoxy-4-hydroxyphenylglycol (MHPG) (20 pg/ μL). Subsequently, every “prepared” sample was injected with a 10 μL volume. The corresponding peaks of the samples, with similar retention times as the standards, were assumed to be one of the monoamines of interest.

Analysis
For the HPLC data a one-way ANOVA, comparing the metabolites between genotypes, was conducted. All data described here was analyzed using IBM SPSS Statistics 23.0 undergoing an outlier analysis. Statistical significance was set at p<0.05 in all tests. Results visualization was performed using GraphPad Prism v.6.0 (GraphPad Software Inc., San Diego, USA).

RESULTS

Study 1

Quantification of mutant TBP aggregates
Aggregation levels of mutant TBP have been measured in the cerebellum and cortex of SCA17 rats at three different ages points (3, 6 and 10 months old), to evaluate the development of aggregates across time. As shown in Figure 1A and 1A1, using the polyQ-specific N-terminal antibody 1C-2 a significantly increased aggregation was observed over time in the cerebellum. One-way ANOVA revealed a significant age effect (F (2,12)= 9.0, p<0.01) and post-hoc analyses indicated a significant increase between 3 and 10 (p<0.01) and between 6 and 10 months old (p<0.05) cerebellar tissues respectively. In the same direction, as shown in Figure 1B and 1B1, when using TBP-specific N-terminal antibody N-12, there was a significant increase in the aggregates in the cerebellum from 3 to 10 months of age (age effect: F (2,12)= 27.2, p<0.0001; post-hoc analysis: 3-6 months old: p<0.05, 6-10 months old: p<0.01, 3-10 months old: p<0.0001). Based on these results, the N-terminal antibody was used in all analyses thereafter for TBP detection (aggregates, mutant, or endogenous). Conversely, cortical analysis did not show any significant aggregation throughout time compared to wildtype tissues (F (2,12)= 0.97, p>0.05) (Figure 1C and 1C1). Finally, an exploratory analysis using only tissues of the last age point in order to investigate any differences of aggregation between cerebellum, cortex and striatum...
**Figure 1** Filter trap analysis of mutant TBP aggregates

SDS-insoluble proteins from cerebellar (A & B), cortical (C) and striatal (D) lysates were trapped on a nitrocellulose membrane and probed with the polyQ-specific (A) and/or TBP-specific N-12 (B, C, D) antibody to quantify the amount of aggregated mutant TBP (A1-C1). Levels of soluble mutant TBP were measured in cerebellar and cortical lysates at the ages of 3, 6 and 10 months via filter trap analysis. (D1) Levels of soluble mutant TBP were measured in cerebellar, cortical and striatal lysates from 10 months WT and SCA17 rats via filter trap analysis. Data are expressed as means ± S.E.M. Two-way ANOVA results are displayed in each graph. Results from post-hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ****p<0.0001).
Molecular changes in the SCA17 rats

The three brain areas that are seemingly the most affected in patients showed a significantly high level of aggregates in the cerebellum compared to the cortex and striatum respectively (tissue effect: $F_{(2,12)} = 57.71$, $p<0.0001$; post-hoc analysis: Ce-Ctx and Ce-Str $p<0.001$, Ctx-Str: $p>0.05$) (Figure 1D and 1D1).

Quantification of TBP, Purkinje cell, reactive gliosis and neuroinflammation markers

Expression levels of several proteins in the cerebellum were investigated by protein gel blot analysis of cerebellar (Figure 2A-C), cortical (Figure 3A-C) and striatal (Figure 4A-B) lysates from wildtype and SCA17 rats at the ages of 3, 6 and 10 months old. In the cerebellum, levels of endogenous TBP were found to be strongly reduced at all three age points tested in the mutant animals in comparison with wildtype rats (3 months old: $t(8)=4.28$, $p<0.01$ (Figure 2A1); 6 months old: $t(8)=8.39$, $p<0.0001$ (Figure 2B1); 10 months old: $t(8)=4.47$, $p<0.01$ (Figure 2C1)). On the contrary, the levels of mutant TBP were as expected significantly higher in the transgenic animals at all three age points we analysed, providing evidence of the validity of the model (3 months old: $t(8)=10.28$, $p<0.0001$ (Figure 2A2); 6 months old: $t(8)=14.60$, $p<0.0001$ (Figure 2B2); 10 months old: $t(8)=9.93$, $p<0.0001$ (Figure 2C2)).

Subsequently, in the cortex, endogenous rat TBP levels were strongly reduced at the ages of 3 and 6 months old but reached no statistical significance at 10 months most probably due to large within group variation in the control group (3 months old: $t(8)=6.64$, $p<0.001$ (Figure 3A1); 6 months old: $t(8)=3.0$, $p<0.05$ (Figure 3B1); 10 months old: $t(8)=1.39$, $p=ns$ (Figure 3C1)). On the other hand, the levels of mutant TBP were as expected significantly higher in the transgenic animals in all three age points we analysed, providing evidence of the validity of the model (3 months old: $t(8)=11.7$, $p<0.0001$ (Figure 3A2); 6 months old: $t(8)=9.56$, $p<0.0001$ (Figure 3B2); 10 months old: $t(8)=8.85$, $p<0.0001$ (Figure 3C2)). Conversely, protein levels analysis in the striatum at 3 and 10 months old revealed that the endogenous and mutant TBP levels remained unchanged at 3 months of age (endogenous: $t(8)=1.69$, $p=ns$ (Figure 4A1); mutant TBP: $t(8)=1.18$, $p=ns$ (Figure 4A2)) with subsequently a significant decrease in the endogenous TBP and a significant increase of the mutant TBP in the SCA17 rats compared to the control group at 10 months old (endogenous: $t(8)=5.94$, $p<0.001$ (Figure 4B1); mutant TBP: $t(8)=2.87$, $p<0.05$ (Figure 4B2)).

Additionally, we performed a comparative analysis of the endogenous and mutant TBP protein levels between cerebellar, cortical and striatal tissues at the ages of 3 and 10 month respectively (Figure 4C-D). At 3 months old, endogenous and mutant TBP levels were significantly higher in the cerebellum compared to cortex and striatum (tissue effect: $F_{(2,12)} = 89.69$, $p<0.0001$; post-hoc analysis: Ce-Ctx and Ce-Str $p<0.0001$, Ctx-Str: $p=ns$) (Figure 4C1 and 4C2). At 10 months old, the same effect was observed (tissue effect: $F_{(2,12)} = 89.50$, $p<0.0001$; post-hoc analysis: Ce-Ctx and Ce-Str $p<0.0001$, Ctx-Str: $p=ns$).
Molecular changes in the SCA17 rats
Finally, comparative analysis of the endogenous and mutant TBP levels in the cerebellum between ages 3, 6 and 10 months (Figure 4E) revealed no significant differences across the ages tested with both proteins being slightly but not significantly increased between the three age points within the transgenic population (endogenous: age effect $F_{(2,12)} = 1.21, p = \text{ns}$, (Figure 4E); mutant TBP: age effect: $F_{(2,12)} = 2.0, p = \text{ns}$, (Figure 4E2)).

These comparative analyses described here, were performed to be able to quantify and directly compare the expression of endogenous and exogenous TBP: (1) at a specific age point between different brain areas and (2) in the cerebellum where the most high expression was noted, between the three different age points we chose to monitor the changes of the TBP expression with the disease development. These measurements were performed additionally to the quantifications described above and presented in figures 2 and 3, in order to be able to directly compare and analyze statistically together the expression in different brain areas or in cerebellum at different ages. Such an approach strengthens the results of the statistical analysis as all samples analysed were blotted in the same gel. This minimizes the risk of finding differences due to technical inconsistencies or artefacts during the western blot analysis. For this reason, results from separate blots (Figure 2 and 3) were analysed separately using a t-test, while results from the comparative blots (cerebellum, cortex, striatum per age point or cerebellum at 3, 6 and 10 months) were analysed using one-way ANOVA.
Molecular changes in the SCA17 rats

A  Rat cortex

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3M

B  Rat cortex

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6M

C  Rat cortex

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10M

Endogenous TBP - 3 M

A1: Signal relative to wild-type

Endogenous TBP - 6 M

B1: Signal relative to wild-type

Endogenous TBP - 10 M

C1: Signal relative to wild-type
Figure 3  Western blot analysis of TBP levels in the cortex of WT and SCA17 rats of 3 (A), 6 (B) and 10 (C) months old

Full-length forms of endogenous (A₁, B₁, C₁) and mutant (A₂, B₂, C₂) TBP were assessed in cortical lysates using the TBP-specific N-12 antibody for the ages of 3, 6 and 10 months old. Black arrowheads ‘fl’= full-length mutant TBP and ‘feg’= endogenous TBP; α-tubulin= loading control agg. TBP= TBP aggregates; SG=Stacking gel; RG=Running gel. Astrocyte activation was assessed using anti-GFAP antibody for the ages of 3, 6 and 10 months old (A 3, B 3, C 3). (‘A’ graphs refer to 3 months old analyses, ‘B’ to the 6 and ‘C’ to the 10 months old respectively). Data are expressed as means ± S.E.M. Student’s unpaired t-test results are displayed in each graph. Results are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; ****p<0.0001).
Molecular changes in the SCA17 rats

A. Rat striatum

Endogenous TBP - 3 M

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B. Rat striatum

Endogenous TBP - 10 M

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Mutant TBP - 3 M

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Mutant TBP - 10 M

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</table>
C1  Endogenous TBP - 3 M

C2  Mutant TBP - 3 M

D1  Endogenous TBP - 10 M

D2  Mutant TBP - 10 M

**Endogenous TBP**

**Mutant TBP**

**WT TBPQ64/Ce**

**TBPQ64/Ctx**

**TBPQ64/Str**

**agg.TBP**

**α-tubulin**

**TBP (N-12)**
Figure 4  Western blot analysis of TBP levels in the striatum (A, B), cerebellum (C, D, E) and cortex (C, D) of WT and SCA17 rats at different age points. Full-length forms of endogenous (A1, B1) and mutant (A2, B2) TBP were assessed in striatal lysates using the TBP-specific N-12 antibody for the ages of 3 and 10 months old. Comparative protein expression analysis in the cerebellum, cortex and striatum of WT and SCA17 rats of 3 (C) and 10 (D) months old. Full-length forms of endogenous (C1, D1) and mutant (C2, D2) TBP were assessed in cerebellar, cortical and striatal lysates using the TBP-specific N-12 antibody for the ages of 3 and 10 months old. Comparative protein expression analysis in the cerebellum at ages 3, 6 and 10 months old (E). Full-length forms of endogenous (E1) and mutant (E2) TBP were assessed in cerebellar lysates using the TBP-specific N-12 antibody for the ages of 3, 6 and 10 months old. Black arrowheads fltg= full-length mutant TBP and fltg= endogenous TBP; α-tubulin= loading control, agg. TBP= TBP aggregates; SG=Stacking gel, RG=Running gel. Data are expressed as means ± S.E.M. Student’s unpaired t-test results are displayed in each graph. Results are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001, ****p<0.0001).
Alterations of the Purkinje neurons within the cerebellum are thought to be relevant for the neurodegeneration observed in the SCA17 disease (Bruni et al., 2004). Calbindin, which is a calcium binding protein abundant in the cerebellar Purkinje cells, was quantified here to further investigate Purkinje cell loss and calcium homeostasis, as calbindin is known to be involved in cellular calcium regulation (Barski et al., 2003). Therefore, we also evaluated calbindin levels in the cerebellum of SCA17 rats with respect to wildtype animals. As shown in Figure 2A–2C, we observed significantly increased calbindin levels at pre-symptomatic stage, followed by a down-regulation at the last stage of the disease (3 months old: $t(8) = 3.16$, $p < 0.05$ (Figure 3A); 6 months old: $t(8) = 0.62$, $p = n.s.$ (Figure 2B); 10 months old: $t(8) = 2.71$, $p < 0.05$ (Figure 2C)).

Reactive gliosis, characterized by intense GFAP staining and a prominent pathological feature of SCA17 patients (Bruni et al., 2004). Neuroinflammation has also been demonstrated in several neurodegenerative diseases, and inhibition of microglial activation could lead to an amelioration of neurodegeneration (Liu, 2003; Sugama et al., 2009). In our study, no changes in GFAP levels were observed in the cerebellum of the SCA17 rats at all ages we analysed (3 months old: $t(8) = 2.03$, $p = n.s.$ (Figure 2A); 6 months old: $t(8) = 0.75$, $p = n.s.$ (Figure 2B); 10 months old: $t(8) = 1.57$, $p = n.s.$ (Figure 2C)). Conversely, increased Iba1 levels at pre-symptomatic stage, followed by a down-regulation at the last stage of the disease were observed in our results (3 months old: $t(8) = 2.6$, $p < 0.05$ (Figure 2C); 6 months old: $t(8) = 0.04$, $p = n.s.$ (Figure 2B); 10 months old: $t(8) = 2.7$, $p < 0.05$ (Figure 2C)). Concerning astrocytes activation in the cortex, GFAP levels were shown to be significantly decreased at 3 and 6 months of age, without reaching statistical significance for the 10 months due to large within groups variation (3 months old: $t(8) = 2.32$, $p < 0.05$ (Figure 3A); 6 months old: $t(8) = 2.93$, $p < 0.05$ (Figure 3B); 10 months old: $t(8) = 0.12$, $p = n.s.$ (Figure 3C)).

Study 2

Monoamine analysis

The metabolic content (picograms/mg wet tissue) of the catecholamines and indoleamines per region, in tissues harvested from 9 months old wildtype and transgenic SCA17 rats were examined. Results showed that the striatum of the SCA17 rats had a significant reduced norepinephrine (NE) levels ($F(1, 18) = 5.69; p < 0.05$) (Figure 5A). In the basolateral amygdala, a statistical trend for a decrease of the 3-methoxytyramine (3-MT) level was obtained ($F(1, 18) = 3.64; p = 0.072$) in SCA17 rats (Figure 5C). Furthermore, the medial prefrontal cortex exhibited a significant decrease of homovanillic acid (HVA) level in SCA17 compared to WT rats ($F(1, 18) = 8.79; p < 0.01$) (Figure 5E). Additionally, the turnover rate of serotonin (5-HT) and dopamine (DA), which was calculated according to (Bichelmeier et al., 2007), indicated a significantly reduced 5-HT turnover rate ($F(1, 18) = 5.69; p < 0.05$) (Figure 5A).
Figure 5  Monoamine analysis in the SCA17 and wildtype rats at 9 months old.

Metabolic content of catecholamines and indoleamines in (A) the striatum (CPu), (C) the basal lateral amygdala (BLA), (E) medial prefrontal cortex (mPFC) and the turnover rates of dopamine and serotonin in (B) the CPU, (D) BLA and the mPFC (F), respectively. Data are expressed as means ± S.E.M. One-way multivariate ANOVA results are displayed in each graph. Results from post hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; #=0.05<p<0.1; ns=not significant).
5.78; \( p < 0.05 \)) in the striatum, but no significant DA turnover rate (\( F_{(1, 18)} = 0.042; \ p > 0.05 \)) (Figure 5B). Conversely, only the DA turnover rate was significantly increased in the basolateral amygdala (\( F_{(1, 18)} = 10.19; \ p < 0.01 \)) in SCA17 compared to the WT (Figure 5D). In contrary to the striatum and the basolateral amygdala, no significant differences between the genotype were found at both DA and 5-HT turnover rates in the medial prefrontal cortex (\( p > 0.05 \)) (Figure 5F). All other metabolites and turnover rates remained unchanged between genotypes (data not reported). Finally, the metabolic content and turnover rates in the cerebellum, raphe nucleus, central amygdala and primary motor cortex did not exhibit any significant genotypic differences (Supplementary Figure 1; data not reported).

**DISCUSSION**

In terms of pathogenesis, SCA17, similar to other polyQ disorders, is suggested to be caused by accumulation of insoluble aggregates (Friedman et al., 2008; Reid et al., 2003). However, although numerous studies have addressed several SCA17 pathomechanisms, whether expanded polyQ tracts affect the function of TBP has yet to be comprehensively addressed. In several polyQ diseases such as in SCA3 for example, an aggravated neurological phenotype has often been characterized by an increased number of nuclear aggregates and accelerated neurodegeneration in the cerebellum (Hubener et al., 2013). Localization of the aggregates in SCA17 patients has been predominantly reported in the cerebellum and moderately in the motor cortex (Stevanin and Brice, 2006). For this reason, in the context of characterization of a SCA17 animal model, the investigation of aggregate levels in different brain areas and age points was considered relevant. Characterization of pathological changes is also important for revealing the in vivo toxicity of mutant TBP. Identification and quantification of soluble and aggregated forms of mutant TBP in different tissues and at different ages would help us to understand how mutant TBP mediates tissue or age specific pathology. Insoluble inclusions were observed in the cerebellum from the age of 3 months old already using the FTA. A subtle increase of signal of mutant TBP from 3 to 10 months old was also shown here, suggesting an aggregate accumulation over disease progression.

Aggregate levels in different brain areas were examined as a method to confirm the validity of this rat model by investigating whether changes seen in the SCA17 rat model are in line with changes seen in the patients. Changes in the accumulation of aggregate levels in the SCA17 rats over time would suggest a link between the formation of aggregates and the behavioural changes reported so far. To that extent, our results are in line with effects seen in patients, where the highest aggregation amounts are seen in the cerebellum. Our results also conform to high aggregate quantities mainly in the cerebellum, reported in several different SCA17 animal models generated such as the
Molecular changes in the SCA17 rats

TBP-71Q and TBP105Q mice with which our TBPQ64 rats share the same mouse prion promoter (Friedman et al., 2007), or the L7-hTBP mice (Chang et al., 2011). These animal models showed aggregated forms also in the cortex and striatum but in lower levels, as also observed in our results.

TBP is known to contain two functional domains: a saddle-shaped C-terminal domain (CTD) responsible for TATA box DNA binding and a polyQ-rich N-terminal domain (NTD) that exhibits transactivation capability (Hsu et al., 2014). In SCA17 the expansion of the CAG/CAA repeats are within the coding region of TBP, leading to an abnormal extension of polyglutamine (polyQ) stretch at the N-terminal domain (NTD). This in turn, affects the dimerization of the TBP with the TATA-box complex formation (Friedman et al., 2008, 2007). Our results support the hypothesis that fragmentation is a prerequisite for aggregation, also known as the toxic fragment hypothesis (Weber et al., 2014; Wellington and Hayden, 1997). This hypothesis states that proteolytically derived fragments of TBP are required for initiating the aggregation process associated with toxicity. Such a phenomenon is also reported in SCA17 transgenic mice which have shown fragmentation and fragment- dependent formation of aggregates (Friedman et al., 2008). However, it is worth mentioning that in vitro assays in literature, failed to show a TBP substrate-specificity for caspases, suggesting different proteolytic enzymes to be involved in truncation of TBP (Wellington et al., 1998).

Concerning the levels of the soluble mutant TBP and endogenous TBP, our results showed a strong reduction in the endogenous rat TBP levels in all three age points analysed, mostly in the cerebellum and consequently also in the cortex and in the striatum only at 10 months old. This may suggest that loss of TBP function contributes to structural and functional defects seen in SCA17 (Hsu et al., 2014). This endogenous TBP reduction, caused by the presence on the mutant TBP, has been also observed in several SCA17 animal models such as the TBP-71Q and TBP-105Q mice from the age of 4 months old (Friedman et al., 2007), as well as the L7-hTBP mice (Chang et al., 2011), the nestin-TBP KI mice (Huang et al., 2011), the inducible TBP KI mice (Yang et al., 2014), the germine-TBP KI mice and the muscle-TBP KI mice (Huang et al., 2015). In addition to the endogenous TBP, the expression of mutant TBP is of equal importance in order to understand how mutant TBP can cause differential pathology in SCA17 animal models. In literature, transgenic animal models have been made to overexpress exogenous mutant TBP in order to mimic SCA17 neuropathology and behavior phenotypes. In our results mutant TBP levels were significantly high from the age of 3 months old already until 10 months old in the cerebellum as well as in the cortex, and in striatum only at the 10 months old age point. Elevated mutant TBP expression levels have also been described in all the aforementioned SCA17 animal models especially in the cerebellum, followed by somewhat lower levels in the cortex, striatum, which provides more evidence to support the validity of our rat model. However, it is worth mentioning that in relation to the lifespan reported in some of these animal models, changes in both the endogenous and
the mutant TBP expression levels were observed at quite later stages of the disease (Friedman et al., 2008, 2007). In this study, both protein expression levels were observed at an early stage, before the manifestation of any of the pathologic symptoms these animals exhibit (Kelp et al., 2013). Conversely, in other polyQ disorders such as HD and SCA3 a tendency for decrease of soluble mutant polyQ proteins during disease progression in the cerebellum has been reported, which is in turn inversely correlated with aggregate formation and phenotypic aggravation (Clemens et al., 2015; Hubener et al., 2013). In this SCA17 rat model, we observed a downregulation in the expression of the endogenous normal TBP caused by the mutant TBP. This observation suggests that although it seems that mutant TBP preserves some important normal functions during early development, it can later cause a gain of toxic function and therefore reduce the level of normal TBP to induce a loss of function of normal TBP (Huang et al., 2015; Rubinsztein et al., 1999). This phenomenon has also been described in all mice models reported above, knock-in or transgenic, except for the L7-hTBP transgenic mouse model (Chang et al., 2011) where the exact opposite case was observed. These findings therefore suggest that TBP is an important transcriptional regulator and its protein level is tightly controlled in the body.

Previously, Purkinje cell degeneration was observed in the TBPQ64 rats when compared with wildtype littermates at 9 months. Here, we used a specific antibody to calbindin, a protein abundant in Purkinje cells, to further investigate the neuronal loss at three different age points. A significant reduction of calbindin expression was observed only at the last disease stage, whereas in younger rats calbindin levels were intact or even higher for the transgenic rats. These data reflect not only the loss of Purkinje cells but also imply a possible calcium homeostasis impairment in these transgenic rats, as calbindin has been shown to be involved in cellular calcium regulation (Barski et al., 2003; Tang et al., 2003). Similar findings have been shown in other mice models (Friedman et al., 2007) supporting the value of our results. The unexpected upregulation of calbindin at the 3 months old age point cannot be fully explained, but it is speculated that it may be attributed to activation of intracellular calcium stores serving as a neuroprotective mechanism to protect against excitotoxicity caused by SCA17 (Ferdinandusse et al., 2008).

Recent studies have demonstrated a strong link between neurodegeneration and chronic inflammation (DeLegge and Smoke, 2008; Krause and Müller, 2010; Salminen et al., 2009; Wüllner and Klockgether, 2003). We thus used the microglia marker, Iba1, to examine whether inflammation occurred in the cerebella of the TBPQ64 transgenic rats. A significant decrease of Iba1 staining was identified in transgenic cerebella compared to wildtype counterparts at 10 months old, in contrast with the 3 months old where an increase was observed. This increase could be a sign of early microglia activation, which is indicative of a neuroinflammatory response. Therefore, microglia modifications can precede the disease onset, as at this early symptomatic stage this phenomenon most
likely reflects an early phase of proliferation and activated microglia. This activation may mainly be of endogenous origin rather than from peripheral recruitment. Similar early microglia activation has been reported in other polyQ animal models (Björkqvist et al., 2008; Cvetanovic et al., 2015; Evert et al., 2001; Lin et al., 2001). The decrease observed at the last disease stage may be attributed to increased microglia degeneration, which can be explained by the microglial dysfunction hypothesis. This hypothesis states that senescence of microglia produces dysfunctional cells, which are losing their neuroprotective abilities. Thus, this loss of microglial neuroprotection is in large part responsible for aging related neurodegeneration and onset of neurodegenerative disease. Subsequently, old neurons which may require greater neuroprotection, die because they are increasingly neglected by weaker microglial cells (Streit, Wolfgang, 2008). However, this reduction in the late disease phase is not in line with findings reported for other polyglutamine diseases where an increase in microglia activation is mostly observed (Björkqvist et al., 2008; Chang et al., 2011; Friedman et al., 2007; Hickey et al., 2008; Orr, 2001) or no changes are observed in the cerebellum at different disease stages (Hübener et al., 2012; Silva-Fernandes et al., 2010). Nevertheless, it is worth mentioning that in almost all studies found in literature no longitudinal results are presented from animal models which makes the comparison of our results with the results reported in literature more challenging. Interestingly in only one mouse model for HD a decrease in density has been observed with age, with the authors suggesting that microglia changes with normal aging and that this process is accelerated in the transgenic brains and that such changes in the dynamic status of microglia may lead to an impairment of their neurosupportive functions (Ma et al., 2003). To analyze potential neuroinflammation even further, cerebellar lysates were also blotted for the inflammatory markers glial fibrillary acidic protein (GFAP) at the ages of 3, 6 and 10 months old. No significant changes in GFAP levels in the cerebellum were observed at any age point tested, suggesting that mutant TBP does not lead to astrocyte activation in the SCA17 rat model. Several mouse models have shown GFAP gliosis in the cerebellum in a relative early age (Chang et al., 2011; Friedman et al., 2007), while also in SCA17 patients astrocyte activation has been observed (Bruni et al., 2004; De Michele et al., 2003). On the contrary, the decreased GFAP levels in the cortex at 3 and 6 months of age may be a possible indication of the atrophic changes present in the cerebral cortex or a reflection of disease-specific alterations in pathways controlling post-translational modification observed in severe psychiatric disorders such as schizophrenia, bipolar disorder and major depressive disorder (Johnston-Wilson et al., 2000). Thus, our results do not recapitulate results of other polyglutamine animal models (Bradford et al., 2009; Cemal et al., 2002; Cvetanovic et al., 2015; Li et al., 2018; Lin et al., 2001), or of SCA17 patients (Bruni et al., 2004; Fujigasaki et al., 2001; Lasek, 2006; Rolfs et al., 2003; Toyoshima et al., 2004) or of other polyQ-disease patients (Evert et al., 2001; Nóbrega, 2018; Schöls et al., 2004) where reactive gliosis (i.e. activation of both astrocytes and microglia) has been reported.
However, due to the limitations of our study, we cannot draw any concrete conclusions on the mechanisms underlying these changes which should be assessed further in future studies.

It is also known that neurotransmitter levels such as DA and 5-HT are changed in neurodegenerative diseases (Morgan et al., 1987). Here we studied the metabolic content of metabolites, with dopamine as precursor, resulting in epinephrine, norepinephrine, DOPAC, MHPG, 3-MT, normetanephrine, HVA, at the terminal stage of the animal (i.e. 9 months). In addition, 5-HT and 5-HIAA were also analyzed, as well as the turnover rates of dopamine and serotonin in several brain areas. Neurochemical analysis by HPLC in the striatum, basolateral amygdala and medial prefrontal cortex of wildtype and SCA17 mutant rats revealed differences in the dopaminergic and serotonergic pathway. Surprisingly, only the CPu and mPFC exhibited a significantly reduced level of NE and HVA, respectively. The changes observed in NE are not in line with literature findings were unchanged levels have been reported in HD transgenic mice (Duan et al., 2004; Reynolds et al., 1999). Furthermore, the lower levels of HVA in the mPFC, are also indicative of motoric impairments which have also been observed in symptomatic HD transgenic mice models (Duan et al., 2004; Mochel et al., 2011; Reynolds et al., 1999) as well as in transgenic mouse models of SCA3 (Bichelmeier et al., 2007). Such a reduction of dopamine metabolites has also been reported in SCA3 (Higgins et al., 1996) and HD patients (Reynolds and Garrett, 1986). Another possible explanation of this decrease may be cognitive impairments in the SCA17 rats as in literature reduced HVA levels in the prefrontal cortex are indicative of impaired performance on cognitive tasks dependent on prefrontal cortex function (Diamond, 1996). Thus, the investigation of cognition may be worth investigating in future studies. Concerning the 5-HT and DA turnover rates, a significantly increased DA turnover rate in the BLA in the SCA17 rats was noted compared to the wildtypes as well as a trend for reduced 3-MT levels. These changes suggest susceptibility to high stress level (Inglis and Moghaddam, 1999) for the SCA17 rats which confirms the behavioural anxiety responses observed in chapters 3 and 4. Moreover, no changes in DA, DOPAC and HVA in the CPu were found, providing similar results as in Huntington’s disease and SCA3 (Bernheimer et al., 1973; Bichelmeier et al., 2007; Huot et al., 2006; Reynolds and Garrett, 1986). This suggests a possible shared DA pathological mechanism between other polyglutamine disorders and SCA17. Furthermore, although 5-HT and 5-HIAA levels in SCA17 rats in the CPu remained unchanged, 5-HT turnover rate was significantly reduced. Unlike observations in transgenic HD mice were serotonin levels have been reported to be decreased in the striatum from an early age already (Mochel et al., 2011) our results are in line with findings reported for SCA3 transgenic mice (Bichelmeier et al., 2007; Hübener et al., 2012). Surprisingly, no significant differences regarding the metabolic content and turnover rates, in the cerebellum, DR, CeC and M1 were found. Future research is therefore required to gain more knowledge on the dopamine and serotonin pathway in the SCA17 rat model.
To summarize, this study revealed new information regarding the molecular profile of the SCA17 transgenic rat model by investigating protein aggregation, neuroinflammation and monoamine levels in this model. We showed specific molecular changes in the SCA17 rats such as accumulation of TBP aggregates and soluble mutant TBP, reduction of endogenous levels of TBP. Additionally, signs of neuroinflammatory responses at the early stages of the disease were also observed which in turn gradually fade out as a result of extensive neurodegeneration. Lastly, we found moderate changes in the dopaminergic and serotonergic pathway which should be further investigated. This study provides the basis for further molecular studies and may present valid readouts for treatment or diagnostic purposes.

**Conclusion**

All in all, our study aimed at characterizing molecularly the SCA17 transgenic rat model. Here we recapitulated several pathological hallmarks of SCA17 by revealing specific differences in several molecular aspects in the SCA17 rat model at different ages and brain areas related to protein aggregation, neuroinflammation and monoamine content. However, further investigations are needed in order to identify further the cause of these changes. Our findings illuminate key endpoints in trials of therapeutic agents as well as starting points for further studies on the function of TBP.

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**Conflict of interest**

The authors E. Kyriakou and Dr. J.E. van der Harst are working for the EU funded “PhenoRat” project of which Noldus Information Technology, the manufacturer of the PhenoTyper device and EthoVision XT 9 software. At the time of the studies, J.E. van der Harst was employed as part-time scientific project advisor for PhenoRat employed by Noldus Information Technology and E. Kyriakou was employed by Noldus Information Technology as part of the PhenoRat project.
Supplementary Figure 1  Monoamine analysis in the SCA17 and wildtype rats at 9 months old

Metabolic content of catecholamines and indoleamines in (A) the cerebellum (Ce), (C) the raphe nucleus (DR), (E) central amygdala (CeC) and the turnover rates of dopamine and serotonin in (B) the Ce, (D) DR, the CeC (F) and the M1 (H), respectively. Data are expressed as means ± S.E.M. One-way multivariate ANOVA results are displayed in each graph. Results from post hoc analyses are indicated with asterisk(s) in case of a significant genotype effect (*=p<0.05; **=p<0.01; ***p<0.001; #=0.05<p<0.1; ns=not significant).
General Discussion
GENERAL DISCUSSION

As Europe’s ageing population is increasing rapidly, and since several neurodegenerative disorders are debilitating and still incurable, there is a medical and societal challenge for the EU society. 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. Thus, bridging the gap between animal model research and clinics is one of the major research challenges of the current century. Accordingly, identifying suitable animal models that recapitulate the pathological and behavioural profile of the human patient situation to its maximum and can serve for the identification of novel therapeutic approaches is an essential research goal (Marsh et al., 2009). Acknowledging the explosion in the generation of animal models of genetic diseases nowadays, the development and validation of (automated) tools for qualitative and quantitative behavioral measurements is a challenge. For this reason, developing tools that have a pure translational value and link directly human behaviours and homologous behaviours in lower animals are of great need and value.

At present, the focus of preclinical research on rodent behaviour lies on the discovery of suitable readout parameters in disease animal models for neurodegenerative diseases, also known as translational research. These animal models are generated and used in order to understand the disease mechanisms with the goal to ultimately develop possible treatments. Nevertheless, there are several critical limitations that translational research faces such as the translational value of the animal models generated, resulting in a very low reproducibility of the research results but also in overstated treatment effects (Barrett, 2015; Belzung, 2014; Braff and Braff, 2013; Fonio et al., 2012; McGonigle and Ruggeri, 2014; Reichlin et al., 2016). Thus, common practices currently used in behavioural neuroscience can play an important role for these challenges. That is due to the use of a limited and simplistic designs for behavioural analyses, as at present, there is a strong preference for fast and easy animal experiments with low maintenance and costs. Thus, instead of adopting a more ethologically based approach, which can give a better understanding of the mechanisms underlying behaviour, current practice is often limited to the analysis of the occurrence of individual behaviours from brief tests.

Since Spinocerebellar Ataxia type 17 (SCA17) belongs to the category of the disorders with currently no available cure, there is an urge for valid animal models to study the pathogenesis and to test novel therapeutic approaches. In order to address this bottleneck, this thesis focuses on a more in-depth behavioural and molecular characterization and validation of the only transgenic rat model known so far for SCA17 and the development of new methods for behavioural classification. The work presented in this thesis aimed at making a step further in order to enable early diagnosis for early targeted treatments. This will subsequently contribute to accelerating progress in finding solutions that can alleviate the symptoms, and lessen the social and economic impact for patients,
families and health care systems. Within this project we selected a range of commonly studied behaviours relevant to human disease. We monitored disease progression in the home environment, anxiety, fine motor control, gait analysis and social interaction. Furthermore, we assessed changes in brain in protein aggregation, monoamine content analysis and protein level determination. Accordingly, the studies described in this thesis were designed to detect features that resemble the SCA17 patients’ situation and to monitor their development by longitudinal testing at different age points.

All experiments presented in this thesis were performed using only male heterozygous SCA17 rats as previous work (performed by Kelp and colleagues in Tuebingen University) has shown that transgenic homozygous animals do not survive beyond birth. Concerning SCA17 patients, thus far homozygosity has been reported only in four cases, as the majority of the diagnosed patients are heterozygous (Hire et al., 2011; Toyoshima et al., 2004; C. H. Zühlke et al., 2003). This supports the use of the heterozygous SCA17 rats in this thesis as appropriate. Additionally, based on literature, no specific sex-dependent differences have been reported between human SCA17 patients, which constitutes the use of only male rats sufficient at this stage of validation of the SCA17 rat model.

In the studies described in this thesis, an effort was made to improve the behavioural experiments in a way that increases the translational value of the SCA17 rat model. This was addressed by adopting an approach in which ethology and automation were taken into account, to increase the biological relevance of the results and allow the measurement of complex behaviours in a more objective less sensitive to errors than by human observation only (Robie et al., 2017; Schaefer and Claridge-Chang, 2012; Spruijt et al., 2014). It was aimed for the behavioural characterization the SCA17 rat model by using high throughput behavioural phenotyping such as an automated homecage environment and other sophisticated automated methods and setups, to assess impairments in motor coordination and gait abnormalities (i.e. CatWalk®). In addition, innovative behavioural paradigms were combined together with classical behavioural paradigms, to achieve a more integrative phenotyping and identify cognitive and emotional impairments (anxiety and alterations in social interaction) typically seen in SCA17 patients (Vaccarino et al., 2011; Zühlke and Bürk, 2007). These novel automated methods for behavioral classification and assessment of transgenic and wild type rats were evaluated, as these often have a higher throughput compared to classical tests, and are thought to provide more valid behavioral readouts for the detection of early onset of specific symptoms and read-out parameters for future preclinical studies.

The SCA17 rats showed characteristic phenotypic features and neuropathological abnormalities comparable to those in SCA17 patients. Although several of these changes have already been described in other SCA17 mouse models, many disease characteristics are still challenging or impossible to reproduce. In the rat model we examined here, some of the key features of SCA17 are better reproduced than the available mouse models, which makes it a valuable model for further research and therapeutic approaches.
In a nutshell, the main objectives of this thesis were to: (1) define the onset of the disease in the SCA17 rat model by detecting at an early stage the first core symptoms so that possible therapies can be more effective in preventing further development of neurodegeneration; (2) extend previous findings regarding the motoric disturbances with more automated and sophisticated tests; (3) identify potential non-motor symptoms in SCA17, such as anxiety and alterations in social interaction which are also reported in patients suffering from this fatal disorder; (4) link relevant behavioural responses to protein aggregation, neuroinflammation, monoamine levels and neuronal activity in relevant brain areas.

KEY FINDINGS

Fine and gross motor control investigations in the SCA17 rat model using automated and classical tests

The use of a battery of motor tests with a mixture automated and traditional behavioural paradigms, allowed us to investigate in depth gross motor function, fine motor control, coordination and muscle strength. Concerning gross motor control, SCA17 rats presented several significant impairments. Balance deficits were seen in the SCA17 rats most prominently at the age of 9 months in CatWalk. Coordination deficits were also confirmed in the SCA17 rats. A lower pressure was applied on both front and hind paws in the SCA17 rats, suggesting an imbalance due to muscle impairments (Vandeputte et al., 2010). This was not discovered in the beam walk test, clearly demonstrating the advantage of using automated gait analysis for the study of motor deficits in the SCA17 rats. As in literature an interdependency has been stated between body weight and a couple of CatWalk parameters (Koopmans et al., 2007; Parkkinen et al., 2013a). The SCA17 rats displayed a shorter stride length, which is indicative of disturbed gait. This phenotype has been described in parkinsonian rats (Westin et al., 2012) and BACHD rats (Huntington’s disease) (Abada et al., 2013). Furthermore, several parameters were significantly influenced by time showing that the SCA17 rats have an impaired inter-paw coordination at 3 and 6 months of age without reaching statistical significance at 9 months. These results are in line with findings in ataxic animal models and patients with neurologically impaired gait (Ebersbach et al., 1999; Vinuez Veloz et al., 2014). Another pronounced difference seen in the SCA17 rats was the significantly increased speed at which the paw loses contact with the glass plate in the CatWalk, also observed in models of brain injury (Mountney et al., 2013) and of neurological diseases such as Huntington’s disease (Vandeputte et al., 2010). Additionally, the generally lower swing speed in the SCA17 rats indicates that for each step a SCA17 rat took, the body was moved a shorter distance during a longer time span. This phenomenon has been also observed in the uncoordinated gait of PD rat model (Westin et al., 2012). Our results recapitulate several features of
shuffling gait seen in SCA patients such as the shorter stride lengths, decreased swing speed, and increased base of support (Bonnet et al., 2012).

Regarding fine motor control, the SCA17 rats showed an impaired phenotype already from the age of 3 months old. This is similar to SCA17 patients as well as patients with cerebellar degeneration patients who are significantly impaired in fine motor skills such as hand transport and shaping, general grasping (Ashizawa and Xia, 2016; Brandauer et al., 2008). Our results therefore suggest a general role of the cerebellum in fine motor control. These results conform observations in rat models of stroke and brain injury (Ramanathan et al., 2006; Whishaw et al., 2008) but also models of neurological dysfunction (e.g. Parkinson’s disease) (Klein and Dunnett, 2012; Vergara-Aragon et al., 2003). To the best of our knowledge this is the first animal model from all the spinocerebellar ataxias’ group that has been tested in this test and has shown such robust effects.

With respect to muscle function, the SCA17 rats showed a significantly decrease in fore limb grip strength already from the age of 3 months old. However, after correction for the body weight, these differences were limited only to the 9 months time point, indicative for a reduced muscle function. This explains that despite the different body composition (smaller in size and reduced body weight) of the SCA17 rats their muscle endurance remained intact until the last stages of disease development. This is in line with observations in different types of SCA patients where it has been shown that patients are able to exert increased grip forces but fail in anticipation of inertial load fluctuations (Rost et al., 2005). Additionally, not all SCA17 patients have been reported to show muscle weakness and those who have, muscle weakness was manifested when full disease symptomatology was displayed (Bruni et al., 2004; Rolfs et al., 2003). Conversely, although SCA patients have been reported to have balance-related abnormalities (Ashizawa and Xia, 2016; Van de Warrenburg et al., 2005), this could not be replicated in the SCA17 rats in the beam walk test, probably due to unsuitability of the version of the beam walk test we chose in our study.

Measuring anxiety-like behaviour in the SCA17 rats using automated and classical paradigms

In SCA17 human situation, psychiatric symptoms are detected before the motoric impairments are reported. The study described in Chapter 4 focused on assessing the development of the anxiety-like phenotype over the lifespan of the SCA17 rat model (at 3, 6 and 9 months old). The data demonstrated an increased risk assessment and anxiety-related behaviour for the SCA17 rats, with the first being more prominent than the latter, as measured in the elevated plus maze (EPM), across the 3 different disease stages. These effects were not caused by locomotor differences or (dis)abilities between the transgenic and wildtype rats, since velocity and distance moved on the apparatus did not show genotype-effects. Increased risk assessment behaviour, as reflected by an increased frequency of head dips was detected already from the age of 3 months old. In addition,
increased anxiety-related behavior, was observed in the SCA17 rats as reflected by the decreased time spent in the open arms at the age of 6 months, although genotype differences were subtle. In the same direction, when introduced into the open field, the SCA17 transgenic rats inclined to stay mainly in the peripheral zone, which is translated as an increased index of anxiety (Bailey and Crawley, 2009). Possible locomotor differences affecting the data were checked by measuring the total distance moved and velocity. We found that these measures remained unchanged between the two genotypes. This also suggests that the exploratory activity is not per se influenced by genotype. Increased anxiety-like behavior as described by the stronger preference to stay in the periphery of the arena rather than exploring the center was prominent already from the age of 3 months and remained steadily high at both 6 and 9 months. We showed for the first time that the anxiety-like phenotype is manifested at a much earlier age than thus far reported (after the 7th month of age (Kelp et al., 2013)). In our study we used an arena of 90 x 90 cm, which provides the animals with sufficient space to express a relatively natural behavioral response to an open area, whereas the automated cages used in the study of Kelp are quite small (standard Makrolon type III (42.5 x 26.6 x 18.5 cm)) for the animals to show their full locomotor activity and exploration. To our knowledge, no other study involving the phenotyping of transgenic SCA17 rodent models has looked into anxiety-related behavior of their models (Chang et al., 2011; Friedman et al., 2007; Huang et al., 2015, 2011; Yang et al., 2014). These results confirm the presence of psychiatric symptoms along the locomotor impairments during the progression of the disease. In addition, it is the first study to show an anxious phenotype at the early age of 3 months and to confirm anxiety as part of the disease progression in a rodent model of SCA17.

The negative correlation that was found between c-fos expression and the anxious-like phenotype in the SCA17 rats might be caused by reduced ability to activate the neurons as a result of the TBP mutation. Due to the presence of a TATAA sequence in the c-Fos promoter, the c-fos gene serves as a binding site for the TBP (Tansey et al., 1994). Hence, altered structure of the mutant TBP may result in a decreased transcription of the c-Fos gene and subsequently in lower protein levels in the SCA17 rats. The negative correlation we found between the c-Fos neuronal activation in the basolateral amygdala after the EPM is puzzling considering that most literature findings state an increase in c-Fos expression in the amygdala accompanied with anxiety behavior in rats triggered by EPM testing (Campeau and Watson, 1997; Hinks et al., 1996; Singewald et al., 2003). Neuronal activation in the amygdala has been suggested to reflect not only immediate responses to stress, but also the fear and memory components of exploration of a novel environment (Hinks et al., 1996). Therefore, a mismatch between the behavioral index of anxiety/stress and the immediate early gene expression in SCA17 rats might imply that the transgenic rats are not able to integrate such information. Our results indicate that the SCA17 rat model displays an anxious-like phenotype already at 3 months of age, and continues across all three age points tested. In translation to patients’ situation, our
results are in line with findings of mood changes including stress and anxiety in early- and late-symptomatic patients.

In a subsequent study, described in Chapter 4, we used an anxiety test implemented in an automated homecage environment, aiming at investigating the behavioural reaction of the SCA17 rat model to this test and the suitability of this test (the “light spot” or LS) to evoke and measure anxiety-like behaviour. More specifically, the focus of this study was to find out whether the SCA17 rats show a stronger, or more subtle anxious phenotype when subjected to a mild aversive stimulus in their homecage (LS) and to verify potential developmental changes in anxiety-traits. Importantly, the additional value of this approach in relation to previous findings, is the benefit that the homecage-based experiments offer.

During the first 15 minutes of the LS, when the strongest reaction occurred, the SCA17 rats travel a longer distance at the ages of 6 and 9 months, which may be indicative of an approach-avoidance conflict. This can be translated to increased risk assessment behaviour, which is in line with our previous findings where risk assessment behaviour was prominent in the SCA17 rats as measured in the elevated plus maze (Kyriakou et al., 2017). The increased mobility observed during the LS may be a sign of evaluating the potential threat from the sudden change in the light conditions in the homecage, together with the conflict to approach the feeder zone and a clear avoidance, since no increased time spent or frequency of visits in the LS zone was seen. In general, both genotypes responded similar to the LS by a decrease in the time spent in the LS zone, making it clear that the LS was able to evoke avoidance responses as expected. However, most likely due to the strength of the avoidance responses no genotype differences in these responses were found. Notably, under baseline conditions the SCA17 rats seem to spend more time in the feeder zone at the age of 6 and 9 months (although this was not statistically significant). Such observation is in line with previous findings that although the SCA17 rats weigh significantly less than their wildtype counterparts already from the age of 5 months and thereafter, the food consumption in relation to their body weight was higher compared to the control group (Kelp et al., 2013). Time spent inside the shelter during the LS was significantly increased for both genotypes, which basically confirms the effectiveness of the LS to evoke avoidance responses. However, in order to eliminate any possibility of confounding effects due to the ataxia related phenotype of the SCA17 rats, the distance travelled in all three ages was measured; no baseline genotype differences were found. Yet, the LS induced a significant increase in the distance travelled compared to baseline confirming that the test was successful in evoking a clear behavioural response.

The LS showed to be a promising test for measuring and evaluating avoidance and responses to negative stimuli in a familiar setting. This is because it provides several advantages such as allowing continuous monitoring of pre- and post-effects and permitting in-depth analysis. Despite the mild nature of the stimulus, the properties of
the test such as the setting and the lack of punitive character, make it a useful tool which can detect subtle or complex anxiety-related traits in rodents.

**Social behaviour in the SCA17 rats**

Measuring social behaviour should be a standard component in studies using animal models for human neuropathologies. In the case of SCA17 in particular, cognitive deficits in situations related to social behaviour have been described in patients (Lasek, 2006). In the context of behavioural characterization of a transgenic rat model for SCA17, in Chapter 5 the social interaction profile of the SCA17 rats was investigated longitudinally, over the course of the disease progression (over three different ages: 3, 6 and 9 months).

The social interaction test revealed no significant genotype differences in in social behaviour, shown by the unchanged total social interaction time. Yet, a significant increase of the mean duration of the social interaction bouts and a statistical trend for a decrease in the frequency of occurrence of social interactions were observed in the SCA17 rats at the age of 9 months, in the test-situation with familiar partners. Furthermore, both total distance moved and velocity were shown to be significantly lower in the SCA17 rats during the 9 months age point. These results suggest that when the SCA17 phenotype and symptomatology is fully manifested, considerable alterations with initiating social interactions with their conspecifics are present. As it has been described, the decrease in the number of contacts may be associated with hypoactivity (Kaidanovich-Beilin et al., 2011), confirmed by the decreased distance moved and the trend for a lower frequency of social interactions in the SCA17 rats of 9 months old. However, this decrease may also be associated with depressive- and/or anxiety-like behavior, with the latter being evaluated and confirmed for the SCA17 rats by using both traditional (Kyriakou et al., 2017) and automated novel behavioural paradigms (Kyriakou et al., 2018). That said, it might eventually be the combination of these two factors that result in a fewer number of contacts of the SCA17 rats with their conspecifics. This is in line with reports that in SCA17 patients social phobia has frequently been observed (Lasek, 2006). The intact total social time between the two genotypes, can be interpreted as an unchanged motivation (social interest) to socialize despite the locomotion deficits of the SCA17 rats (chapter 2). On the contrary, our results suggest that despite the prominent difference in the total distance moved and velocity, this did not result in social withdrawal, as measured by the unchanged total social interaction time and solitary time of the SCA17 rats compared to the wildtype pairs. Furthermore, SCA17 rats spent significantly less time in each other’s proximity at the age of 9 months. This behavioural category reflects approaching, following and moving away behaviours. Behaviours that required physical contact showed no differences between the two genotypes. Concerning social approach, SCA17 rats were significantly less motivated than the control group to approach and move away from their conspecifics only at the age of 9 months, while following showed only a decrease with age from 3 to 9 months old. This lower approaching in the SCA17
rats may suggest a social anxiety phenotype in the SCA17 rats, or is caused by the lower activity level and compromised condition of the SCA17 rat model. However, the similar levels of total social interaction, suggest that social motivation to interact remained intact in the SCA17 rats.

When introducing an unfamiliar partner, SCA17 rats showed no difference compare to wildtype in the total time spent on social interaction. Additionally, the mean duration of social interaction bouts was increased in the SCA17 rats when encountering an unfamiliar partner, as it was also observed with familiar pairs. Conversely, in SCA17 rats, non-contact social behaviours were decreased in the unfamiliar context, whereas the in-contact social behaviours were significantly increased, compared to the control group. The frequency of social interaction in the unfamiliar setting were significantly decreased in the SCA17 rats at 9 months, enlarging the slightly decrease observed in the test with the familiar test partner. Additionally, the significant decrease in the duration of both approach and following, when using an unfamiliar partner, provides supportive information to the speculated social anxiety profile of the SCA17 rats, although again the decreased activity can be the main cause of this. In a different way, the significantly increased allogrooming in the test with the unfamiliar partner could also be an indicator of anxiety (either social anxiety or related to the environmental stressors). Concerning social novelty, our results showed no alterations in transgenic animals, supported by the lack of genotype differences in the total social interaction time in the test with the unfamiliar partner that was conducted at the age of 9 months. However, the diversity of effects seen in both type of social interaction tests, and the fact that (un)familiarity seemed to enlarge certain effects is interesting to explore further.

Investigation of molecular changes in the brain of the SCA17 rat model
Localization of the aggregates in SCA17 patients has been predominantly reported in the cerebellum and moderately in the motor cortex (Stevanin and Brice, 2006). In the SCA17 rats, insoluble inclusions were observed in the cerebellum from the age of 3 months old already, with an increased mutant TBP signal from 3 to 10 months old, suggesting a strongly increased aggregate accumulation over disease progression. The cerebellum is one of the most affected brain areas in SCA17. The results presented in this thesis are in line with effects seen in patients, where the highest aggregation amounts are seen in the cerebellum. This suggests that the immunoreactivity of the C-terminal TBP epitope is not compromised by aggregate conformation. On the contrary, these data indicate that mutant TBP fragments lacking an intact C-terminal DNA-binding domain can form aggregates in the SCA17 brain. Furthermore, we showed a strong reduction in the endogenous rat TBP levels in all three age points analysed, mostly in the cerebellum and also in the cortex and in the striatum only at 10 months old. These results suggest that loss of TBP function contributes to structural and functional defects of SCA17 pathogenesis (Hsu et al., 2014). In addition, mutant TBP levels were significantly high
from the age of 3 months old already until 10 months old in the cerebellum as well as in the cortex and in striatum only at the 10 months old age point. This supports the validity of our rat model. Additionally, we observed a downregulation in the expression of the endogenous normal TBP, caused by the mutant TBP. This observation suggests that although it seems that mutant TBP preserves some important normal functions during early development, it can later on cause a gain of toxic function and therefore reduce the level of normal TBP to induce a loss of function of normal TBP (Huang et al., 2015; Rubinsztein et al., 1999). Furthermore, a significant reduction of calbindin expression was seen only at the last disease stage, whereas in younger rats calbindin levels were intact or even higher for the transgenic rats. These data reflect not only the loss of Purkinje cells but also imply a possible calcium homeostasis impairment in these transgenic rats, as calbindin has been shown to be involved in cellular calcium regulation (Barski et al., 2003; Tang et al., 2003). Recent studies have demonstrated a strong link between neurodegeneration and chronic inflammation (DeLegge and Smoke, 2008; Krause and Müller, 2010; Salminen et al., 2009; Wüllner and Klockgether, 2003). In this respect, our results showed a significant decrease of Iba1 staining in transgenic cerebella at 10 months of age. In contrast, at 3 months, an increase was observed. This increase is a sign of early microglia activation, an indication of a neuroinflammatory response. Therefore, microglia modifications can precede disease onset, as at this early symptomatic stage this phenomenon most likely reflects an early phase of proliferation and activated microglia. This activation may mainly be of endogenous origin rather than resulting from peripheral recruitment. The decrease at the last disease stage may be attributed to increased microglia degeneration. Furthermore, no significant changes in GFAP levels in the cerebellum were observed at any age point tested, suggesting that mutant TBP does not lead to astrocyte activation in the SCA17 rat model. In SCA17 patients on the other hand, astrocyte activation has been observed (Bruni et al., 2004; De Michele et al., 2003). In the cortex, on the contrary, the decreased GFAP levels at 3 and 6 months of age may be a possible indication of the atrophic changes present in the cerebral cortex. Thus, our results do not recapitulate results of SCA17 patients (Bruni et al., 2004; Fujigasaki et al., 2001; Lasek, 2006; Rolfs et al., 2003; Toyoshima et al., 2004).

Neurochemical analysis by HPLC of the neurotransmitters, dopamine and serotonin, and the metabolites in the striatum, basolateral amygdala and medial prefrontal cortex of the SCA17 rats revealed differences in the dopaminergic and serotonergic pathway. Surprisingly, only the striatum (CPU) and medial prefrontal cortex (mPFC) exhibited a significantly reduced level of norepinephrine (NE) and homovanillic acid (HVA), respectively. Such a reduction of dopamine metabolites was also observed in human SCA3 (Higgins et al., 1996) and HD patients (Reynolds and Garrett, 1986). Additionally, the serotonin (5-HT) and dopamine (DA) turnover rates analysis, indicated a significantly increased dopamine turnover rate in the basolateral amygdala (BLA) in the SCA17 rats as well as a trend for reduced 3-methoxytyramine (3-MT) levels. These changes in dopamine
in the amygdala suggest susceptibility to stress (Inglis and Moghaddam, 1999), which is already behaviourally observed in the SCA17 rats. Furthermore, although serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) levels in SCA17 rats in the striatum (CPu) remained unchanged, serotonin (5-HT) turnover rate was significantly reduced. Surprisingly, no significant differences, regarding the metabolic content and turnover rates, in the cerebellum, dorsal raphe nucleus (DR), amygdala (CeC) and motor cortex (M1) were found. Future research is therefore required to gain more knowledge on the dopamine and serotonin pathway in the SCA17 rat model.

Methodological, ethological and other considerations
Moving towards automation in behavioural neuroscience has been an attractive topic of discussion over the past years (De Visser et al., 2006; Richardson, 2015; Schaefer and Claridge-Chang, 2012; Spruijt and DeVisser, 2006). For example, several options for home cage monitoring are commercially available for both mice and rats. Quite often in literature the PhenoTyper® homecage but also other automated homecage setups are only used for extracting measurements such as general locomotor/ambulatory activity, velocity, food and water consumption monitoring etc. It is not often the case to see specific tests such as anxiety tests to be implemented within such automated cages. It is argued that this would not only make better use of large and sophisticated laboratory setups, but also adds ethological value to the behavioural tests (Spruijt and DeVisser, 2006) by offering the animals the opportunity to display biologically relevant behaviour in a familiar setting. Having useful and biologically relevant screening tools that allow the thorough quantification of a behavioural phenotype that covers specific behavioural domains (e.g. anxiety) is quite essential, especially when respective ethological techniques are integrated (Rodgers, 1997). Repeated testing in novel environments and inherent human handling and disturbance, can cause a high level of discomfort which can interfere with the results and cause a negative impact on animal welfare (Burn, 2008). Moreover, in comparison to the classical short-lasting behavioural tests, automated home-cage tests often have the advantage of having baseline measurements of the same animals allowing within-group analysis. A test conducted in a homecage environment after habituation, continuous monitoring and high sampling-rate provides great opportunities with respect to the within-group comparison to baseline and for in depth analysis tools to optimally use the detailed data acquired. Therefore, attention should be given to the ethological and construct validity of a behavioural test (Hånell and Marklund, 2014; Lister, 1990; Rodgers et al., 1997). However, one should also keep in mind that despite the benefits of baseline measurements of the same animals, this may also be a weakness due to the large variation within the population in the responses of each animal.

The implementation of an ethological approach together with the use of automated technology can significantly contribute to a better understanding of an animal model and thus increase its translational value (Brain et al., 1991; Chaouloff, 2013; Fonio et al.,
Both the setups and the automated behavioural analyses can provide numerous behavioural categories which can be sensitive markers for changes induced by genetically manipulated disease models. Most importantly, as seen also in our results, subtle differences can be revealed in several behavioural domains while the use of too simplistic behavioural tests may prohibit the assessment of the functional meaning of behavioural changes or differences within and/or between groups. As Peters (2018) also explained in her thesis, one of the main features of an ethological approach is the activation of more than one motivational systems at the same time. Thus, the employment of classical battery of tests for the analysis of single parameters with each test can have enormous limitations as it ignores the complexity of behaviour as a result of several behavioural systems working together. The employment of automated phenotyping techniques such as testing in an automated homecage provides numerous advantages such as integrated measurements within the homecage, clear differentiation between novelty-induced and baseline behaviours thanks to the possibility of long-term recordings in a familiar setting without disturbing the animals by handling or transfers. For the same reason, investigation of long-term effects of experimental manipulations also becomes feasible. Furthermore, it can address multiple behavioural domains that otherwise can only be assessed with the use of a single setup. The generation of large amount of data per animal using automated techniques allow the use of mathematical modelling to extract additional measures (Lorbach et al., 2018; Lorbach, 2017). This in turn, can provide refinement of behavioural measures which may increase between-laboratories reproducibility. Thus, this may improve the validity of automated testing as it yields more objective behavioural measures. Nevertheless, despite these valuable advantages, some limitations should also be taken into consideration. The use of automated tests entails mostly (longer) individual housing and/or testing, which is a less preferable approach from an animal welfare point of view and inherent effect on the translational value of the data generated this way. It produces a great amount of data that can make analysis rather complex and time consuming. Additionally, although in theory such types of automated approaches aim to increase between-laboratory reproducibility with respect to standard tests, this has not yet been strongly proven. However, this change in the mindset regarding the measurements of rodent behaviour is still a slow process. However, over the past decades large amounts of data have been generated by using this minimalistic approach in terms of parameters analysed and testing condition used. Therefore, it is a challenge to change the way animal behaviour is investigated and analysed in such an established conventional field.

With respect to the anxiety phenotype we investigated extensively in chapters 3 and 4, it is worth mentioning that in literature a distinction has been reported between ‘state’ and ‘trait’ anxiety. As anxiety is a complex behaviour that can be expressed by a wide variety of responses in both humans and animals, several scientists have defined ‘state anxiety’ as mostly context- and/or environment-dependent, while ‘trait anxiety’
often refers to a persistent, long-lasting feature of an individual that is more influenced by genetic components rather than the context and/or environment (Belzung and Berton, 1997; File, 1991; Lister, 1990). Based on these definitions, it becomes clear that the specific setup of behavioural tests can also be distinguished between measuring state or trait anxiety. For example, tests such as the elevated pus maze and the light-dark box that expose the subjects to stressogenic, or even anxiogenic, and unfamiliar situations are suitable for measuring state anxiety, while tests such as the light spot which measure anxiety in a stress-free familiar environment and under baseline conditions are more suitable for measuring trait anxiety (Belzung and Berton, 1997; Griebel et al., 1993). Thus, in our studies we have covered an extensive anxiety spectrum for measuring anxiety in the SCA17 rat model which might also explain some of the observed differences in anxiety displayed in different test situations. In addition, although fear is often disassociated from anxiety, it is still the reflexive response to an immediate threatening stimulus (Davis, 1998; Lang et al., 2000). Therefore, when testing for anxiety with the light spot test, fear may be also measured as the response seen immediately after the bright light is turned on as this is the response to the specific change of the environment that may be associated with direct danger. In the same line of reasoning, the light spot test may also provide the opportunity to measure pathological anxiety in rodents as an extreme avoidance response without any habituation signs to the aversive stimulus can be indicative of pathological expression of avoidance behaviour (Ohl et al., 2008). An in-depth data analysis might be able to provide a behavioural distinction between fear, anxiety and pathological anxiety in the light spot test. The same applies for other automated tests that collect data over a longer period of time, where a conscious choice can be made to obtain these data in either a familiar or unfamiliar setting, or to compare in both settings.

Concerning the three validities (construct, face and predictive) described in the introduction, based on which an animal model is often being judged, the experiments presented in this thesis contributed substantially in increasing the face validity of the SCA17 transgenic rat model. All key findings summarized in the paragraphs above, provide evidence to support the increase in face validity of the SCA17 rat model. That is based on several behavioural deficits and neuropathology that were shown by our results, which solidly recapitulate the symptoms and phenotypes related to the human patients’ situation. As in the research performed in this thesis, the construct of the transgenic rat model remained unchanged, our results did not further contribute to the construct validity on the SCA17 rat model. Furthermore, no treatment approaches were applied in the research presented in this thesis which can conform or increase the predictive validity of this rat model.
**Strengths and limitations of cross-sectional and longitudinal studies**

In this thesis, a combination of cross-sectional (chapter 3, 4 and 6) and longitudinal (chapter 2 and 5) was used depending on the objectives and goals of each study. However, both study designs offer advantages and disadvantages that are important to be considered, especially applicable on the work presented in this thesis.

Typically, cross-sectional studies tend to be time-efficient as they capture a specific point in time with no long waiting periods in between. Additionally, it allows researchers to compare many different variables at the same time without the effect of previous experiences and repeated measurements influencing the outcomes. On the other hand, clear relationships between causes and effects are not always possible to be extracted from cross-sectional studies (Pfefferbaum and Sullivan, 2015). This is because the single time point examined in this case, may not be fully representative of the situation without considering information before or after the time point that was tested. It is believed that cross-sectional studies are subject to confounding cohort differences, including small changes in housing, timing and testing circumstances, which may tend to exaggerate age differences.

In longitudinal studies on the other hand, the same subjects are being investigated several times over a (long) period of time. One of the main advantages of a longitudinal study is the detection of changes or developments of changes over time, at a group as well as an individual level (in our case the age of the subjects at the point of measurement), as the subjects serve as their own control (Caruana et al., 2015). Especially in case of disease progression over time this is highly relevant. Other important advantages of longitudinal studies are that they facilitate the determination of causality, they eliminate any confounding factors due to possible cohort effects and can distinguish differences in aging rates between individuals (Guralnik and Kritchevsky, 2010). Therefore, the data gathered are unique which in turn, offers high validity levels, as subjects are being followed over time (Pfefferbaum and Sullivan, 2015). Such an approach makes the data collected accurate but also flexible, as additional time points can be added if necessary due to unanticipated patterns or meaningful relationships observed in the data. Flexibility for shifts in focus can also occur whenever an interesting effect is found in the data. However, longitudinal studies have also limitations, such as the underestimation of age differences due to selective subject attrition, but also the effect of previous experiences and repeated measurements (Martinez and Kesner, 1998). Subjects drop-out at any point of the study due to several unforeseen reasons is also a risk that is always present. From a data quality point of view, longitudinal studies may not always provide accurate or reliable data, as one subject can inadvertently change a long-term outcome in addition to the large sample sizes required to make results meaningful (García-Peña et al., 2015). In neuroscience research, when longitudinal studies are performed until a late age of the subjects, it is important to account for the mild age-associated impairments which normally occur as subjects get older. In the case of the experiments presented in this
thesis, the wildtype rats used as a control group grow old along with the transgenic SCA17 rats, which is a helpful way to account for age-related differences. Therefore, the need for research aimed at distinguishing between normal nonpathological and pathological changes, is of great importance (Penner and Barnes, 2007).

**Future directions**

The results presented in this thesis have contributed significantly to the face validity of the SCA17 transgenic rat model, as it has been shown to recapitulate most of the patients’ symptoms. If considered necessary, further validation can be performed in the future in the areas of cognition and psychiatry, such as depression and anhedonia. Concerning the predictive validity of the SCA17 rat model, it can be argued that while light has been shed onto several aspects of SCA17, the underlying pathomechanisms are not yet fully understood. Nevertheless, the results presented in Chapter 6 point to the direction of fragmentation as a prerequisite for aggregation, supporting one of the most commonly known concepts: the toxic fragment hypothesis (Weber et al., 2014; Wellington and Hayden, 1997). This hypothesis supports that proteolytically derived fragments of TBP are required for initiating the aggregation process associated with toxicity. In polyQ diseases, mutant fragments derive from proteolytic cleavage of the full-length protein and several classes of proteolytic enzymes have been identified to mediate this process, including calpains, caspases, cathepsins and matrix metalloproteinases. The fragmentation of the mutant TBP by yet unidentified proteases is therefore suspected to contribute to the toxicity of the mutant protein in affected neurons. A common hallmark of many neurodegenerative diseases such as Parkinson disease, Huntington’s disease or SCA3 and calpains, a family of calcium-dependent proteases, is the role of proteolysis of mutant proteins which was found to play an important role (Esteves et al., 2010; Gafni et al., 2004; Gafni and Ellerby, 2002; Haacke et al., 2007; Hubener et al., 2013; Samantaray et al., 2008). Therefore, the investigation of proteolytic cleavage of mutant TBP and the potential contribution of calpains in this process are interesting follow up research directions. Such research may provide answers on interesting questions such as if the protein fragments are indeed toxic and if proteases are overactivated due to mitochondrial dysfunction and calcium derangements. Preliminary investigations in one of our labs showed the occurrence of specific calpain-dependent fragments of wildtype and mutant TBP after addition of exogenous calpains or after triggering activity of endogenous calpains. Additionally, pharmacologic inhibition of calpains reduced baseline cleavage of the disease protein and corresponding TBP fragments in the cerebellum of SCA17 rats were identified (Anger et al., 2016). Therefore, since there is preliminary evidence that calpains can be involved in the cleavage of TBP, ways to reduce fragmentation of TBP in vivo can also be investigated further. In this regard, olesoxime, a small molecule drug candidate, has recently attracted attention due to its significant beneficial effects in models of several neurodegenerative disorders including Huntington’s
disease. Its neuroprotective effects have been focused on the suppression of the activation of the calpain proteolytic system but also by positively influencing the mitochondrial function (Clemens et al., 2015; Weber et al., 2016). That said, if calpain overactivation can be conformed in SCA17, olesoxime would be a promising therapeutic approach that can be further investigated in the transgenic SCA17 rats using the readouts provided by this thesis.

Concluding remarks

The validation of a relatively new animal model for the detection of early onset symptoms of SCA17, presented in this thesis, suggests several directions for future research that seem promising and worth being investigated further. Additionally, the work described here highlights the importance of a holistic approach when validating the symptoms of SCA17 at a preclinical level, to cover the complexity of all disease features and symptomatology. Such an approach contributes significantly to our understanding of the neuropathological mechanisms underlying SCA17 and can help to develop valuable animal experiments for subsequent therapeutic studies.

This thesis presents new information about the onset and development of several different disease symptoms from motoric and psychiatric phenotypes to molecular changes in the brain of the SCA17 rat model. For the purpose of designing innovative interventional therapeutic studies in the future, the results can be useful for choosing and identifying proper intervention time windows as well as providing information about the presence and development of specific neuropathological and behavioural phenotypes and brain changes. Ultimately, all research results combined (see Figure 1), will hopefully contribute to the development of successful therapies for this devastating neurodegenerative disease.
Figure 1 Summary of key findings presented in this thesis
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Appendices
ENGLISH SUMMARY

Nowadays, Europe is facing a great medical and societal challenge as several neurodegenerative disorders are still incurable and the ageing population ageing is increasing rapidly. Spinocerebellar Ataxia type 17 (SCA17) is a rare and severe autosomal-dominant neurological disorder with currently no available cure. SCA17 shows generally a late-onset, commonly observed during the second and third decade of life. The SCA17 clinical description consists of progressive gait and limb ataxia (95%), cognitive dysfunctions and dementia (~90%), and involuntary movements (extrapyramidal features) (~70%), including chorea, spasticity, parkinsonism and dystonia, as well as neuropsychiatric symptoms, pyramidal signs, and rigidity. Despite the large variation within patients, ataxia and psychiatric abnormalities are frequently the initial findings followed by involuntary movement, parkinsonism, dementia, and pyramidal signs. SCA17 is caused by a CAG/CAA repeat expansion of 45 or more in the TATA-box protein (TBP) gene.

One of the most important bottlenecks for finding more effective drugs for treatment of brain disorders is the development of model systems that recapitulate the human pathology and can provide prognosis of clinical efficacy. Thus, bridging the gap between animal model research and clinics is one of the major research challenges of these days. At present, there is an urge for valid animal models and thus, the focus of preclinical research on rodent behaviour lies on the discovery of suitable readout parameters in disease animal models for neurodegenerative diseases, also known as translational research. Nevertheless, there are several critical limitations that translational research faces such as the translational value of the animal models generated, resulting in a very low reproducibility of the research results but also in overstated treatment effects.

In order to address this bottleneck, this thesis focuses on a more in-depth behavioural and molecular characterization and validation of the only transgenic rat model known so far for SCA17 and the development of new methods for behavioural classification. Hence, the work presented here aims at making a step further in order to enable early diagnosis for early targeted treatments. This will subsequently contribute to accelerating progress in finding solutions that can alleviate the symptoms, and lessen the social and economic impact for patients, families and health care systems. Within this project I selected a range of commonly studied behaviours relevant to human disease. Accordingly, the studies described in this thesis were designed to detect features that resemble the SCA17 patients’ situation and to monitor their development by longitudinal testing at different age points. The SCA17 rat is a recently generated transgenic rat model that carries the full-length human TBP-cDNA with an abnormal CAG/CAA repeat of 64 codons expressed under the control of the murine prion promoter (Prp).

In the studies described in this thesis it was attempted to improve the behavioural experiments in a way that increases the translational value of the SCA17 rat model. This
was addressed by adopting an approach in which ethology and automation were taken into account, to increase the biological relevance of the results and allow the measurement of complex behaviours in a more objective way than by human observation only. Although several changes have already been described in other SCA17 mouse models, many disease characteristics are still challenging or impossible to reproduce. In the rat model we examined here, some of the key features of SCA17 are better reproduced than the available mouse models, which makes it a valuable model for further research and therapeutic approaches.

In chapter 2, a thorough characterization of the different motor function aspects in the SCA17 rat model is given, using both traditional and automated behavioural paradigms. Both gross and fine motor control aspects were assessed longitudinally at 3, 6 and 9 months of age in wildtype and SCA17 transgenic rats. Gross motor function was evaluated using the CatWalk® gait analysis system and the grip strength and beam walk tests. Fine motor control was measured using the pellet reaching test. Our results were not only in accordance with previous motoric phenotyping results reported, but revealed also a strong impairment in the pellet reaching task and inter-paw coordination on the CatWalk at already 3 months of age. Ataxic and uncoordinated gait were also observed in transgenic animals together with an affected muscle strength most prominently at the last stage of the disease. The parameters that showed the most pronounced differences were related to fluidity of movement and body support. In translation to the patients’ situation, the results are in line with impaired prehension kinematics and grasping forces and characteristic ataxic gait seen in early and/or late-symptomatic patients. Thus, the SCA17 rat model recapitulates several features of the SCA17 patients like muscle weakness, ataxic uncoordinated gait and loss of hand dexterity. These clear deficits observed in several aspects of the motor function contribute to the validity of the SCA17 transgenic rat model.

Besides motor symptoms, anxiety is a commonly described feature of several polyglutamine diseases and thus also an important aspect for the face validity of the SCA17 rat model. In chapter 3, risk assessment and anxiety-like traits were characterized in 3, 6 and 9 months old male SCA17 rats and their wildtype littermates using the standard behavioural test elevated plus maze (EPM). In addition, c-Fos immunostainings in the basolateral amygdala evaluated neuronal activation in correlation to the behavioural responses. The most prominent behavioural effect was a higher level of risk assessment in the transgenic rats. In addition, an increase in anxiety-related behaviour in these rats was found and the genotype effect on risk-assessment and thigmotactic behaviours continues across all three age points tested. This indicates that psychiatric symptoms may develop before and along the locomotor deficits in the SCA17 rat model. Although the EPM caused no overall effect on c-Fos expression, a negative correlation with the anxiety-like behavioural response was observed. In translation to patients’ situation, our results were in line with findings of mood changes including stress and anxiety in early-
and late-symptomatic patients. However, it is difficult to say the same for the pre-symptomatic phase considering that very little or no information about the psychiatric status of the patients is usually known before the manifestation of the first motor symptoms. Our results suggest that the SCA17 rat model displays an anxious phenotype already at 3 months of age resembling the generalized anxiety in early symptomatic SCA17 patients.

In chapter 4, a method is described for measuring anxiety in a reliable manner in the SCA17 rats. An automated tool for assessing anxiety within the home cage was used in this study which can minimize human intervention, stress of handling, transportation and novelty. The anxiety test “light spot” (LS) (white led directed at the food-hopper shortly after the switch from light to dark when rats usually become active and start eating) was applied to our transgenic SCA17 rat model in the PhenoTyper 4500® to verify an anxiety-like phenotype at three different disease stages (3, 6 and 9 months old). Locomotor activity was increased in SCA17 rats at 6 and 9 months during the first 15 min of the LS, potentially reflecting increased risk assessment (approach-avoidance). The LS successfully evoked avoidance and shelter-seeking in rats: both genotypes responded to the test with lower duration in the LS zone and higher time spent inside the shelter compared to baseline. SCA17 rats showed a stronger approach-avoidance conflict reflected by increased activity in the area outside the LS. This homecage test, continuously monitoring pre- and post-effects, provides the opportunity for in-depth analysis, making it a potentially useful tool to detect subtle or complex anxiety-related traits in rodents. We have presented the first data of a rat model subjected to the LS, which can be considered more biologically relevant than a traditional test as it measures anxiety in a familiar situation. Therefore, the LS test is a promising tool to measure and evaluate avoidance and responses to negative stimuli in general by allowing continuously monitoring of pre- and post-effects and providing the opportunity for in-depth analysis.

Chapter 5 continues with exploring potential alterations in social behaviour in the transgenic SCA17 rat model using the social interaction test in a spacious arena (PhenoTyper 9000). Several social interaction parameters were assessed longitudinally with disease progression, at the ages of 3, 6 and 9 months in SCA17 transgenic rats and their wildtype littermates. Animals were tested with their familiar cagemate after a short isolation of 24h prior to the social interaction test. At the age of 9 months, an extra social interaction test was performed with unfamiliar pairs of the same genotype in order to investigate social novelty at the last stage of the disease development. Symptomatic SCA17 rats (9 months old) displayed specific changes in their social behaviour when reunited with their cagemate, or when encountering an unfamiliar SCA-rat after the isolation period.

Specifically, the decrease in frequency of interactions as well as the decrease in total time approaching the test-partner, suggest an inhibition in initiating social interactions with their conspecifics. This may be indicative of social anxiety, a feature also reported in SCA17 patients. However, despite the prominent decrease in the total distance moved.
and velocity, this did not result in social withdrawal as the total time of social interaction remained intact and the mean duration of each social interaction bout was longer in the SCA17 rats compared to the wildtype control group. It cannot be excluded that the overall lower activity level of the SCA rats at this age plays a role here. Unfamiliarity between the interaction pair resulted in unchanged total time spent interacting, while the mean duration of social interaction bouts was also here increased in the SCA17 rats when encountering an unfamiliar conspecific, similarly to the test situation with the familiar rat. Our results showed thus alterations in social contact and motivation in the SCA17 rats at the age of 9 months.

The parameters that showed the most pronounced differences were related to initiating a social interaction with a familiar or an unfamiliar animal. This suggests that despite these difficulties, which may have a basis in the general activity level, the motivation (social interest) for social interaction was unchanged in the SCA17 rats.

Finally, in chapter 6, several different molecular aspects of the transgenic SCA17 rats combining different molecular technique were investigated. Brain tissues from wildtype and transgenic animals were collected from different age points (3, 6 and 9 or 10 months old). To measure aggregate levels, brain lysates were analysed using the filter trap assay whereas for quantification of TBP protein levels (mutant soluble and endogenous) lysates were analysed using the western blot assay. Monoamine levels were also investigated using HPLC, but only at final disease stage. Molecular characterization of the SCA17 rat model revealed strong aggregate accumulation, high levels of soluble mutant TBP, reduced levels of endogenous TBP and neuroinflammation responses at early disease stages, especially in the cerebellum and less in the cortex. Moderate neurotransmitter changes were also observed, mostly in the striatum and amygdala. Our comprehensive characterization approach has revealed certain changes in the SCA17 rat model that recapitulate features of SCA17 and other polyglutamine diseases’ patients such as polyQ- and age-dependent protein aggregation, intranuclear inclusions and neurotransmitter alterations. Together, these results add to the validity of this transgenic rat model.

In chapter 7, all research findings summarized above are being thoroughly discussed and considerations to be taken into account as well as future directions are being presented.

In sum, the work presented in this thesis aims at the validation of a relatively new animal model for the detection of early onset symptoms of SCA17 and suggests several directions for future research that are promising to be further investigated. New information about the onset and development of several disease symptoms from motoric and psychiatric phenotypes to molecular changes in the brain of the SCA17 rat model were presented. For the purpose of designing innovative therapeutic studies in the future, the results can be useful for identifying suitable intervention time points, which ultimately will hopefully contribute to the development of successful therapies for this devastating neurodegenerative disease.
Momenteel staat Europa voor een grote medische en maatschappelijke uitdaging omdat verschillende neurodegeneratieve aandoeningen nog steeds ongeneesbaar zijn, terwijl de leeftijd van de populatie snel toeneemt. Spinocerebellaire ataxie type 17 (SCA17) is een zeldzame en ernstige autosomaal-dominante neurologische aandoening waartegen op dit moment geen behandeling mogelijk is. SCA17 uit zich over het algemeen pas op volwassen leeftijd, vaak rond het twintigste tot dertigste levensjaar. Het klinische beeld van SCA17 bestaat uit progressief verlies van de loopcoordinatie en de coordinatie van de ledematen (ataxie, 95%), cognitieve achteruitgang en dementie (~90%), en onwillekeurige bewegingen (extrapiramidale kenmerken, ~70%), waaronder chorea, spasticiteit, parkinsonisme en dystonie, evenals neuropsychiatrische symptomen, piramidale symptomen en starheid. Ondanks de grote variatie tussen patiënten zijn ataxie en psychiatrische afwijkingen vaak de eerste bevindingen, gevolgd door onwillekeurige bewegingen, parkinsonisme, dementie en piramidale kenmerken. SCA17 wordt veroorzaakt door een CAG/CAA repeat expansie van 45 of meer herhalingen in het gen wat codeert voor het TATA-box eiwit (TBP).

Een van de belangrijkste beperkende factoren voor het vinden van meer effectieve medicatie voor de behandeling van hersenziekten is de ontwikkeling van modelsystemen die de humane pathologie reflecteren en een prognose van de klinische werkzaamheid kunnen bieden. Het overbruggen van de kloof tussen onderzoek in diermodellen en klinisch onderzoek is dus een van de belangrijkste uitdagingen van deze tijd. Er is op dit moment een noodzaak voor valide diermodellen en daarom ligt de focus van preklinisch onderzoek naar knaagdiergedrag op het vinden van geschikte uitleesmaten van neurodegeneratieve ziekten, ook wel bekend als translationeel onderzoek. Desalniettemin zijn er verschillende kritieke beperkingen waar het translationele onderzoek mee te maken heeft, zoals de translationele waarde van de gegenereerde diermodellen, wat resulteert in een erg lage reproduceerbaarheid van onderzoekresultaten, maar ook in een overschatting van effecten van behandeling.

Om dit knelpunt aan te pakken, richt dit proefschrift zich op het diepgaand karakteriseren en valideren van gedragskenmerken en moleculaire aspecten van het tot nu toe enige bekende transgene ratmodel voor SCA17, en de ontwikkeling van nieuwe methoden voor gedragsclassificatie. Daarom is het doel van het hier gepresenteerde werk om een stap voorwaarts te maken in het stellen van een diagnose en het mogelijk maken van gerichte behandelingen in een vroeg stadium van de ziekte. Dit zal vervolgens bijdragen aan een versnelde voortgang van de zoektocht naar oplossingen die symptomen kunnen verlichten en de sociale en economische impact voor patiënten, families en gezondheidszorgsystemen kunnen verminderen. Binnen dit project selecteerde ik een reeks van frequent bestudeerde gedragsmaten die relevant zijn voor humane ziekten. Zodoende zijn de beschreven studies in dit proefschrift ontworpen om
kenmerken te vinden die gelijkenis vertonen met de situatie van SCA17 patiënten en om de ontwikkeling hiervan te monitoren door het longitudinaal testen op verschillende leeftijden. De SCA17 rat is een recent gegenereerd transgeen ratmodel wat voor de volledige lengte het humane TBP-cDNA draagt met daarin een abnormale CAG/CAA herhaling van 64 codons, en welke tot expressie komt onder controle van de murine prion promoter (Prp).

In de in proefschrift beschreven studies is getracht de gedragsexperimenten te verbeteren om zo de translatele waarde van het SCA17 ratmodel te verhogen. Dit werd gedaan door een benadering waarin etiologie en automatisering een belangrijke rol spelen, om zo de biologische relevantie van de resultaten te bevorderen en om de meting van complexe gedragsmaten op een objectievere manier uit te voeren in vergelijking tot menselijke observatie. Ondanks dat enkele veranderingen al eerder beschreven waren in andere SCA17 muismodellen, zijn veel ziektekenmerken nog steeds lastig of onmogelijk te repliceren. In het ratmodel dat wij hier bestudeerd hebben zijn sommige van de kenmerken van SCA17 beter gereproduceerd dan in de beschikbare muismodellen, wat het een waardevol model maakt voor verder onderzoek en het testen van mogelijke behandelingen.

In hoofdstuk 2 wordt een uitgebreide karakterisatie van de verschillende aspecten van motorische functie in het SCA17 ratmodel gegeven, waarbij zowel traditionele als geautomatiseerde gedragsmetingen worden toegepast. Grove en fijne motoriek werden longitudinaal getest met metingen op leeftijden van 3, 6 en 9 maanden in wildtype (controle) en transgene SCA17 ratten. Grove motoriek werd geëvalueerd met het CatWalk® loopanalyse systeem, een test voor grijpsterkte en een looptest op een balk. Fijne motoriek werd gemeten met behulp van een reiktest waarbij een voedselpellet gegrepen moest worden. Onze resultaten waren niet alleen in overeenstemming met eerder gerapporteerde resultaten van motorische fenotypen, maar lieten ook een sterke beperking zien in de pellet reiktest en in de coördinatie tussen de poten in het CatWalk® experiment op een leeftijd van slechts drie maanden. Ataxie en ongecoördineerd voortbewegen werden ook geobserveerd in transgene dieren, samen met een aangetaste spierkracht in het laatste stadium van de ziekte. Parameters die de meest uitgesproken verschillen lieten zien hielden verband met de mate van vloeiend bewegen en lichaamsondersteuning. In de vertaling naar de situatie van de patiënt corresponderen de resultaten met beperking van de grijpbeweging, verminderde grijpkracht, en een karakteristieke loop bij vroege en/of laat symptomatiche patiënten. Het SCA17 ratmodel omvat dus verschillende kenmerken van de SCA17 patiënt, zoals verminderde spierkracht, een ongecoördineerde loop (ataxie), en verminderde handvaardigheid. Deze duidelijke beperkingen zoals waargenomen in de verschillende aspecten van motorisch functioneren dragen bij aan de validiteit van het transgene SCA17 ratmodel.

Naast motorsymptomen is angst een veel beschreven kenmerk van verschillende polyglutamine ziekten en dus ook een belangrijk aspect voor de gezichtsvaliditeit van het
SCA17 ratmodel. In hoofdstuk 3 werden risicobeoordeling en angstachtige kenmerken gekarakteriseerd in 3, 6 en 9 maanden oude SCA17 ratten en hun wildtype nestgenoten met een standaard gedragstest, een verhoogd plusvormig doolhof (elevated plus maze, EPM). Daarnaast werd neuronale activatie in de basolaterale amygdala in relatie tot de gedragsreacties geëvalueerd met c-Fos immunokleuringen. Het meest prominente gedrageffect was een hoger niveau van risicobeoordeling in de transgene ratten. Verder werd er een toename van angst-gerelateerd gedrag in deze ratten gevonden, en het genotype effect op risicobeoordeling en thigmotactisch gedrag was zichtbaar op alle geteste leeftijden. Dit laat zien dat psychiatrische symptomen zich kunnen ontwikkelen vóór en gedurende de ontwikkeling van bewegingsstoornissen in het SCA17 ratmodel. Ondanks dat de EPM geen algeheel effect op c-Fos expressie veroorzaakte werd er een negatieve correlatie met angstachtig gedrag geobserveerd. In de vertaling naar de situatie van de patiënt komen onze resultaten overeen met gevonden stemmingswisselingen, waaronder stress en angst in vroeg- en laat-symptomatische patiënten. Het is echter moeilijk om hetzelfde te kunnen zeggen over de presymptomatische fase, gezien het feit dat er normaal gesproken weinig tot geen informatie over de psychiatrische status van de patiënten beschikbaar is voor de manifestatie van de eerste motorische symptomen. Onze resultaten suggereren dat het SCA17 ratmodel al op een leeftijd van 3 maanden een angstfenotype vertoont wat een gelijkenis heeft met gegeneraliseerde angst in vroeg-symptomatische SCA17 patiënten.

In hoofdstuk 4 wordt een methode beschreven voor het betrouwbaar meten van angst in de SCA17 ratten. In deze studie werd een geautomatiseerd hulpprogramma voor het testen van angst binnen de eigen thuiskooi (in plaats van een testkooi) gebruikt, om zo het effect van humane interventie, stress van het hanteren, transport en een vernieuwde omgeving te minimaliseren. De angsttest „light spot” (LS) (een witte led gericht op de locatie van het voedsel net na de overgang van licht naar donker, wanneer de ratten actief worden en beginnen met eten) werd toegepast op het transgene SCA17 ratmodel in de PhenoTyper 4500® om een angstachtig fenotype gedurende drie fases van de ziekte (3, 6 en 9 maanden oude dieren) te verifiëren. Locomotie was verhoogd in SCA17 ratten na 6 en 9 maanden gedurende de eerste 15 minuten van de LS test, wat potentiële verhoogd risico beoordelend gedrag reflecteert (benadering-ontwijking). De LS test induceerde met succes ontwikkeld gedrag en het zoeken naar een schuilplaats in de ratten: beide genotypen reageerden op de test met een kortere doorgebrachte tijd in de LS zone en een langere doorgebrachte tijd in de schuilplaats vergeleken met de controleconditie. SCA17 ratten lieten een sterker conflict zien tussen benaderen en ontwijken, gereflecteerd in een toegenomen activiteit in het gebied rondom de LS. Deze test in de eigen thuiskooi, waarin effecten continu gemonitord worden, biedt de mogelijkheid voor een diepgaande analyse wat het potentiële nuttig maakt om subtiele en complexe angst-gerelateerde kenmerken in knaagdieren te onderzoeken. We hebben de eerste data van een ratmodel onderworpen aan de LS test beschreven, welke gezien
kan worden als biologisch relevanter dan de traditionele test omdat het angst in een voor het dier vertrouwde omgeving meet. Daarom is de LS test in het algemeen een veelbelovend hulpmiddel om ontwikkend gedrag en de reactie op negatieve stimuli te meten en te evalueren door het monitoren van effecten voor- en na het blootstellen aan de stimulus en het bieden van de mogelijkheid voor diepgaande analyse.

**Hoofdstuk 5** vervolgt het onderzoek naar potentiële veranderingen in sociaal gedrag in het transgene SCA17 ratmodel door gebruik te maken van een sociale interactie test in een ruime arena (PhenoTyper 9000). Verschillende sociale interactie parameters werden longitudinaal met het ziekteverloop gemeten, op de leeftijden van 3, 6 en 9 maanden in transgene SCA17 ratten en hun wildtype nestgenoten. Dieren werden getest met een bekende kooigenoot na een korte isolatie gedurende 24 uur voorafgaand aan de sociale interactie test. Op de leeftijd van 9 maanden werd een extra sociale interactie test uitgevoerd met onbekende paren van hetzelfde genotype om sociale vernieuwing in het laatste stadium van ziekteontwikkeling te onderzoeken. Symptomatische SCA17 ratten (9 maanden oud) lieten specifieke veranderingen in hun sociaal gedrag zien wanneer ze herenigd werden met een kooigenoot, of wanneer ze een onbekende SCA-rat tegenkwamen na een periode van isolatie.

In het bijzonder was er een afname in de frequentie van interacties en een afname in de totale tijd gespendeerd aan het benaderen van de testpartner, wat een inhibitie in het initiëren van sociale interactie met soortgenoten suggereert. Dit kan indicatief zijn voor sociale angst, een kenmerk wat ook beschreven is in SCA17 patiënten. Echter, ondanks de prominente afname in de totale afgelegde afstand en de snelheid van bewegen, resulteerde dit niet in een sociale terugtrekking aangezien de totale tijd van sociale interactie onveranderd bleef ten opzichte van de wildtype controlegroep. Het kan niet uitgesloten worden dat het algeheel lagere activiteitsniveau van de SCA ratten op deze leeftijd hier een rol in speelt. Onbekendheid tussen een interactiepaar resulteerde in een onveranderde totale tijd gespendeerd aan interactie, terwijl de gemiddelde duur van de sociale interacties ook hier was toegenomen in de SCA17 ratten wanneer zij een onbekende soortgenoot tegenkwamen, gelijk aan de testsituatie met de bekende rat. Onze resultaten laten veranderingen zien in sociaal contact en motivatie in de SCA17 ratten van 9 maanden oud.

De parameters die de meest uitgesproken verschillen vertoonden waren gerelateerd aan het initiëren van sociale interactie met een bekend en onbekend dier. Dit doet vermoeden dat ondanks deze moeilijkheden, welke een basis kunnen hebben in het algemene activiteitsniveau, de motivatie (sociale interesse) voor interactie onveranderd was in de SCA17 ratten.

Tot slot worden in **hoofdstuk 6** verschillende moleculaire aspecten van de transgene SCA17 rat onderzocht door toepassen van een combinatie van moleculaire technieken. Hersenweefsels van wildtype en transgene dieren werd verzameld op verschillende leeftijden (3, 6, 9 en 10 maanden oud). Om eiwitaggregaten te meten werden hersenlysaten
Nederlandse samenvatting


In hoofdstuk 7 worden alle onderzoeksbevindingen uitvoerig bediscussieerd en worden overwegingen en toekomstige richtingen voor onderzoek besproken.

Kortom, het in dit proefschrift gepresenteerde werk heeft als doel het valideren van een relatief nieuw diermodel voor de detectie van vroege symptomen van SCA17 en suggereert verschillende richtingen voor toekomstig onderzoek die veelbelovend zijn en die verder bekeken moeten worden. Nieuwe informatie over het ontstaan en ontwikkelen van verschillende ziektesymptomen, van motorische en psychiatrische fenotypes tot moleculaire veranderingen in het brein van de SCA17 rat, werden gepresenteerd. Met het oog op de ontwikkeling van toekomstige innovatieve therapeutische strategieën, kunnen deze resultaten nuttig zijn in het identificeren van geschikte interventietijdstippen, welke hopelijk uiteindelijk zullen bijdragen aan de ontwikkeling van succesvolle therapieën voor deze verwoestende neurodegeneratieve ziekte.
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Οικογένεια Κυριακού: θεία Πενύ, θείε Γιώργο, θείε Νίκο, Νανά, Λένα, θείε Γιώργο, θεία Κατερίνα, Νίκο, Μιχάλη, Δημήτρη, Γιώργο και Μαριέλα. Ευχαριστώ πολύ για όλη τη στήριξη σας όλα αυτά τα χρόνια σε όλα μου τα βήματα και όλη την αγάπη και ζεστασία μου μου δείχνετε ακόμα και αν δεν βλεπόμαστε συχνά πια.

Μαμά, μπαμπά και μικρέ μου αδερφέ Νίκο: η ζώση δεν έχουν εφευρεθεί ακόμα οι λέξεις σε καμία γλώσσα του κόσμου για να περιγράψω την απέραντη, ατελείωτη, ανεξάρτητη αγάπη και ευγνώμονη που νιώθω για εσάς. Τόση στήριξη σε κάθε βήμα μου, τόση συμπαράσταση σε όλα, τόση επένδυση και πίστη σε μένα ακόμα και τις στιγμές που ούτε εγώ δεν πίστευα σε μένα. Είστε για μένα τα πάντα όπως ξέρω ότι είμαι και εγώ για σας. Τότε από όλα αυτά δεν θα είχε συμβεί χωρίς εσάς. Σας αγαπώ και σας ευχαριστώ για όλα.

Dick, alleen maar voor jou zou ik de poging doen om dit in het Nederlands te schrijven. Gebroken, maar toch vind ik het de moeite waard. Dit proefschrift zou een titel, een cover opvatting, een nederlandse sammenvatting en zoveel andere missen zonder jou. Misschien zou het niet eens hebben bestaan zonder jou. Ikzelf, ben zoveel dank verschuldigd dat er geen woorden genoeg zijn om te beschrijven, voor alle steun, begrip,
begeleiding, advies en vooral liefde tijdens deze lange hobbelige weg. Je geloofde in mij, je gaf me ruimte en je had eindeloos geduld totdat ik kon afmaken waar ik aan begon. Je overtuigde me dat ik het kon toen ik dacht dat ik zou stoppen. Om deze en vele andere redenen, hou ik steeds meer van jou. Je bent mijn partner, beste vriend, familie geweest en dat is wat jou zo speciaal voor mij maakt. We hebben al veel stappen samen gezet, nu met ons eerste huis samen, en ik hoop dat er nog veel meer stappen zullen volgen.
LIST OF PUBLICATIONS


CURRICULUM VITAE

Elisavet Kyriakou was born on 7 June 1988 in Kalamata, Greece. After finishing the lyceum in 2006, she started studying Biology at University of Patras in Greece. In 2012 she obtained her Bachelor's degree and continued her studies in the University of Edinburgh in Scotland in the master program Integrative Neuroscience where she obtained the title Master of Science by Research. During two large internships, she acquired experience in research, with special focus on behavioural neuroscience. Her first internship was in the University of Patras where under the supervision of Dr. M. Margarity she studies the toxic effects of different dose levels of lead on cognition, memory and learning administered in the drinking water. Her second internship took place in the University of Edinburgh in Scotland. Under the supervision of Dr. M. Nolan, she participated in research on spatial navigation in virtual environments and investigation of Arc expression in the enthorinal cortex of behaving animals in virtual environments. After obtaining her master's degree in 2013, her interests in behavioural neuroscience led her to start her PhD studies at the CRO Delta Phenomics in Schaijk, the Netherlands under the supervision of Dr. J. van de Harst. PhenoRat is a European-funded project under which she worked and was an intersectoral PhD program (Marie Curie Initial Training Network). The consortium included Delta Phenomics, Noldus IT, Radboud University and the University of Tuebingen. During her first year, she spent 10 months at Delta Phenomics and after that she was transferred and continued her research at the Department of Cognitive Neuroscience at the Donders Institute for Brain Cognition and Behaviour in Nijmegen, under the supervision of Prof. dr. B. Roozendaal, Dr. J. Homberg and Dr. J. van de Harst. In February 2016, she continued and completed her work at the University of Tuebingen at the Institute of Medical Genetics and Applied Genomics under the supervision of Dr. med. H.H.P. Nguyen. Her research presented in this thesis was conducted between November 2013 and October 2016. The findings included in this book were presented at different national and international meetings including the Measuring Behavior conferences in Wageningen and Dublin and the Dutch Neuroscience meeting in Lunteren. Her interests in biology, behavioural neuroscience and preclinical research will remain important factors in her future career.
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.

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