Validating cognitive impairments found in the BACHD rat model of Huntington disease through the use of control tests that account for the animals' reduced motivation

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“During the past few months we have conducted several behavioral tests to evaluate how far the European tree frog can jump. During initial trials we trained frogs to jump when given the command “Jump frog, jump!”. We then used a tabletop setup with a start line to carefully measure their jumping distance. The frogs reliably responded to the command, and jumped on average 0.52 meters. Next, we surgically removed the frogs’ hind legs, and placed them back on the test setup. When given the command “Jump frog, jump!” the frogs now only waddled around aimlessly. We have from this concluded that the surgery made the frogs deaf.”

- Internet

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Summaries

Summary (English)

Huntington disease is an autosomal-dominantly inherited, neurodegenerative disease that is caused by a specific mutation in the gene encoding the huntingtin protein. Expression of the mutated protein results in extensive neuronal loss throughout the brain, although certain brain regions are more heavily affected. The resulting clinical symptoms include a range of motoric, psychiatric, cognitive, and metabolic changes that progress until the patients are unable to care for themselves, and ultimately result in an early death. There is currently no disease-modifying treatment available. Thus, continued efforts in both clinical and preclinical research are of importance.

Several animal models of Huntington disease have been established following the discovery of the huntingtin gene and the disease-causing mutation. Each model has strengths and weaknesses, and their combined use is of importance for preclinical research concerning disease mechanisms and potential therapeutics. Thorough characterization of a given animal model is important in order to understand to what extent it models the actual disease and how to work with it in an appropriate way.

The current thesis includes a series of studies focusing on the characterization of a recently established rat model for Huntington disease, called the BACHD rats. These rats carry a transgenic construct, which expresses the full-length mutated protein that causes Huntington disease. The project included the assessment of body size and body composition as well as investigations of motivational and cognitive phenotypes. Results indicated that male BACHD rats were obese, while simultaneously showing discreet developmental deficits. These phenotypes might be caused by neuropathology of the hypothalamus, which has also been noted among Huntington disease patients. Assessment of the BACHD rats' performance on a test of motivation suggested that the rats' altered body composition might affect their interest in working for food rewards. Strategies to circumvent this influence were evaluated, as motivational differences might confound investigations of other behavioral aspects. Through the use of control tests, robust phenotypes of cognitive impairments among the BACHD rats were characterized. Similar phenotypes have been found among rats with fronto-striatal lesions, suggesting that disease-related neuropathology might be causing the BACHD rat's phenotypes.

Ultimately, the work presented in the current thesis served to further the research on how to work with Huntington disease models in general, and the BACHD rats in particular. In addition, the noted phenotypes would likely be suitable in future preclinical testing of potential therapeutic agents, although specific investigations to determine the underlying neuropathology are still of importance.

Zusammenfassung (Deutsch)

Die Huntington Erkrankung ist eine autosomal dominant vererbte, neurodegenerative Erkrankung, die durch eine spezifische Mutation im Gen des Huntington-Proteins verursacht wird. Die Expression des mutierten Proteins führt zum dramatischen Verlust von Nervenzellen im gesamten Gehirn, wobei bestimmte Hirnareale stärker betroffen sind als andere. Die daraus resultierenden klinischen Symptome beinhalten eine Reihe motorischer, psychiatrischer, kognitiver und metabolischer Beeinträchtigungen, die mit voranschreitender Krankheit zunehmen, bis die Patienten nicht mehr in der Lage sind, sich um sich selbst zu kümmern und schließlich frühzeitig versterben. Zurzeit gibt es keine krankheitsmodulierende Therapie, weshalb die präklinische und klinische Forschung von enormer Wichtigkeit sind.

Seit der Entdeckung des krankheitsauslösenden Gens wurden zahlreiche Tiermodelle für die Huntington Erkrankung etabliert. Jedes Tiermodell besitzt Vorzüge und Nachteile, und für die präklinische Forschung hinsichtlich Krankheitsmechanismen und potentieller Therapeutika ist die Ausschöpfung aller Modelle gemeinsam von enormer Wichtigkeit. Die gründliche Charakterisierung eines jeden Tiermodells ist maßgeblich, um den Grad der Übereinstimmung mit der menschlichen Erkrankung einschätzen zu können und zu wissen wie mit den Tieren gearbeitet werden sollte.

Die vorliegende Arbeit beinhaltet eine Reihe von Studien, die sich auf die Charakterisierung des jüngsten Rattenmodells der Huntington Erkrankung, die BACHD-Ratte, beziehen. Diese Tiere tragen ein transgenes Konstrukt, welches das gesamte, mutierte Huntingtin-Gen exprimiert. Die Arbeit beinhaltet die Untersuchung der Körpergröße und Körperzusammensetzung sowie der Ausprägung von Verhaltensphänotypen hinsichtlich Motivation und Kognition. Die Ergebnisse zeigen, dass die BACHD-Ratten fettleibig sind und ein Wachstumsdefizit aufweisen, was möglicherweise auf eine Pathologie im Hypothalamus zurückzuführen ist wie sie bei Huntington-Patienten vorliegt. Ein Test zur Untersuchung der Motivation deutete ferner darauf hin, dass die Fettleibigkeit der BACHD-Ratten womöglich zu einem verminderten Interesse führt für Futterbelohnungen zu arbeiten und somit möglicherweise andere Verhaltensparameter beeinflusst. Daraufhin wurden Vorgehensweisen getestet, um dies zu unterbinden und eine unverfälschte Verhaltenscharakterisierung zu ermöglichen. Durch den Einsatz von Kontrolltests konnten schließlich robuste, kognitive Phänotypen beschrieben werden. Ähnliche Einbußen sind von Ratten mit fronto-striatalen Läsionen bekannt, was darauf hindeutet, dass eine krankheitsbedingte Neuropathologie zugrunde liegen könnte.

Der Nutzen der vorliegenden Arbeit liegt insbesondere darin, die Forschung an Huntington-Tiermodellen, insbesondere der BACHD-Ratte, im Hinblick auf deren adäquate Nutzung voranzutreiben. Die beschriebenen Phänotypen können weiterhin in präklinischen Studien zur Evaluierung von potentiellen Therapeutika von Nutzen sein. Untersuchungen zur zugrundeliegenden Neuropathologie wären nachfolgend von Wichtigkeit.

Publications

List of publications

Published articles are listed in a specific order to fit the narrative of the thesis. Equal contribution of two or more authors is indicated by an "&" sign linking the authors' names. The author's name is highlighted. Please note that the author changed his last name between 2014 and 2017.

I. **E K H Jansson** & L E Clemens, O Riess, H P Nguyen. Reduced motivation in the BACHD rat model of Huntington disease is dependent on the choice of food deprivation strategy. PLoS One, 2014; 9(8): e105662, doi: 10.1371/journal.pone.0105662

II. **E K H Clemensson**, L E Clemensson, B Fabry, O Riess, H P Nguyen. Further investigation of phenotypes and confounding factors of progressive ratio performance and feeding behavior in the BACHD rat model of Huntington disease. PLoS One, 2017; 12(3): e0173232, doi: 10.1371/journal.pone.0173232

III. **E K H Clemensson**, L E Clemensson, O Riess, H P Nguyen. The BACHD rat model of Huntington disease shows signs of fronto-striatal dysfunction in two operant conditioning tests of short-term memory. PLoS One, 2017; 12(1): e0169051, doi: 10.1371/journal.pone.0169051

Statement of personal contribution

Statements are given according to the order of publications listed above

I. Main investigator together with L E Clemens. Equal share in planning and performing experiments, analysis and manuscript preparation.

II. Main investigator. Planned and performed experiments, analysis and manuscript preparation. It should, however, be noted that collections of blood samples were made together with L E Clemens, and subsequent leptin ELISAs were run by B Fabry.

III. Main investigator. Planned and performed experiments, analysis and manuscript preparation.

Abbreviations

BAC	Bacterial artificial chromosome
BACHD	BAC-containing full-length huntingtin
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DNA	Deoxyribonucleic acid
GABA	γ-amino butyric acid
GPe	Globus pallidus pars externa
GPi	Globus pallidus pars interna
HD	Huntington disease
IGF-1	Insulin-like growth factor 1
IT15	Interesting transcript 15 / Huntingtin gene
PR	Progressive ratio
TG5	BACHD rat, transgenic line 5
TG9	BACHD rat, transgenic line 9
WT	Wild type
YAC	Yeast artificial chromosome

Introduction

Huntington disease

Epidemiology and cause of disease
Huntington disease (HD) is an autosomal-dominantly inherited neurodegenerative disease, which affects approximately 6 out of 100,000 people in Europe, North America and Australia[1,2]. Although genetic modifiers have been identified[3,4] the sole genetic cause of the disease is the expansion of an unstable CAG repeat sequence in the protein-coding region of the *IT15* gene (consequently termed the Huntingtin gene, for the huntingtin protein), on chromosome 4[5]. Alleles with up to 34 CAG repeats are considered to lie in the normal range, and do not confer any disease risk[6,7]. Alleles with longer repeat sequences have been found to cause HD, although full penetrance is primarily seen for alleles with more than 42 CAG repeats[6]. In addition to being the primary cause for disease development, the length of the CAG repeat sequence is known to affect the age at onset of HD[7–10]. Thus, patients carrying an allele with 40 CAG repeats have a 50% probability to develop the disease around the age of 60, while this decreases to an age of 40 and 30 for alleles with about 45 and 50 CAG repeats respectively[6]. Patients who show an age at onset younger than 20 are considered to have juvenile HD, which is often associated with CAG repeat lengths above 60[11,12]. Still, CAG repeat length only explains about 50% of the variation in the age at onset of HD[8,10]. Thus, although it is crucial to the development of HD, additional genetic and environmental factors appear to contribute to the appearance of the disease[3,4,13,14]. It should also be noted that in contrast to its clear effect on age at onset, the effect of CAG repeat length of the rate of clinical progression is somewhat unclear[15–19].

Most HD patients carry one mutated allele, which they inherited from one of their parents. The incidence of *de novo* mutation has been estimated to be about 0.1% for transmission from a father with a high, but normal, CAG repeat sequence[20]. Still, *de novo* mutations are estimated to account for up to 10% of diagnosed patients[21]. Interestingly, intergenerational changes in CAG repeat lengths often concern expansions rather than contractions, particularly when being inherited paternally[22,23]. Although there currently are symptomatic treatments that can reduce the disease burden for patients[24], there is no disease-modifying treatment available. Thus, HD is at this time invariably fatal.

Protein function and neuropathology
The huntingtin gene is expressed in most tissues of the body with highest protein levels found in testes and neurons[25–28]. The protein is present both in the cytoplasm and nucleus of cells[29,30], where it is thought to function as a scaffold protein, as it has been found to interact with several other proteins[31–33]. Through these interactions huntingtin appears to take part in a range of cellular processes, including endocytosis, vesicle transport, synaptic plasticity, gene transcription, cell metabolism, mitosis and apoptosis[31–33]. As noted, the mutated form of the huntingtin gene has an elongated CAG repeat sequence, which in the translated protein gives an elongated stretch of glutamine amino acids. This is thought to confer both toxic gain of function and disruption of the protein's normal function[31–34]. Although the exact interplay between these aspects is not clear, both are likely important for shaping the specific pathology of HD[34].

The neuropathology of HD primarily affects the basal ganglia, although several other brain regions are involved during the late stages of the disease[35–37]. The basal ganglia comprise several subcortical nuclei within the cerebrum, which together play a crucial role in coordinating various kinds of behaviors (Figure 1-4). In brief, the basal ganglia are thought to inhibit inappropriate behaviors while promoting appropriate ones[38–42]. This is primarily thought to function through different neuronal signaling loops, where cortical neurons convey information concerning the current situation and possible behaviors to the basal ganglia. As the basal ganglia receive input from most parts of the cortex it offers an anatomically convenient location where the diverse information can be weighed. Ultimately, signals promoting appropriate (or at least the selected) behaviors will be relayed back to the cortex via the thalamus, while inappropriate behaviors are silenced[38–42]. The signaling loops that connect the cortex and basal ganglia are thought to be arranged in a parallel manner, with some level of cross-communication. As separate loops connect different regions of the cortex and basal ganglia, they are also thought to govern different behaviors[43,44]. Within the basal ganglia it is primarily the projection neurons in the striatum that directly receive the excitatory glutamatergic signals from the cortex[42] (the aforementioned signaling loops are thus generally referred to as cortico-striatal loops). These neurons (known as medium-sized spiny neurons) have in turn axonal connections with other nuclei within the basal ganglia, and form one of the primary sites of basal ganglia signal modulation[41,45]. Notably, the striatum is the main site of HD pathology, where there is extensive loss of medium spiny projection neurons[35-37,46]. More specifically, the most striking pathology is found in the dorsal striatum, which is composed of two interconnected but distinct nuclei called the caudate and putamen. Neuronal loss is first evident in the tail of the caudate nucleus, and later extends in caudo-rostral, dorso-ventral and medio-lateral directions to include both the body and the head of the caudate nucleus[35-37,46]. Neuronal loss within the putamen shows a similar progression, and occurs largely in parallel to the involvement of the body and head of the caudate nucleus[37,46]. As noted, it is primarily projection neurons that are lost within the striatum, while interneurons remain largely spared[47–53]. In addition, different projection neuron populations are lost at different points of disease progression[35,36,50]. Thus, neurons that synapse on the globus pallidus pars externa (GPe) and contain dopamine 2 (D2) receptors are the more susceptible than neurons that synapse on the globus pallidus pars interna (GPi) and contain dopamine 1 (D1) receptors. This selective neuropathology is not in line with the ubiquitous expression of mutant huntingtin and the exact cause for it is not yet clear. Recent hypotheses suggest that the medium spiny neurons might be particularly sensitive to excitotoxicity, due to the extensive glutamatergic input they receive in combination with several effects of mutant huntingtin[47,54,55].

Clinical symptoms of HD

HD presents with a range of clinical symptoms that include motoric, psychiatric, cognitive and metabolic disturbances. The motoric symptoms are diverse, and concern

both difficulties with voluntary and involuntary movements. Thus, patients often display a mixture of chorea (irregular involuntary movements classically likened with dance-like movements), dystonia (involuntary twisting and repetitive movements caused by co-contractions of opposing muscle groups), rigidity and bradykinesia (slowed movement)[56–58]. Due to this, several aspects of normal life are affected for HD patients. One aspect that is of particular relevance for the work presented in this thesis concerns difficulties with eating, where patients have problems with moving food towards their mouths, chewing, and swallowing[59–62].

Psychiatric symptoms of HD frequently include apathy, depression, irritability, anxiety and obsessive-compulsive disorder. Psychosis (i.e. delusions and hallucinations) is, on the other hand, rare[63–68].

Cognition is a broad term that is used to refer to a range of mental processes that concern the acquisition, storage, manipulation and retrieval of information[69]. It thus relates to several higher functions of the central nervous system, including perception, memory, language, problem solving and abstract thinking[70]. Several aspects of cognitive function are impaired in HD[71–73]. First, HD patients have repeatedly been shown to have a reduced psychomotor speed[74–80] (mental aspect of reaction time). In addition, HD patients show deficits in both episodic[81–83] (events and experiences) and semantic[84,85] (facts and concepts) memory functions. These deficits are generally thought to be due to patients having difficulties with efficiently retrieving information rather than forgetting it[81–90], although this hypothesis has been questioned[91,92]. In relation to their general memory problems, HD patients have difficulties to acquire both motor-related and non-motor related skills[90,93,94]. A final memory-related aspect of HD concerns their impaired working memory[89,95–101]. Working memory is commonly considered to be the cognitive function that allows for temporary storage and online manipulation of information[102]. Due to its complexity, there are several aspects of the information or task at hand that affect the overall strain that is put on the system. Among other things it includes temporal (the time something needs to be remembered) and span (the amount of information that needs to be remembered) aspects[102]. HD patients have shown consistently impaired span capacity[89,96,98–101] while temporal capacity appears to be less impaired[97,99]. Working memory is considered to be one of the components of the central nervous system's executive function[103]. This is in turn a function that is thought to be fundamental for optimizing and maintaining appropriate behaviors in general[103]. In addition to working memory, it incorporates cognitive flexibility (the ability to adjust attention, strategies and behaviors) and inhibitory control (includes selective attention, and inhibition of inappropriate responses and behaviors). These functions then allow for higher cognitive processes such as the ability to plan responses or behaviors[103]. There is extensive data indicating that several aspects of executive control is impaired in HD, including working memory (as noted above), cognitive flexibility[96,104–106], selective attention[106,107] response inhibitition[106,108–110] behavioral inhibition (not extensively reported and changes in risk-taking are unclear[111])[112] and planning[113,114]. A final cognitive aspect of HD, which does not necessarily fit into the categories of symptoms described above, is that patients frequently have difficulties to recognize emotions, particularly negative ones[74-79,115–117].

The main metabolic symptom found among HD patients is considered to be extensive weight loss[118–122]. It is generally thought that this is due to a loss of both adipose and

muscle tissue[120,121,123], although this has not been extensively investigated. Thus, although there are clear indications of muscle dysfunction in HD, the extent of actual muscle atrophy is not yet clear[124]. The exact cause of weight loss has also not been fully elucidated yet, but is likely multifaceted. It has been argued that due to their problems with eating, HD patients might have difficulties to consume enough food to maintain a stable body weight[125]. Still, HD patients have been found to lose weight when consuming diets with comparable[126] and higher[120] caloric content than healthy persons. There are also indications that HD patients have higher energy expenditure due (in part) to their choreatic movements[127,128]. Still, weight loss has also been found among patients that do not suffer from overt choreatic movements[118,121]. Thus, although reduced food intake and chorea are likely to affect the symptoms, recent hypotheses suggest that the body weight loss is primarily caused by an underlying hypermetabolic state[121,123,129]. This has, in turn, been suggested to be due to neuropathology of the hypothalamus, although it is likely that pathology in peripheral tissues also play a role[123]. Finally, it should be pointed out that progressive weight loss is not always present in HD patients[130,131].

Clinical progression of HD

As noted, HD is a progressive disease. Thus, the symptoms listed above initially appear as discreet impairments and then progressively worsen with time. The appearance of choreatic movements was initially used to mark the onset of the disease, although this has been replaced by a scoring method that takes several motoric aspects into account[132,133]. Still, more discrete motoric impairments, as well as psychiatric and cognitive symptoms are known to be present in earlier stages of the disease (see below). Neuronal loss and dysfunction within the striatum is likely apparent at even earlier stages[75,78,132,134].

Motor symptoms appear to start as discreet impairments in oculomotor function[135,136], and movement correction abilities[137] (7-10 years before clinical onset). This is closely followed by the appearance of chorea and bradykinesia (which commonly coincides with the clinical onset of the disease), while dystonia appears slightly later[135] (2 to 4 years after clinical onset). In late stages of the disease, the chorea subsides, leaving the patients largely akinetic[138–140]. A notable exception to this clinical progression is seen among patients with juvenile HD, as they present primarily with stiffness and akinesia from the start, and only rarely display choreatic movements[141]. It should still be noted that similar clinical progression is also seen in a subset of adult-onset HD patients[138,142].

All aforementioned psychiatric symptoms of HD have been found to be present before clinical onset of the disease (up to at least 10 years) [134,143,144]. Although the exact time course of their development is unclear there are indications that apathy progressively worsens with general progression of HD, while depression does not[78,145–147]. Thus, apathy might be closely related to the progressive neuropathology.

Several cognitive symptoms are also present long before clinical onset of HD, although once again the exact time course for their development is uncertain[71,73,78,79,148,149]. Still, impaired psychomotor speed, cognitive flexibility and emotion recognition appear to be among the earliest symptoms[78,79,104,149] (10-15 years before clinical onset). As the disease progresses, these impairments become more apparent and additional cognitive symptoms manifest (described above). Ultimately, HD patients develop a general dementia[71,73].

As described above, the neuronal loss in HD is first present in the tail of the caudate nucleus. It then develops in caudo-rostral, dorso-ventral and medio-lateral directions to encompass the body and head of the caudate as well as the putamen[37,46]. As different parts of the caudate and putamen are involved in different cortico-striatal loops[43,44], this gradual neuronal loss should have some connection to the time course of symptom development. It is, however, important to note that HD patients appear to suffer from additional discreet neuronal dysfunction, which might show different temporal and spatial progression[150]. In addition, the exact function of cortico-striatal loops in relation to behavior is not fully elucidated[151]. Still, it is worth mentioning that the caudate nucleus is thought to be strongly linked to cortico-striatal loops involved in cognitive and oculomotor function, while putamen is more linked to sensorimotor tasks[151]. Thus, the current consensus of cognitive and oculomotor symptoms being among the earliest behavioral changes in HD patients is in line with the early appearance of caudate nucleus pathology in HD progression.

Models of Huntington disease

General introduction
A multitude of model systems are used in HD research. These include cell-based models such as transient or stable transfection of mammalian cell lines[152,153], cell lines established from genetically engineered animal models[154] (see below), stably transfected embryonic stem cells[155], transient transfection of primary cultures[156] and inducible pluripotent stem cells from HD patients[157]. In addition, there is a range of HD animal models using both invertebrates (Caenorhabditis elegans[158] and Drosophila melanogaster[159]) and vertebrates (primarily mammalian, such as mouse[160–167], rat[168–174], sheep[175], pig[176] and monkey[177]). Rodent models are among the most frequently used ones, and will be the main focus of this introduction section.

Rodent models of HD
Before the identification of the disease-causing gene, rodent models of HD were primarily based on various forms of striatal lesions[54,168–172,178]. Although crude, this research was central in shaping the excitotoxicity-based hypothesis of HD's neuropathology[168–172]. Following the discovery of the huntingtin gene, however, a range of animal models based on different forms of genetic manipulation were established[178,179]. It is thus of interest to note that wild type (WT) alleles of huntingtin homologue genes in mice and rats contain seven and eight CAG repeats respectively[180].

The first transgenic rodent models that were established used constructs that only expressed a fragment of the disease-causing gene[160,161,173,178]. For these models, the transgene rarely exceeds past huntingtin's first exon, where the elongated CAG repeat sequence resides. The models generally confer strong and early phenotypes[54,178] (see below). Common models include the R6/1[160], R6/2[160] and N171-82Q[161] mouse models, as well as the TgHD[173] rat model. The models are generally referred to as fragment models, due to the nature of their transgene. There are also rodent models that carry transgenic constructs that express the full huntingtin gene[162–164,174]. These models generally show milder phenotypes than the fragment models[54,178] (see below). The genetic constructs used to create these models used high capacity DNA vectors called yeast and bacterial artificial chromosomes (YAC and BAC respectively), due to the large size of the huntingtin gene. This is referenced in the names of common full-length models, such as the YAC128[163] and BACHD[164] mice, as well as the BACHD[174] rats. The aforementioned models were created through classical transgenic methods, where genetic material is injected into fertilized oocytes, whereupon it incorporates at random position in the host animal's genome[181]. Thus, the disease-related transgene is expressed in addition to the two endogenous WT huntingtin alleles of the host animal. A final kind of animal models was established with the aim to more closely model the genetic aspects of HD. Rather than introducing an exogenous genetic material through a transgenic construct, these models are based on specifically modifying the length of the CAG repeat sequence of the endogenous huntingtin alleles[165–167]. These models are generally thought to confer more subtle phenotypes compared to full-length models[54,178,179], although careful characterization still reliably reveals them (see below). This kind of model, known as knock-in model, has so far only been established in mice. Commonly used strains are the HdhQ92-111[165], HdhQ140[166] and HdH(CAG)150[167] mice.

As noted, the general consensus is that fragment models confer the strongest phenotypes, while full-length models show milder ones, and knock-in models show very discreet ones[54]. It should, however, be noted that models are frequently established on different genetic backgrounds, use different promoter sequences to drive the expression of their genetic construct, have different numbers of CAG repeats, and are differentially susceptible to positional mutagenesis[160-167,173,174]. Naturally, these factors are likely to influence the overall phenotype, just like the specific nature of the genetic modification itself (i.e fragment, full-length or knock-in). It is also noteworthy that several of the genetically modified rodent models carry constructs with repeat lengths that exceed the ones commonly found in HD patients (80 to 150 CAG repeats for most models[160-167,173,174] compared to about 40 to 50 CAG repeats for adult onset HD[6]). As noted above, patients with repeat lengths above 60 commonly develop juvenile HD, which differs somewhat from adult onset HD[11,12]. One of the exceptions is the TgHD rat, which carries 51 CAG repeats[173].

Disease-related phenotypes of genetic HD rodent models
All genetic rodent models of HD show a widespread expression of their respective disease-related protein product[160-167,173,174]. Indications of HD-related neuropathology have also been found in most models, although the onset and extent of neuropathology varies. Specifically, fragment mouse models have been found to show early onset (2-5 months of age) of progressively reduced striatal volumes and numbers of striatal neurons[182–189]. Similar phenotypes have been found for the TgHD rats[190,191], although they appear at an older age (9-12 months) and seem to be somewhat difficult to reproduce[192,193]. HD-related neuropathology has also been found in the YAC128[163,194–197] and BACHD[164,197] mice, although the phenotypes appear at older ages (6-12 months of age) compared to fragment mouse models, and extensive neuronal loss has not been found in BACHD mice[164,197]. In addition, the neuropathology of BACHD mice does not appear to be reliably reproducible[198,199]. BACHD rats also appear to have mild neuropathological phenotypes with late onset[174] (see below). Finally, knock-in mouse models were initially not thought to have any strong neuropathology at all[165-167], although more recent studies have found loss of striatal volume and neurons at advanced ages[200,201] (12-26 months).

As described above, HD is a fatal disease. This does, however, not appear to be a general phenotype among genetic rodent models[54]. Fragment models generally show a reduced life span, although this is more apparent in the mouse models[160,161,183] (life span of about 3-10 months) compared to the rat model[173] (life span of at least 15 months). The life span of full-length and knock-in models is generally reported to be normal[54]. It should, however, be noted that many characterization studies do not specifically investigate life span, but rather follow the animals until a certain specified age and collect tissue sample at this arbitrarily chosen end point[162-167,174,194-201]. Some authors have specifically noted that there is no apparently reduced survival among knock-in and BACHD mice, up to two years of age[198,202]. Still, a reduced life span has been noted for YAC128 mice[203] (becoming apparent at about one year of age), although it is not generally reported[163,194-197]. Regardless, the current consensus is that life span does not constitute a useful readout for full-length or knock-in models of HD.

Motoric impairments of some form are found in all types of genetic rodent models of HD. It should, however, be noted that the motor tests used for rodents do not necessarily

evaluate impairments that directly relate to symptoms seen in patients. Thus, genetic rodent models commonly show hind limb clasping, meaning that they do not properly stretch out their limbs when being suspended by their tails[160-162,167,174,183,189,202,204] (this can be caused by striatal dysfunction, although other brain regions might also play a part[205]). Another common test where genetic rodent models of HD show impaired performance is the Rotarod test[161,163,164,167,173,174,183,189-190,194,197-200,202-204,206–210]. In this test, animals are trained to walk on a rotating rod where, commonly, the rotation speed gradually increases. The number of times that animals fall off the rod, and/or the latency and rotation speed at their first fall, serves as the main readouts of the test[211]. In addition to these phenotypes, genetic animal models of HD frequently show a disturbed gait when walking[161,162,166,167,174,200,202,209,212–215]. Notably, chorea-like motoric phenotypes are rare, and have so far primarily been found and investigated in the TgHD rats[173,216–218].

Characterization of psychiatric phenotypes found in genetic rodent models of HD has primarily focused on characterization of depression- and anxiety-related phenotypes. Thus, several models have shown indications of anhedonia or depression-like behaviors[197,199,219–225], which would be in line with symptoms found in patients. Reduced locomotor activity has also been found in several models[163,174,198,203,206,208,226–232], although it is unclear to what extent these phenotypes are caused by psychiatric phenotypes (such as apathy) rather than motoric impairments. There are several behavioral protocols for assessing anxiety in rodents[233]. However, research on genetic HD models has primarily focused on tests of exploration anxiety[173,174,190,192,199,200,204,214,220,221,224,226,231,234–238]. Findings from these studies have indicated both increased[199,200,214,220,221,224-226], decreased[173,174,190,192,204,234-237] and unchanged[220,231] anxiety among HD models. Importantly, models with the same kind of genetic modification (i.e fragment, full-length of knock-in) do not necessarily show the same anxiety phenotype. It should also be noted that some HD models have been found to show inconsistent anxiety phenotypes between studies[190,200,221,226,234,236,237].

Cognitive impairments are also frequently present among genetic rodent models of HD. The discovered deficits include impaired motor learning[197,199,207,208,238,239], procedural learning[192,207,240,241], attention[216,212,242–246], spatial learning[247–251], memory retention[207,228,236,237,252–256], reversal learning[195,199,204,207,251], strategy shifting[195,257,258], working memory[173,190,259,260], reaction time[239,243,244], and impaired performance on tasks that are dependent on fronto-striatal circuits[216,218,245,261–264]. In general, it is unclear if models with the same kind of genetic modification show comparable phenotypes, due to the range of different behavioral tests that have been used. Similarly, it is not necessarily possible to give general comments concerning which animal model shows the most severe phenotypes. However, it should be noted that the more slowly progressing full-length and knock-in models are generally better suited for extensive cognitive characterization, as certain behavioral protocols can take weeks or months to complete (which is not always manageable with fragment models due to their short life-span).

The main metabolic phenotypes vary between the genetic rodent models of HD. Fragment mouse models generally show comparable growth to WT mice during early life, but develop a phenotype of progressive weight loss as they deteriorate and approach death[160,161,183,209,220,225,226,232,235,249,258,259,265]. TgHD rats were initially reported to show a stunted growth[173], although later studies indicated largely unchanged growth

and weight[192,266,267]. Inconsistent body weight phenotypes have been found among knock-in models, indicating unchanged weight[201], stunted growth[167,226,268–271], or progressive weight loss at older ages[202,225,236,270–273] (some of these discrepancies are likely due to differences in CAG repeat length and gender among the animals used in the studies). Full-length mouse models, on the other hand, commonly show an increased body weight compared to their WT littermates[163,164,197-199,208,214,220,222,224,226,274–276] (although this is known to depend on the genetic background[208,248]). This appears to be primarily due to an increase in adipose tissue[274,275]. Interestingly, there are indications that fragment mouse models also carry high amounts of adipose tissue both prior to and during early stages of weight loss[277–279]. Thus, an increase in adipose tissue might be a quite general phenotype in genetic rodent models of HD. The weight loss seen among fragment models ultimately includes loss of both adipose and lean mass[278,279].

Behavioral characterization projects with genetic rodent models of HD have to be planned, performed and interpreted carefully due to the wide range of behavioral phenotypes that can be expected. One aspect to consider is that although many behavioral tests are considered to be highly specific, performance frequently relies on several cognitive processes (as an example, see[280] for discussion on spatial navigation, which is often referred to as a hippocampal function, although several processes and brain regions are involved). Another important aspect is that some phenotypes can confound the readouts used for assessing other phenotypes (as an example, see[281] for a discussion on the influence of body weight and body size on Rotarod performance). Thus, behavioral characterization of HD models should utilize suitable control tests in order to ensure valid interpretations (see[198,199,208,220,226,282] for examples concerning better standards for motor characterization in relation to[281]).

The BACHD rat

The project presented in the current thesis focused on characterization of the BACHD rat. Because of this, this animal model will be given a more detailed introduction.

The BACHD rat model is a recently established transgenic rat model of HD[174]. The transgenic construct that was used to create it is identical to the one used in the BACHD mouse[164]. This construct contains the genomic sequence for the entire human huntingtin protein in addition to human huntingtin's endogenous 5' and 3' flanking sequences (about 20 and 50 kilo basepairs respectively). These regions are thought to contain the majority of the endogenous transcription regulatory sequences, and thus drive the expression of mutant huntingtin in the BACHD rodent models. The construct does not contain a pure CAG repeat sequence, but rather a CAG/CAA mixed sequence. Both codons are translated to glutamine during protein synthesis and this genetic setup should not have a major impact on the disease modeling aspects of the rats. However, the CAG/CAA mixed sequence is more stable when inherited, meaning that BACHD rodent colonies are unlikely to have large variations in repeat lengths between generations (which is an aspect to consider in other HD models[160,165,200]). In total, the CAG/CAA sequence of the BACHD construct is 97 repeats long. A final interesting aspect of the construct itself is that exon 1 (which contains the CAG/CAA sequence) is flanked by LoxP sites, enabling it to be turned into a non-HD related huntingtin allele through use of the Cre recombinase. Through this, the specific involvement of individual brain regions in a given phenotype can be investigated.

There are currently twelve publications available on the BACHD rat[174,204,206,283–291]. The work presented here concerns three of those publications[289-291]. As this work was performed largely in parallel to the other published studies, the introduction will focus on the background information that was available at the outset of the current project. This largely encompasses the original publication[174] and some unpublished results. Additional publications of interest will be discussed in the results and discussion section.

Two of the initial BACHD rat founders were kept for further breeding, and used to establish two separate BACHD rat lines[174]. The TG5 line was kept due to its high expression level of mutant huntingtin, while the TG9 line was kept as its expression level of mutant huntingtin was similar to that of the already established BACHD mice. Both lines overexpress mutant huntingtin relative to the endogenous rat hungtintin. TG5 and TG9 rats show approximately 4.5 and 2.5 fold higher expression of the transgene respectively[174].

The transgene is heavily expressed throughout the central nervous system[174,204], although the rats do not appear to suffer from extensive neuronal loss. Still, BACHD rats display dispersed degenerated neurons that are not found among WT rats[174], and there are volumetric changes found in the brain[204]. Specifically, the volume of cerebrum, striatal volume, and thickness of frontal cortex are all reduced among BACHD rats[204]. It should, however, be noted that longitudinal MRI studies have indicated that these volumetric changes are likely related to developmental deficits, rather than progressive neuronal loss (unpublished data). However, loss of D2 receptor binding has been noted among 18 months old rats[174], indicating that progressive neuronal degeneration might occur at advanced ages.

The BACHD rats still display clear and early behavioral changes, despite their discreet neuropathology. Specifically, they show an impaired performance on the Rotarod already at one to two months of age[174,204,206]. More discreet gait impairments, however, appear to be present first when rats are older than a year[174,206]. BACHD rats show reduced anxiety in certain exploration anxiety tests[174,204]. This phenotype is also present at young ages (from about four months and onwards) and becomes more apparent with age[174]. Initial investigation into metabolic aspects indicated that BACHD rats showed unchanged body weight, despite having an obese appearance and consuming less food than WT litter mates[174]. Unpublished results also showed that BACHD rats carried a larger amount of adipose tissues compared to WT rats.

Aims of the current project

The project presented in the current thesis had several aims. These aims, and their backgrounds, are summarized below. Briefly, aims I-III focus on how to work with BACHD rats in food-based behavioral tests, while aim IV focuses on obtaining proof of concept results for the strategies developed under aims I-III.

I.
Confirm previous results concerning metabolic phenotypes of BACHD rats
As described in the introduction, BACHD rats had initially been noted to have similar body weights as their WT littermates, despite having an obese appearance and consuming lower amounts of food[174]. From this initial data our research group formed a hypothesis that BACHD rats were indeed obese but that presence of other phenotypes ultimately resulted in unchanged body weights. To evaluate this, we performed detailed dissections on rats of several different ages in order to obtain measurements of the rats' body composition. In addition, food and water consumption was evaluated as initial data was obtained through an automated homecage system with debatable validity[292]. The results from this are summarized in Publication I and Appendix I.

II.
Evaluate if metabolic phenotypes might confound results from food-based operant conditioning protocols
As described above, some phenotypes might confound proper measurement of other phenotypes. An overarching aim in our research group was to assess BACHD rats' performance in several tests that evaluated cognitive functions, all of which were based on training rats to perform tasks in exchange for food rewards. However, if BACHD rats indeed were obese and consumed less food than WT rats, they might also be less motivated to perform food-based behavioral tests. Such motivational differences might result in profound differences in behavior, which could be misinterpreted as cognitive phenotypes[293]. To evaluate whether BACHD and WT rats were differently motivated to perform food-based behavioral tests we investigated their performance in a progressive ratio protocol for Skinner boxes. The results from this are summarized in Publication I and II.

III.
Evaluate strategies for circumventing motivational differences between BACHD and WT rats
If WT and BACHD rats were indeed differently motivated to perform the progressive ratio test, one could reasonably assume that similar motivational differences would be present in other tests of cognitive function. As noted, such motivational differences can result in behavioral changes, which might in turn be misinterpreted as cognitive phenotypes[293]. Because of this, a general strategy of how to handle motivational differences would be needed in order to ensure that valid behavioral characterization of the BACHD rats could still be performed. One such strategy would be to work with unconventional food restriction protocols in order to better match the hunger and motivation among WT and BACHD rats. Further tests with the progressive ratio protocol were performed to evaluate whether or not this approach was suitable. The results from this are summarized in Publication I and II.

IV.

Use the developed strategies to obtain valid behavioral characterization data of BACHD rats

As noted, a major aim for our research group was to assess BACHD rats' performance in several food-based tests of cognitive function. Thus, once a strategy for how to work with BACHD rats in these tests had been developed (through completing the aims described above), the aim was to continue with the main characterization. Although several behavioral protocols were initially included in this characterization, only results from the short-term memory and inhibitory control protocols will be considered here. The results from this are summarized in Publication III, Appendix II and Appendix III.

Ethical statement

All tests of the current thesis that involved animals were approved by the local ethics committee (Regierungspraesidium Tuebingen) and carried out in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU). All procedures were carried out by persons with appropriate training in order to minimize stress and suffering among animals.

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Further details regarding funding are described in the individual publications.

Results and discussion

I.
Male BACHD rats are obese, consume less food and water than WT rats, and show signs of discreet developmental deficits

Current findings
As described, initial characterization of the male BACHD rats noted that they appeared to be obese, while showing unchanged body weight and decreased food consumption[174]. These results were further investigated in both unpublished dissection studies performed at our institute, as well as the dissection study presented in Publication I of the current thesis.

In short, our results have indicated that male BACHD rats of the line TG5 are obese and suffer from discreet developmental deficits. These phenotypes counteract each other and frequently result in the transgenic rats showing comparable body weights to their WT littermates (Publication I). It should, however, be noted that male BACHD rats are on occasion found to be lighter than their WT littermates, while we have so far not encountered litters where the opposite phenotype is present (unpublished results). It thus appears as if the developmental deficits are on occasion more apparent than the obesity. The developmental deficits appear to primarily result in an overall growth impairment among male BACHD rats, as evident from their smaller body sizes, reduced muscle and bone mass, as well as generally smaller organs (see Appendix I). However, certain tissues appear to be particularly affected, as their reduced weight was disproportionate to the BACHD rats' smaller body size. This concerned the BACHD rats' brain, kidneys, muscle tissues, pancreas and testicles. Finally, it should be noted that while BACHD males appeared to consume more food than their WT littermates until the age of about three months, the opposite phenotype was present at older ages.

Connection to other HD models, and HD patients
Genetic animal models of HD differ in terms of body weight phenotypes. In general, fragment models[160,161,183,209,220,225,226,232,235,249,258,259,265] and knock-in models[202,225,236,270-273] appear to show phenotypes of progressive weight loss while full-length models show stably increased body weights[163,164,197-199,208,214,220,222,224,226,274-276]. Further, unchanged body weights have been reported for the fragment rat model of HD[192,266,267], and in some studies of knock-in mice[201]. HD patients are primarily thought to gradually lose weight[118-122], which relates well to findings in fragment and knock-in models. It should, however, be noted that a recent study of HD patients failed to reveal a progressive weight loss, although lower body weights and a clear deficit in reliably gaining weight was found[130]. Notably, the BACHD rats have so far primarily been found to display unchanged body weights, which is similar to the fragment rat model, and some reports on the knock-in mice. However, a clear outcome from our initial study of the BACHD rats is that body weight is a quite limited parameter to work with, as it does not necessarily reflect differences in body composition.

The BACHD rats were found to carry an increased amount of adipose tissue compared to their WT littermates. Similar phenotypes have been found in full-length mouse

models[274,275], but also among fragment[277-279] and knock-in[277] mouse models, during ages where clear weight loss is not yet present. In line with this, a recent study of body composition in HD patients found a trend suggesting that premanifest patients have increased amount of subcutaneous fat[294]. Thus, the increase in adipose tissue might be a quite general component in both animal models and HD patients, at least during initial stages of the disease.

As noted, fragment and knock-in mouse models, as well as HD patients eventually develop weight loss symptoms, which is thought to be due to a combined loss of adipose and muscle tissues[120,121,123,278,279,295]. Full-length models on the other hand maintain a stably increased body weight, which is in line with them carrying an increased amount of adipose tissues, while having unchanged lean mass[275]. Thus, overt muscle atrophy does not appear to be a phenotype among full-length mouse models. In contrast, male BACHD rats were found to show a specific developmental deficit, resulting in disproportionally lower muscle mass (Publication I). Although this phenotype is not in line with the general idea of HD patients suffering from progressive muscle atrophy, it should be noted that the presence and development of this symptom has been questioned[124]. Specifically, while there are several indications of functional abnormalities in myocytes[296–300], the studies indicating muscle atrophy are generally old (i.e. published before the identification of the disease-causing gene) and/or used low-tech measurements[119,120,295] (i.e. arm circumferences). Thus, it is unclear how the BACHD rat phenotype relates to patient symptoms.

BACHD rats also showed specific malformation of brain, testicles, kidneys and pancreas (Appendix I). Specific investigation of brain development has, to our knowledge, not been extensively studied in either HD patients or animal models. Still, studies focused on the function of huntingtin have indicated a role during brain development[301,302], although mutant hungtintin appears to retain this developmental function[301]. Smaller testicles have been found among YAC128 mice[203,275,303], R6/2 mice[304,305] and HD patients[303]. The phenotype appears to be due to a progressive degeneration[303,304]. Although testicle size is also affected in BACHD rats, it should once again be emphasized that the current phenotype appears to concern an impaired growth rather than a progressive degeneration. Renal function or pathology has to our knowledge not been extensively investigated in HD, although kidney weight has been reported to be unchanged in YAC128 mice[275], and to progressively deteriorate in R6/2 mice[304]. Pancreas function has been investigated in greater details, although the main emphasis appears to be on its endocrine rather than exocrine function[306]. Thus, insulin secretion appears to be impaired in both R6/2 mice[307] and HD patients[308]. In line with this, diabetes mellitus has been reported to be more prevalent among both HD patients[309] and R6/2 mice[307,310] compared to their respective controls. Still, the findings among patients are debated[311,312]. Finally, it should be noted that despite the apparent pancreatic pathology, we have found BACHD rats to show normal glucose tolerance in resting and challenged states (unpublished data).

In addition to specific pathologies of certain tissues, the BACHD rats showed an overall growth deficit (Publication I). Similar phenotypes have been noted for knock-in mice[167] as well as the fragment rat model of HD[173], and are not surprising given that huntingtin appears to be important for embryonic development[313,314]. In line with this, discreet developmental deficits have been found among HD patients as well[315,316].

BACHD rats appear to consume more food than their WT littermates until the age of about three months (Publication I). At older ages, they have consistently been found to consume less food and water than their WT littermates (Publication I, ref. 174). Notably, this is in contrast to their obesity, although the eventually reduced food consumption might be in line with their overall smaller body size. Other HD models show different food consumption phenotypes, with BACHD mice[274] and fragment rats[266] consuming more food than their WT littermates, while others have been found to show largely unchanged food consumption[236,275,278].

It should be noted that the current results only concern male BACHD rats, and that detailed analysis of the same parameters in female BACHD rats has not been performed yet. However, we have in several cohorts found that BACHD females are heavier than their WT littermates, suggesting a slightly different phenotype from the transgenic males (unpublished data). Interestingly, the body fat and body weight phenotype of BACHD mice also appears to be more apparent among females[274].

Possible mechanisms
As increased food consumption does not explain the obesity among BACHD rats, the cause for this phenotype could be reduced home cage activity, or an underlying metabolic condition. Home cage and exploratory activity has been investigated in the BACHD rats, although no conclusive phenotype has been found[174,204,206]. Still, the results do not at this point appear to indicate that BACHD rats show strongly reduced activity in their home cages, suggesting that the likely cause of obesity in BACHD rats is an underlying hypometabolic state. The hypothalamus is known to be important for systemic metabolism[317–319] and hypothalamic pathologies have been suggested to play a role in metabolic symptoms of HD[320–323]. In addition, the obesity phenotype of BACHD mice appears to be dependent on expression of mutant huntingtin in specific hypothalamic neuronal populations[274,324–326]. Although the BACHD mice can be argued to show largely different metabolic symptoms than HD patients (see above), there are indications that they might suffer from dysfunction of overlapping brain regions[322,323,326]. Thus, it is also possible that the BACHD rats' metabolic phenotypes are caused by hypothalamic impairments. Interestingly, lesions to the arcuate nucleus of the hypothalamus have been found to result in obesity despite a reduced or unchanged food intake[327–330]. This specific nucleus is important for several metabolic processes, including the systemic release of growth hormone and central governing of hunger and satiety[331–333]. Interestingly, reduced growth hormone levels, and blocking of specific receptors of arcuate nucleus derived signaling molecules, result in animals displaying growth deficits, unchanged or reduced food intake, and obesity[334–339].

In addition to its direct peripheral effects, growth hormone stimulates the release of insulin-like growth factor 1 (IGF-1) from the liver[340]. These two factors play major roles in growth and postnatal development[341–345]. Interestingly, male BACHD rats have been found to show reduced IGF-1 levels (unpublished data), which suggest a reduced level of

growth hormone. As noted, a growth hormone deficit could explain their physiological and metabolic phenotypes. Importantly, other studies have also indicated connections between HD and growth hormone. Studies on the YAC mouse model have indicated that full-length huntingtin induces growth of specific tissues through increased IGF-1 levels. [275,276]. Other studies have repeatedly shown increased levels of growth hormone in HD patients[346,347]. This might be an important component to their apparent hypermetabolic state, as growth hormone is known to induce lipolysis[340].

Summary and Outlook

The BACHD rats show a range of physiological phenotypes that are to some degree comparable to phenotypes in other models, and symptoms in HD patients. As noted, pathology in the arcuate nucleus of the hypothalamus might be central in the BACHD rats' phenotypes. As histological methods have not revealed extensive neuronal loss in the BACHD rats, specific immunohistochemistry of the arcuate nucleus is unlikely to offer conclusive results regarding its involvement in the current phenotypes. A more suitable approach would be to inactivate the expression of mutant huntingtin within the hypothalamus and arcuate nucleus, through the use of Cre-expressing viruses. If the resulting rats show normalized physiology, it would suggest that the hypothalamus is central to the phenotypes. If so, further investigations should put specific emphasis on the growth hormone/IGF-1 axis.

II.
BACHD rats are less motivated than WT rats in the progressive ratio test, although the nature of the phenotype is unclear

Current findings
Before starting a large-scale behavioral characterization project we sought to investigate if BACHD and WT rats were differently motivated to perform a simple food-reinforced test. The main aim was to better understand how to efficiently work with the rats, considering the presence of their metabolic phenotype. The results from our work on this topic are presented in detail in Publication I and II of this thesis.

In short, we followed a group of male BACHD rats from line TG5, and assessed their performance in a progressive ratio (PR) protocol for Skinner boxes (see Figure 5) at four different ages. In this protocol, rats are taught that pushing a lever results in the delivery of a small food pellet. During each daily session, the number of pushes required for food pellet delivery is initially low, but gradually increases as the rats obtain rewards. Because of this, the rats will eventually lose interest in performing lever pushes, and will take gradually longer breaks from doing it. These breaks serve as the main readout of the test. Thus, rats that take breaks of a given duration at an earlier point in the progression of required lever pushes (i.e. after obtaining fewer rewards) are considered to be less motivated than rats that performs breaks of the same duration at later points in the progression.

We consistently found that BACHD rats were less motivated than their WT littermates to perform the PR test (Publication I). The phenotype did not noticeably change with age, and was not dependent on BACHD rats becoming satiated or fatigued (Publication II). It was, however, clearly dependent on which food restriction strategy that was used. Apart from the lower motivation, BACHD rats were also found to have an increased tendency to perform perseverative lever responses (unnecessary lever responses performed after pellet delivery) and were slower at retrieving the reward pellets (Publication II). Importantly, these phenotypes were not dependent on which type of food restriction strategy that was used.

Connection to other HD models, and HD patients
The phenotype of reduced motivation is interesting on its own, as it can be argued to constitute an apathy- or depression-related phenotype. As noted, apathy and depression are common psychiatric symptoms among HD patients[63-68,144-147]. Reduced motivation to perform the progressive ratio test has also been found in BACHD[348] and knock-in[245] mouse models of HD. Although similar trends have been seen for TgHD rats[349], these findings are not consistent[267]. Still, it is interesting to note that TgHD rats have also been found to be slower at retrieving the reward pellets[349].

Possible mechanisms
The neurobiological basis of PR performance has not been fully elucidated, and several brain regions appear to be involved in governing the rats' behavior. In this regard, lesions to the hippocampus[350], subthalamic nucleus[351] and ventral striatum[352] have been found to increase motivation. Interestingly, lesions to the dorsal striatum (i.e. the region most heavily degenerated in HD) do not appear to have overt effects on motivation, although lesioned rats were found to perform an increased number of perseverative

lever responses[353]. Specific lesions to the dorsolateral striatum have in addition been shown to result in increased latency to retrieve reward pellets[353]. More careful manipulations of specific signaling pathways have offered additional insights, indicating that dopamine signaling in both dorsal[354–357] and ventral striatum [357–359] are important for maintaining proper motivation on the progressive ratio test.

As noted, a reduced motivation could also be argued to be a depression-like phenotype. However, the influence of serotonin on progressive ratio performance is somewhat unclear. A general increase[360] or decrease[361] in signaling has been found to result in reduced and increased motivation respectively (although it has been suggested to be due to changes in general activity[360]). Thus, reduced motivation on the PR test does rather not connect to a lower serotonin level (i.e. the expected depression-related change of serotonin). Still, specific serotonin receptor agonists within the dorsal striatum have been found to increase motivation, suggesting that there might be some connection[362].

All in all, it is possible that all phenotypes found among BACHD rats' performance in the progressive ratio test are caused by striatal impairments. This is further supported by the fact that similar phenotypes have been found in other animal models of HD (see above).

However, the metabolic phenotypes described in the previous section constitute potential confounding factors for the PR test, and also have to be addressed. First, obesity has been found to result in reduced availability of D2 receptors in the stiatum[363]. Second, adipose tissue has certain secretory functions, and is in particular secreting the protein leptin[364,365]. Leptin secretion increases with adipose mass, and acts on neurons within the hypothalamus to reduce food intake, and govern metabolism[364,365]. Leptin receptors are also available at several additional sites of the central nervous system[366,367], and apart from its function in hypothalamic control of metabolism it has been implicated as an important modulator of neuron excitability in the hippocampus, central reward circuits and mood regulation[368–374]. In line with this, changes in leptin signaling have been found to have an effect on PR performance[375–378]. Specifically, acute administration of leptin has been found to reduce motivation[375-377], while knock-down of leptin receptors has been found to increase motivation[378]. BACHD rats have, in line with their obesity, been found to have higher serum leptin levels compared to their WT littermates (unpublished data, Publication II). Thus, their lower motivation on the PR test could also be explained by their metabolic phenotypes. We further evaluated this by adding additional control tests to our study.

Most protocols for food-based cognitive tests use food restriction in order to ensure that rats are motivated to perform the test at hand. For long-term Skinner box-based tests, it is common to restrict daily food intake to a point where animals weigh 80-95% of their free-feeding body weight. For our initial tests we maintained all rats at 85%. However, this would likely mean that BACHD rats still have higher serum levels of leptin than WT rats, as they have a higher fat mass at baseline (this was confirmed by data gathered and displayed in Publication II). Thus, a difference in leptin levels and motivation might still be present despite the food restriction. A possible method for dealing with this has been used when working with BACHD mice[264,348], and was also suggested to us in open

discussions during HD conferences. The basic idea is to first investigate if BACHD rats appear to be less hungry than their WT littermates, by measuring the amount of food they consume during a brief moment of free access. Several studies have used similar tests as control test for PR performance[267,379–381]. It was then suggested that in case there is a difference between WT and BACHD rats' food consumption rates, the food restriction should be adjusted until this difference is no longer present. Thus, the food restriction would be based on matching the apparent hunger of WT and BACHD rats, rather than their relative body weights. During our studies we have repeatedly found that BACHD rats consume less food than WT rats in these brief control tests, when both genotypes are food restricted to 85% of their free-feeding body weights (Publication I and II). Adjusting the food restriction so that WT and BACHD rats display similar food consumption rates (i.e. apparent hunger) reliably resulted in the two genotypes showing equal motivation in the PR test. However, the food restriction adjustment did not affect the higher tendency of performing perseverative lever pushes, or the longer pellet retrieval latencies seen among BACHD rats (Publication I and II). Thus, BACHD rats displayed a set of phenotypes comparable to rats with lesions to the dorsolateral striatum when maintained on the alternative food restriction protocol[353].

Ultimately, while the reduced motivation seen among BACHD rats could have been the consequence of HD-related neuropathology of the striatum (as discussed above), our control tests indicated that it might also be related to their metabolic phenotypes. In contrast, the more discreet phenotypes concerning perseverative lever pushes and pellet retrieval are likely unrelated to motivational or metabolic phenotypes. Still, these control tests were not without their own shortcomings. First, the food restriction adjustment did not fully resolve the difference in serum leptin levels (Publication II). Second, the control tests were based on consumption speed, and it is possible that the BACHD rats' lower food consumption rate was caused by motoric impairments, rather than a difference in hunger. The rats' food consumption behavior was investigated in further detail, and we did indeed find indications that non-hunger related factors likely influenced the BACHD rats' lower consumption rates (Publication II), although the exact nature of this deficit remains uncertain. It should also be noted that these impairments were very discreet, and it is unclear if they in the end had any significant effect on the consumption rate in the control tests (discussed in detail in Publication II).

Finally, it is interesting to note that animal models of obesity have shown both increased[382–388], decreased[389–391] and unchanged[392] motivation in the PR test. The exact reason for this discrepancy in motivation phenotypes is not clear, although it is likely based on differences concerning the cause of their obesity phenotype, and the specific set of metabolic impairments that follow (as an example, models that display hyperphagia frequently show increased motivation in the PR test[382-386]). Thus, like most other behaviors, motivation to work for a food reward is governed by several processes. In order to understand the exact nature of the BACHD rats' motivational phenotype, and the potential involvement of metabolic phenotypes, more extensive investigation of the rats' metabolic phenotypes are needed.

Summary and Outlook

The BACHD rats consistently showed a reduced motivation in the PR test, although the phenotype was dependent on the specific food restriction protocol being used. In addition, the rats displayed an increased tendency to perform perseverative lever

responses, and slowed pellet retrieval latencies. These phenotypes were, in contrast to the motivational phenotype, independent of the food restriction protocol. The latter phenotype has been present in almost all Skinner box based tests performed at our institute (a total of nine tests, each using a 12 vs 12 setup for WT and BACHD rats). The exact nature of the BACHD rats' motivational phenotype is still unclear and might involve both hypothalamus-driven metabolic phenotypes, and striatum-driven psychiatric phenotypes. The other noted phenotypes are, however, unlikely to be related to metabolic phenotypes and suggest that striatal dysfunction is present among BACHD rats. Further studies are, however, necessary to better investigate the underlying neuropathology. It is unlikely that histology-based investigations will be of great benefit, due to the discreet neuropathology among BACHD rats. Thus, a more suitable approach would be to inactive the expression of mutant huntingtin within selected brain regions, to investigate how the PR phenotypes are affected. These studies should be conducted together with investigations of the BACHD rats' metabolic phenotypes. Specifically, if switching off the expression of mutant huntingtin in the hypothalamus is found to resolve the BACHD rats' metabolic phenotypes (as discussed above), the resulting lean rats should be assessed in the PR test. Subsequent inactivation of mutant huntingtin expression in the dorsolateral striatum should be performed if some or all of the noted PR phenotypes persist among the lean rats.

III.
The optimal strategy for working with BACHD rats in food-based tests concerns the use of appropriate control tests rather than unconventional food restriction protocols

Background
As discussed above, there is a consistent phenotype indicating that BACHD rats are less motivated than WT rats in the progressive ratio test when a standard food restriction protocol is used. This phenotype constitutes a potential problem for further behavioral characterization of the BACHD rats, regardless of whether it is caused by psychiatric or metabolic disturbances. Specifically, based on the current results one should expect BACHD rats to be less motivated than WT rats to perform food-based tasks in general. Although other protocols might involve less physical effort than the PR test, there would always be the chance of discreet motivational differences being present. Because of this, results from food-based tests should be interpreted carefully, as motivational differences can have strong influences on readouts such as success rate and choice of strategy[293]. However, the exact influence that motivational differences might have on a given test's readouts is often not known.

When we interpreted our initial findings we argued that the alternative food restriction protocol (where apparent hunger, rather than relative body weight, was matched for WT and BACHD rats) could be used for reliably achieving an experiment setting where the motivational difference between WT and BACHD rats was minimal. Such a setting would likely improve the overall validity of our characterization work, as it would minimize motivation-based artifacts in our data. This idea was published in Publication I. However, as we later found that BACHD rats suffered from an underlying phenotype of impaired food consumption, this idea was abandoned. Thus, the ideas concerning how to optimally work with the BACHD rats in food-based tests were revised, and the more recent ideas were published in Publication II.

Suggested work strategy
As neither the standard or alternative food restriction strategy is entirely optimal, all characterization tests of the BACHD rats that use food-based protocols should include appropriate control tests to evaluate which readouts are affected by differences and changes in motivation.

The suggested approach includes an initial behavioral evaluation using a standard food restriction protocol (i.e. with both WT and BACHD rats restricted to 85% of their free-feeding body weight, meaning that a motivational difference is likely present). This is followed by a step where the daily amount of food given to WT rats is increased, so that they reach roughly 95% of their free-feeding body weight. By comparing the WT rats' performance on the two baselines one should be able to evaluate which readouts from the given test that are affected by a shift in motivation. BACHD rat phenotypes that are based on readouts that are sensitive to motivational shifts should be deemed less reliable than phenotypes in readouts that are not sensitive to motivational shifts. Taking the current results from progressive ratio performance as an example, the number of perseverative lever pushes and the latency to retrieve reward pellets likely constitute actual cognitive and/or motoric impairments, as the parameters were not sensitive to a motivational shift.

The strategy described above is primarily suited for tests where baseline performance, rather than training and learning, is being evaluated. For the latter it would be necessary to include separate control groups with different motivation levels (i.e. restriction levels), that all go through the same learning process. An alternative approach could be to maintain WT and BACHD rats on different restriction levels (95% and 85% respectively) and after evaluating their performance in the given test of interest assess their motivation in a PR test.

The remaining two sections of current results deal directly with detailed behavioral characterization of the BACHD rats. Both projects included some evaluation of the possible influence of motivational differences, using strategies and control tests discussed here.

IV.
BACHD rats show indications of impaired fronto-striatal function in two tests of short-term memory

Current findings

Once a reasonable method for working with the BACHD rats had been established, we aimed to assess the rats' performance in a series of cognitive tests. Two of these were commonly used protocols for assessing short-term memory in rodents, called delayed alternation and delayed non-matching to position. As noted in the introduction, HD patients are thought to largely retain the temporal aspects of their working memory[97,99]. However, general performance in the delayed alternation and non-matching tests are known to be dependent on fronto-striatal circuits[393–401], and thus they are still suitable for evaluating the presence of HD-related neuropathology.

Both test protocols used Skinner box setups, where an interactive wall was set up with one centrally placed food pellet receptacle and two retractable levers (one on each side of the pellet receptacle) (Figure 5). The two protocols followed similar structures, with sessions split into discrete trials where the levers were inserted and available to the rats, separated by inter-trial intervals where levers were retracted. In the delayed alternation test, all trials contained a single step where both levers were inserted and the rats were allowed to make one response. On the first trial of each session, the rats were rewarded for pushing either lever. On all subsequent trials, the rats were rewarded for responding to the lever they did not respond to on the previous trial, forcing them to alternate their responses on the two levers. Once the rats were performing at a high success rate, delays were added in order to vary the trials' temporal spacing in a structured manner. Through this, the rats' short-term memory was assessed. In the delayed non-matching to position test, each trial consisted of two steps. During the first step, only one of the levers was inserted. Once the rats had responded to it, it retracted, without a reward being delivered. During the second step, both levers were inserted. The rats were rewarded for responding to the lever that was not presented to them during the first step of the trial. Once the rats were performing at a high success rate, delays of structurally varied durations were added between the two steps in order to assess the rats' short-term memory function. Both behavioral protocols were designed so that rats were triggered to perform repeated head entries into the pellet receptacle during delays.

BACHD rats showed impaired performance in both tests (Publication III). The phenotypes were present at early ages (2-4 months), but did not appear to progressively worsen as the rats grew older (three additional ages were assessed, with the oldest age being 17-19 months). BACHD rats were found to have difficulties learning the basic alternation behavior, while learning the non-matching was unimpaired. During the delayed alternation test, BACHD rats continued to show indications of having problems with the general alternation task, while their short-term memory appeared to be intact. Despite being unimpaired when performing the basic non-matching task, BACHD rats showed a discreet general impairment during the delayed non-matching task. Although the deficit was dependent on the presence of delays, it did not worsen with longer delays, indicating once again that BACHD rats had intact short-term memory. Performance in both tests was evaluated using both a standard food restriction protocol, and a control setting where the WT rats' restriction level had been adjusted to 95%.

None of the phenotypes were sensitive to this motivational shift, indicating that they were likely unrelated to metabolic and/or motivational changes among BACHD rats.

To further investigate the behavioral basis of the phenotypes, video recordings of the rats' performances were made, and scored manually. These indicated that rats used certain strategic movements during the delays of both tests, to aid their responses. The use of these strategies was especially pronounced in the non-matching test. However, there were no clear differences between WT and BACHD rats in this regard. The videos further revealed that rats of both genotypes displayed specific behaviors when they had to make a decision regarding which lever to push (i.e. at the end of the delay, when both levers were inserted again). In the delayed non-matching test, almost all responses were made without hesitation or apparent interest in the other lever. This was most likely connected to the rats having well-developed strategies for remembering which lever to push. In contrast, the rats in the delayed alternation test frequently showed a correction behavior. During this, the rats would initially move towards one lever, but abruptly change their focus and ultimately respond to the other one. This was considered a correction behavior as it resulted in a correct response in over 90% of the cases. Interestingly, WT rats showed this behavior more frequently than BACHD rats. Further, investigation of hypothetical data where all rats responded according to their initial lever focus indicated no performance difference between the genotypes. Thus, although the behavioral basis for the BACHD rats' impairment in the delayed non-matching test remains unclear, the cause of their general alternation deficit was likely related to a reduced ability to inhibit ongoing erroneous motor responses. This could in turn be related to impairments in various sub processes, such as impaired attention (i.e. failure to realize that they are about to make a mistake), or more directly impaired motor inhibition (i.e failure to inhibit a response, once the rat has realizes that it is erroneous). As described in detail in the publication, there were additional behavioral changes among BACHD rats, although their contribution to the overall impairment was likely small.

Connection to other HD models, and HD patients

An overall reduced performance, without indications of impaired short-term memory, has previously been seen for knock-in mouse models of HD in both the delayed alternation and the non-matching tests[262,263]. No detailed investigation of the exact behavioral basis of these phenotypes was made. Delayed alternation performance has also been assessed in the TgHD rats, although they were found to not be impaired[402].

The two specific test protocols applied here are rarely used in studies on humans, due to their simplicity. However, HD patients have been assessed in the arguably similar pattern matching to sample test[97]. In this test, patients are first shown an abstract pattern and are then asked to select it from a set of four patterns, displayed after a certain delay. Similar to the current results, HD patients appeared to be generally impaired in the pattern-matching task, while showing intact short-term memory[97].

The ability to stop an initiated motor response can be specifically assessed with stop-signal tests[403–407]. Briefly, test subjects are first trained to perform a specific movement (or series of movements) during a set of training trials. Afterwards, their ability to stop the movement is assessed by presenting a stop signal, while the movement is being

performed. The test usually involves presenting the stop signal during both early and late phases of the movement, to estimate the test subject's reaction time. Interestingly, there are indications that HD patients are impaired in such tests, suggesting a similar pathology as the one found for the BACHD rats[108].

Possible mechanisms

Performance in the delayed alternation and delayed non-matching to position test have been found to be sensitive to lesions of several brain structures, including the prefrontal cortex, striatum and hippocampus[393–401]. Lesions have been found to result in both delay-dependent and delay-independent deficits, likely depending on the extent of the lesions and the specific neuron populations that are affected. However, the exact neuronal circuits responsible for optimal performance in the tests are not clear. More detailed findings have been made on stop-signal protocols adapted for rodents, where performance appears to be governed by circuits involving the orbitofrontal cortex, dorsomedial striatum and subthalamic nucleus[403-407]. Thus, the BACHD rats' performance deficit in the delayed alternation test might stem from pathology in these brain regions.

As BACHD rats were found to be specifically impaired on delayed non-matching to position trials, rather than the basic non-matching task, we hypothesized that the behavioral basis of their impairment would relate to a specific change in their behavior during the delay steps. As noted, both WT and BACHD rats used specific body movements during the delays, which clearly indicated which lever they intended to respond to. Successful trials were generally connected to rats maintaining a strong focus on the correct lever. Only limited research has been made regarding mediating behaviors and strategies used by rats in the delayed alternation and non-matching tests. However, one study described similar behaviors as the ones discussed here for the delayed non-matching to position test[408]. The authors further noted that an overall impaired performance was connected to the rats having an increased frequency of changing their focus from one lever to another during the delays. Such a behavioral change could explain the generally reduced success rate among BACHD rats, although it was not apparent in the video scoring results. It is, however, possible that the current analysis simply failed to reveal it due to the limited data available and the subtle success rate phenotype.

Summary and Outlook

The BACHD rats showed impaired performance in both the delayed alternation and the delayed non-matching to position tests. Neither deficit was affected by a shift in motivational state among WT rats, suggesting that the phenotypes were caused by cognitive differences, rather than motivational. The impaired alternation behavior among BACHD rats appeared to be caused by an inability to stop ongoing motor responses, which is similar to symptoms found in HD patients. The behavioral basis of the impairment seen in the delayed non-matching to position test is still unclear, although it likely differs from the delayed alternation impairment.

Further work should focus on the delayed alternation phenotype, as that was the more apparent one. Inactivation studies should be performed to evaluate if expression of mutant huntingtin in the orbitofrontal cortex, dorsomedial striatum and subthalamic

nucleus might be causing the BACHD rats' impaired performance. In addition, the rats' performance in an actual stop-signal test should be assessed.

V.
BACHD rats show impaired response inhibition in specific situations

Current findings
Response inhibition can be divided into two separate processes, withholding responses and stopping already initiated responses[405,409]. These aspects can be assessed with different Skinner box-based behavioral protocols[405,410–420]. The ability to withhold responses can be evaluated in different versions of differential reinforcements of low-rates of responding (DRL) protocols[410-415], and Go/No-Go protocols[264,416-418]. As noted above, the ability to stop initiated motor responses is commonly assessed in stop signal tests[403-406,419,420].

In DRL protocols the Skinner boxes are typically set up with one lever being inserted and available to the rat during the full test session[410-415]. At the start of the session, pushing the lever once will result in a reward pellet being delivered. Afterwards, the lever is inactive for a predetermined and fixed length of time. Pushing the lever during this inactive phase does not result in the delivery of a reward pellet. Instead, it restarts the timer for the inactive phase. Thus, in order for the lever to once again be active, the rats have to withhold lever responses for the full duration of the inactive phase. Notably, the active/inactive status of the lever is generally not signaled to the rat, meaning that they have to rely on their internal time-assessment abilities[410-415]. We have evaluated the BACHD rats' performance in different DRL protocols (Appendix III). Our initial study used a protocol where the lever's active status was indicated with a cue light, and the inactive phase was set to five seconds. The BACHD rats showed a stable impaired performance in the test, suggesting that they had general problems withholding lever responses. However, the rats had been given extensive training on a continuous reinforcement protocol (where each lever push results in pellet delivery) prior to DRL training. Thus, it was unclear if the BACHD rats' impaired performance represented a difficulty in strategy adjustment, rather than a general inhibitory control deficit. In a follow-up study we gave rats only a brief training on the continuous reinforcement protocol (some training is necessary as a part of shaping the basic lever response), before presenting them with a DRL protocol that used cue lights and inactive phases of varied duration. Interestingly, the BACHD rats showed comparable performance to WT rats, with very high success rates, despite the inactive phases being up to 20 seconds long. Thus, BACHD rats did not appear to have general problems to withhold lever responses, although such phenotypes might become apparent when rats need to apply inhibitory control to situations that have previously not required it.

The results from our DRL tests are largely in line with results from a Go/No-Go test that was performed in parallel (Appendix II). Go/No-Go tests also evaluate the rats' ability to withhold lever responses, but through a slightly different approach. In general, Go/No-Go protocols are based on training animals to discriminate between two different cues[416] or conditions[264,415,417,418]. These indicate if responses will be rewarded (Go condition/cue) or not (No-Go condition/cue). Protocols differ in terms of their specific trial structure, and whether withholding responses during No-Go condition/cues is reinforced[415,416] or not[264,417,418]. In addition, the protocols generally switch between Go and No-Go conditions regardless of the rats' responses[264,416-418]. We assessed the BACHD rats' performance in a symmetrically reinforced Go/No-Go test (Appendix II), using the same Skinner box setup as described for the short-term memory tests. The test sessions

were divided into several separate trials. The response levers remained retracted and unavailable to the rats between trials. Each trial followed a similar structure, where one of two light cues was first presented for five seconds. This was followed by the insertion of one lever. One of the cues was used to signal Go trials, meaning that the rats needed to respond to the lever in order to obtain a food reward. The other cue was used to signal No-Go trials, meaning that the rats needed to withhold a lever response in order to obtain a food reward. Time limits were set during the lever presentation phase, so that rats had to perform a lever response within a certain amount of time, or withhold responses for a certain amount of time, in order to obtain rewards. The rats were first given brief training aimed at shaping a reliable response on Go trials. Afterwards, sessions contained an equal number of Go and No-Go trials. Due to their initial training, rats frequently responded to the lever during No-Go trials. In order to facilitate association of the No-Go cue with the possibility of getting a pellet reward if no response was performed, the duration of No-Go trials was initially kept short. Through this, the rats would frequently not manage to respond to the lever before the trial was over and were thus presented with several accidental successes. This still only resulted in a success rate of roughly chance level, and rats had to learn to discriminate the two cues and respond accordingly to reach higher success rates. Once the rats achieved this, they progressed trough a series of protocols where the duration of No-Go trials gradually increased. Our findings indicated that BACHD rats were reliably impaired during the initial stages of this training, where they had to learn to discriminate the two light cues and respond accordingly. The impairment was primarily evident as a failure to withhold responses during No-Go trials. However, once they had learned to do this, they appeared to be unimpaired when forced to withhold responses for longer periods of time.

The control test used in the progressive ratio, delayed alternation and delayed non-matching to position tests (i.e. assessing WT rats' performance on two different food restriction levels) was not suitable to evaluate the influence of motivation on the Go/No-Go test's readouts. This was primarily due to the former tests focusing on baseline behaviors, while the latter focused on learning. Instead, WT and BACHD rats were maintained on different restriction levels from the outset of the test (95% and 85% respectively). The rats were assessed in a progressive ratio test at the end of the study, in order to evaluate if this had indeed avoided motivational differences. The results of this indicated that WT and BACHD rats were equally motivated to work for a food reward (Appendix II).

Ultimately, our results indicated that BACHD rats are not generally impaired concerning withholding lever responses, although they appear to have some deficits in applying inhibitory control to settings that previously did not require any. The exact point where this becomes apparent appears to differ between protocols (i.e. reliably present at the outset of the Go/No-Go test, but for DRL tests only when extensive lever training has been given), which likely relates to differences in where the main inhibitory challenge lies in the given protocols. Notably, the transient nature of this phenotype is different from the BACHD rats' likely impairment in inhibiting ongoing motor responses (as discussed for the delayed alternation test), as that phenotype appears to be a stable baseline phenotype rather than a learning impairment.

Connection to other HD models, and HD patients
Only limited amounts of work has been performed on inhibitory control and HD animal models. Still, one study found subtle impairments in a Go/No-Go protocol among knock-in and fragment mouse models, while the performance of BACHD mice was unimpaired[264]. The particular protocol used in that study was, however, not directly comparable to the one used in our study. HD patients have been found to show impaired abilities to withhold responses on a Go/No-Go protocol more similar to the one used by us[109]. However, only a brief test was performed in that study, and it is unclear if patients could have reached normal accuracy if given enough training.

Others have assessed the BACHD rats in a series of inhibitory control tests, including the DRL test[287,288]. Their study used a more classical DRL protocol, with a fixed inactive phase for the lever, and without light cues. Interestingly, they did not find any impairment during the initial training, when the inactive phase was set to five seconds, but only when the rats were switched to a protocol where the inactive phase was set to ten seconds. Although their interpretation was that the phenotype indicated a general deficit in response inhibition, their study did not include any control tests for evaluating if the phenotype was caused by a deficit in strategy adjustment (as indicated by our DRL results).

Possible mechanisms
As noted, the ability to stop an initiated motor response appears to be dependent on circuits involving the orbitofrontal cortex, the dorsomedial striatum, and the subthalamic nucleus[403-405,407,419]. The involvement of the orbitofrontal cortex in performance on Go/No-Go protocols is unclear[405,407], while the subthalamic nucleus appears to be involved[403,421,422]. As noted, BACHD rats appear to have a general deficit in stopping ongoing motor responses but largely intact ability to withhold inappropriate responses. Thus, it is possible that the neuropathology of the orbitofrontal cortex and dorsomedial striatum is more pronounced that pathology of the subthalamic nucleus in the BACHD rats. Involvement of the striatum in Go/No-Go protocols is unclear[405,407], while DRL performance is primarily governed by the ventral striatum[412-414]. As no overt DRL impairment appears to be present among BACHD rats it is likely that pathology in the ventral striatum is also limited.

As noted, the BACHD rats' deficits concerning withholding lever responses appear to be dependent on situations where they have to apply inhibitory control to situations that previously have not required it. This deficit might relate to the impaired performance during attentional set-shifting tasks, which has been seen among HD patients[104,105]. During these tests, patients are trained to respond to one kind of visual stimuli, but ignore another kind. At certain points the protocol changes, so that patients have to respond to the previously ignored stimulus, while ignoring the previously important one. The processes of learning to respond to a previously unimportant stimulus while at the same time inhibiting responses to the previously important stimulus are thought to be dissociable[105]. Interestingly, HD patients appear to show specific impairments in the latter process[105]. Similar deficits have been found in rats with lesions to the dorsomedial striatum, medial prefrontal cortex, and orbitofrontal cortex[423–425]. Still, as discussed in Appendix II, the BACHD rats' impaired performance in the Go/No-Go protocol could also be due to deficits in attention and visual acuity.

Summary and Outlook

The BACHD rats' exact impairment in inhibitory control is still largely unclear, although our results indicate a general impairment in inhibiting ongoing motor responses, combined with slowed learning to withhold responses in certain situations. Still, the validity of this hypothesis has to be evaluated. The Go/No-Go study was well designed, but proper studies of the rats' performance in DRL and stop-signal tests are needed. In addition, control tests that specifically evaluate the rats' attention and visual acuity have to be performed. Once, this has been achieved, and the phenotypes have been determined, inactivation studies of brain regions such as the orbitofrontal cortex and dorsomedial striatum might be of interest.

VI.
Concluding remarks

Phenotype overview

In summary, the current project achieved several aspects of the initial aims. The overarching metabolic phenotype of male BACHD rats was confirmed, suggesting HD-related pathology of the hypothalamus in general, and of the arcuate nucleus in particular. Strategies for how to efficiently work with BACHD rats in food-reinforced operant conditioning tests were evaluated, concluding that the use of control tests is critical, as there is likely no optimal food restriction protocol available. The use of such control tests aided the identification of phenotypes that are likely to be caused by HD-related pathology of fronto-striatal circuits. These concerned slowed pellet retrieval and an increased tendency to perform perseverative lever pushes in the progressive ratio test, which might indicate pathology of the dorsolateral striatum. It further concerned an overall impaired performance on the delayed alternation test, which appeared to be connected to BACHD rats' inability to stop ongoing motor responses. As noted, this impairment could be caused by HD-related pathology of the orbitofrontal cortex and dorsomedial striatum.

Lack of progressive phenotypes

As HD is a progressive disease one would expect that disease-related phenotypes of BACHD rats would also worsen with age. This was, however, not generally seen for the phenotypes described above (Publication I-III, Appendix II). Progressive change in phenotype severity among BACHD rats has been found in several tests, including the Rotarod[174,206], Elevated plus maze[174], gait analysis[174,206], and Open field activity[206]. These studies used age-spans comparable to what was used in the current project, so a progression was expected. The current studies did, however, involve intense training in operant conditioning protocols, which could be argued to constitute a kind of environmental enrichment. As environmental enrichment has been found to have strong therapeutic effects in both HD[14,258,261,426,427] and other neurodegenerative diseases[428,429], it is possible that the BACHD rats' intense training had a prophylactic effect on disease progression. It should also be considered that no overt cell loss has been found in BACHD rats, and the noted progressive neuropathology primarily concerns gradual accumulation of huntingtin aggregates and a late-onset loss of D2 receptors[174]. Thus, while the neuropathology causing the noted progressive phenotypes might be affected by the gradual accumulation of aggregates, this process might not have any impact on the phenotypes discussed in the current thesis. Such non-progressive pathologies might instead be due to developmental deficits. Finally, it should be considered that the apparent age progression seen in other behavioral tests might be confounded by other factors. As an example, BACHD rats that are trained on the Rotarod have been found to become increasingly anxious with repeated exposure, and the apparent age progression could be caused by psychiatric rather than motoric phenotypes (unpublished data). Similarly, Open field activity has been assessed by repeatedly exposing rats to a specific arena, and the apparent age progression might be influenced by differences in habituation.

Limitations and weak points

The current project is not without shortcomings and weak points. A major limitation is that only male BACHD rats were assessed. This was primarily due to convenience, and the fact that all characterization made prior to the start of this project had focused on

males. Current efforts are being made to also characterize females, which have so far indicated that their metabolic phenotype differs somewhat from males' (unpublished data). Similar discrepancies have been seen in BACHD mice[274]. Another clear shortcoming is that although the discussion above has pointed out several brain regions of interest, there was no possibility to properly investigate their involvement in the noted phenotypes. This was primarily due to time constraints. The major weak point of the study is that no controls were made to evaluate if phenotypes were caused by insertional mutagenesis (i.e. the process of transgenic constructs affecting the expression of genes at their insertion site[430–432]). Previous studies have included rats from the TG9 line as controls[174], as phenotypes caused by mutant huntingtin expression should be present but weaker compared to the TG5 line. This was omitted in the current study due to time and space limitations. However, the behavioral phenotypes found in the current study were largely in line with literature concerning other HD models and animals with HD-related brain lesions. Thus, it is unlikely that insertional mutagenesis had a major influence.

BACHD rats as a model for HD

A large part of the characterization work of any disease model aims to evaluate to what extent it models the actual disease. In this aspect, the BACHD rats show both similarities and differences according to the discussion above. It is important to note that symptoms vary strongly between HD patients[56–58,64] and it is arguable that an animal model based on inbred animals is likely to only model a subgroup of patients. Unfortunately, there has to our knowledge not been extensive studies done on subtyping HD patients based on their symptoms. Thus, specific knowledge of which patient group might be well modeled by the BACHD rats is unclear.

Another important aspect is to understand the strengths and weaknesses of a given animal model. BACHD rats were established primarily due to rats having certain benefits over mice in general. In brief, rats' larger size means that imaging techniques and intracranial injections can be made with greater ease[433–435]. In addition, larger volumes of tissue samples can be gathered[433,436]. Finally, rats are more convenient to work with in operant conditioning tests[262,393,433]. These factors can to a large extent not be refuted, and thus constitute clear benefits of the BACHD rats. A weakness of a similar kind is that rats require more space, and thus put higher demands on housing facilities. When considering strengths and weaknesses in terms of behavioral work, BACHD rats are likely comparable to other animal models of HD. Thus, when assessing activity one would always have to consider that a reduced activity in an HD model could be influenced by both motoric and psychiatric impairments. The same would be true for motivational phenotypes in the progressive ratio test. Similarly, a reduced food consumption rate does not necessarily mean that rats are less hungry. This list of examples can be made long. Ultimately, although the current thesis has focused much on the difficulty of obtaining valid results for BACHD rats, the possible confounding factors that have been considered are quite general to HD models. Thus, the current project has benefitted the BACHD rat project as a whole, and put us ahead of the characterization work of many other models, as these topics are only rarely brought up[282].

General outlook

As noted, the BACHD rats show several phenotypes that could be of use in preclinical evaluation of HD treatments. However, the exact validity of these phenotypes still has to be confirmed by evaluating the underlying neuropathology. Histological analysis is likely of little use for this purpose, as BACHD rats do not show any extensive neuropathology[174]. Instead, further research should focus on evaluating behavioral performance in BACHD rats, following an inactivation of mutant huntingtin expression in the brain regions of interest.

Figures

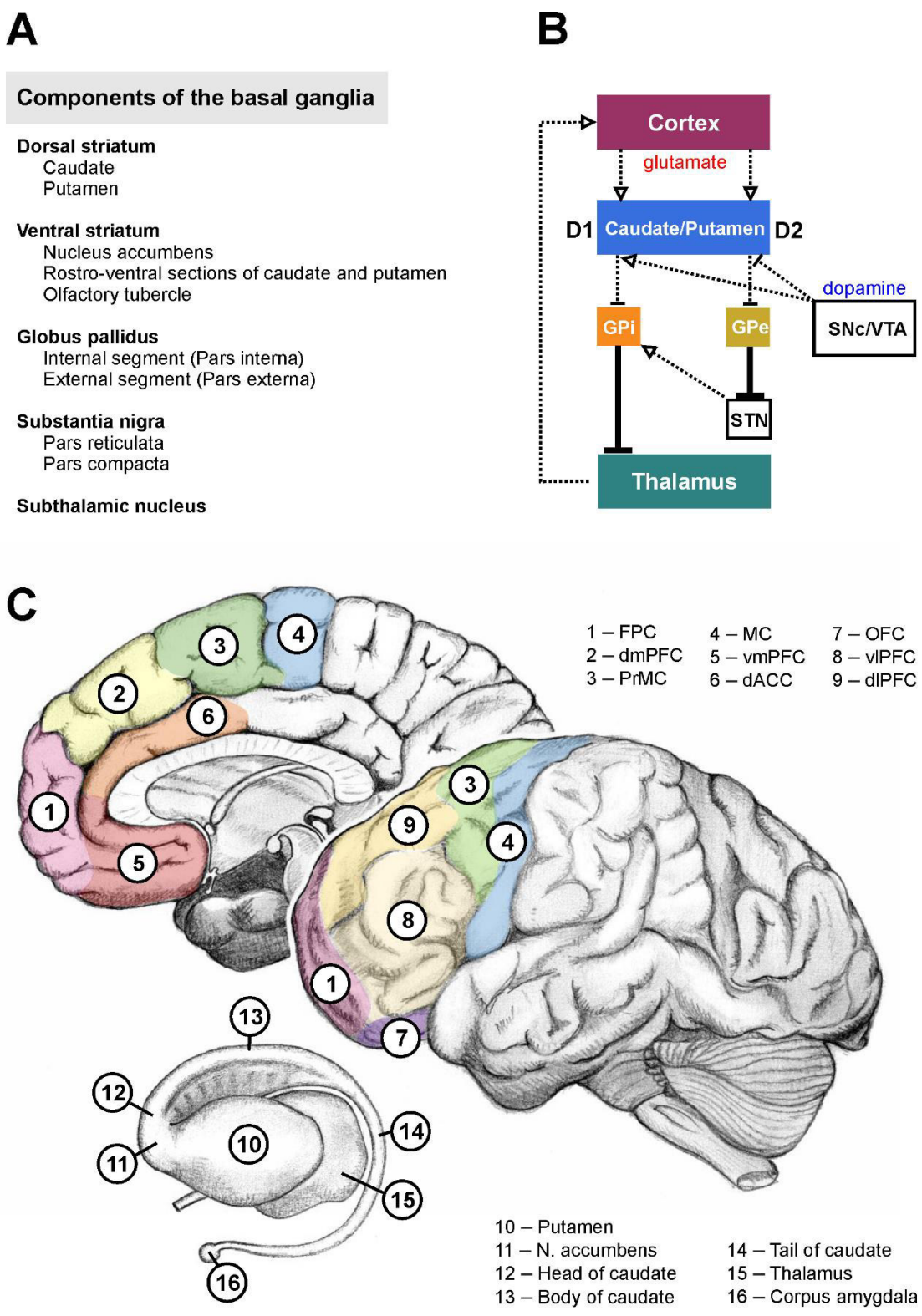

A
Components of the basal ganglia
Dorsal striatum
Caudate
Putamen
Ventral striatum
Nucleus accumbens
Rostro-ventral sections of caudate and putamen
Olfactory tubercle
Globus pallidus
Internal segment (Pars interna)
External segment (Pars externa)
Substantia nigra
Pars reticulata
Pars compacta
Subthalamic nucleus
B
Cortex
glutamate
D1
Caudate/Putamen
D2
dopamine
GPi
GPe
SNc/VTA
STN
Thalamus
C
1 – FPC
2 – dmPFC
3 – PrMC
4 – MC
5 – vmPFC
6 – dACC
7 – OFC
8 – vlPFC
9 – dlPFC
10 – Putamen
11 – N. accumbens
12 – Head of caudate
13 – Body of caudate
14 – Tail of caudate
15 – Thalamus
16 – Corpus amygdala

Figure 1. The human basal ganglia and frontal cortex, part I.

A displays a list of the different neuronal nuclei that make up the basal ganglia of humans [1,2]. **B** displays the classic concept of how neuronal signals pass through the basal ganglia. Solid arrows indicate tonic signals, while transient signals are indicated with dotted arrows. Excitatory signals are indicated with pointy arrowheads, while inhibitory signals are indicated with blunt arrowheads. Neuronal signaling through the basal ganglia can be considered to start with the excitatory glutamatergic signals that come from cortical neurons and target the medium spiny projection neurons of the striatum. Stimulation of these neurons results in subsequent inhibition of the internal and external segments of the globus pallidus, targeted by D1- and D2-containing neurons, respectively. These signals serve to reduce the tonic inhibition that the internal and external segments of globus pallidus exert on the thalamus and subthalamic nucleus, respectively. The D1 neurons' signaling to the internal segment of globus pallidus makes up the so-called direct pathway. The D2 neurons' signaling through the external segment of globus pallidus and subthalamic nucleus makes up the so-called indirect pathway. These two pathways counteract each other, as stimulation of the direct pathway results in reduced inhibition of the thalamus, while stimulation of the indirect pathway results in increased inhibition of the thalamus, through the disinhibition of the subthalamic nucleus, which subsequently stimulates the external segment of globus pallidus. A given action/movement is promoted when the ultimate result of signaling through the basal ganglia results in reduced inhibition of the thalamus. As such, increased signaling through the direct pathway promotes actions/movements while increased signaling through the indirect pathway inhibits it. The striatum also receives dopaminergic input from the substantia nigra pars compacta and ventral tegmental area. This serves an important modulatory effect, as dopamine stimulates D1 neurons (i.e. promotes signaling through the direct pathway) and inhibits D2 neurons (i.e. inhibits signaling through the indirect pathway) [1]. **C** displays anatomical sketches of various brain regions that are of interest for the current thesis [1]. Abbreviations (in chronological order): D1 – Dopamine receptor 1, D2 – Dopamine receptor 2, GPi – Globus pallidus pars interna (internal segment), GPe – Globus pallidus pars externa (external segment), SNc – Substantia nigra pars compacta, VTA – Ventral tegmental area, STN – Subthalamic nucleus, FPC – Frontal pole cortex, dmPFC – dorsomedial Prefrontal cortex, PrMc – Premotor cortex, MC – Motor cortex, vmPFC – ventromedial Prefrontal cortex, dACC – dorsal Anterior cingulate cortex, OFC – Orbitofrontal cortex, vlPFC – ventrolateral Prefrontal cortex, dlPFC – dorsolateral Prefrontal cortex, N. accumbens – Nucleus accumbens.

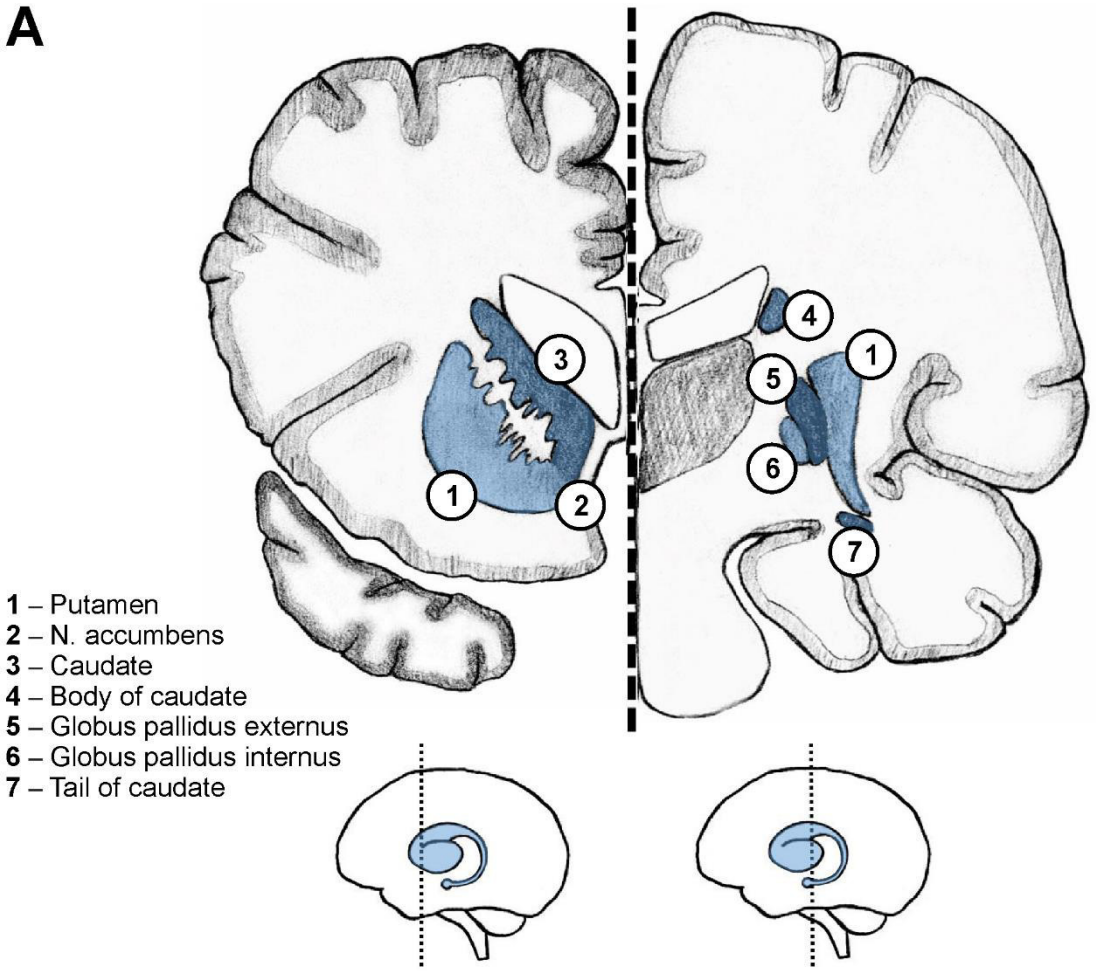

A
1 – Putamen
2 – N. accumbens
3 – Caudate
4 – Body of caudate
5 – Globus pallidus externus
6 – Globus pallidus internus
7 – Tail of caudate
B
1 – Caudate
2 – N. accumbens
3 – Putamen
PrM/M
dPFC
dACC
OFC
vmPFC
C
PFC functions of interest
dlPFC Various aspects of executive function, e.g. planning [2]
Executive component of motivation [3]
Response inhibition [4]
vlPFC Inhibiting ongoing motor responses [5]
vmPFC Affective and emotional component of motivation [3]
Impulsive choice [4]
dACC Response inhibition [4]
Error detection [6]
Divided attention [6]
ORF Behavioral/social inhibition [2]
Response inhibition [4]
Impulsive choice [4]

Figure 2. The human basal ganglia and frontal cortex, part II.

A displays anatomical sketches of two coronal sections taken at different locations along the rostro-caudal axis of the human brain. The locations of various basal ganglia components are indicated. **B** displays a sketch indicating where cortical projection neurons from different regions of the prefrontal cortex synapse on striatal neurons. Note that the sketch displays the rostral striatum, comparable to the leftmost sketch in **A** [1]. **C** displays a short list of suggested functions for different regions of the prefrontal cortex that are of interest for the current thesis. Abbreviations (in chronological order): N. accumbens – Nucleus accumbens, PrM – Premotor cortex, M – Motor cortex, dPFC – dorsal Prefrontal cortex, dACC – dorsal Anterior cingulate cortex, OFC – Orbitofrontal cortex, vmPFC – ventromedial Prefrontal cortex, dlPFC – dorsolateral Prefrontal cortex, vlPFC – ventrolateral Prefrontal cortex.

A

Components of the basal ganglia

Dorsal striatum*
- Caudate
- Putamen

Ventral striatum
- Nucleus accumbens
- Rostro-ventral sections of caudate and putamen
- Olfactory tubercle

Globus pallidus**

Entopeduncular nucleus***

Ventral pallidum

Substantia nigra
- Pars reticulata
- Pars compacta

Subthalamic nucleus

* In rats, the caudate and putamen are not separated by the internal capsule and form one anatomical structure
** Generally thought to represent the rodent homologue to the external segment of globus pallidus in primates
*** Generally thought to represent the rodent homologue to the internal segment of globus pallidus in primates

B

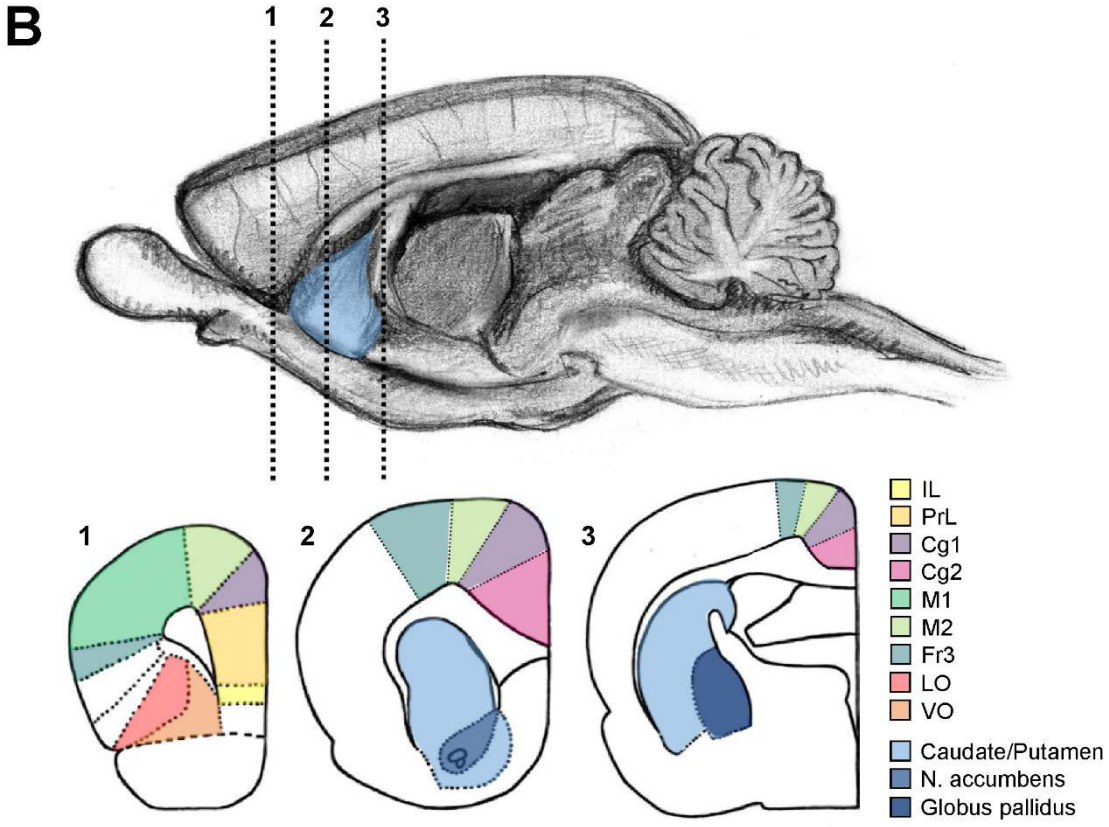
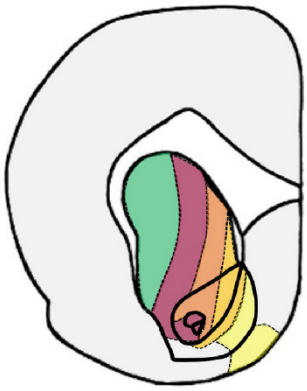


Figure 3. The rat basal ganglia and frontal cortex, part I.

A displays a list of the different neuronal nuclei that make up the basal ganglia of rats. Notable differences between the rat and human anatomy are described [1]. **B** displays a series of sketches that indicate the anatomical location of various cortical regions of interest, and basal ganglia components, along the rostro-caudal axis of the rat brain [2]. Abbreviations (in chronological order): IL – Infralimbic cortex, PrL – Prelimbic cortex, Cg1 – Cingulate cortex area 1, Cg2 – Cingulate cortex area 2, M1 – Motor cortex 1, M2 – Motor cortex 2, Fr3 – Frontal cortex area 3, LO – Lateroorbital cortex, VO – Ventroorbital cortex, N.Accumbens – Nucleus accumbens.

A



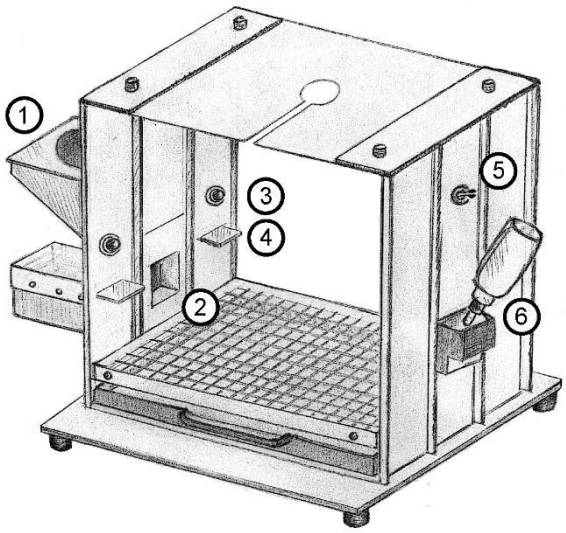
B

PFC functions of interest	
mPFC	Motivation in progressive ratio test [2]
	Accurate performance in DRL test (likely specific to prelimbic cortex) [3,4]
	Accurate performance in operant delayed alternation test [5,6]
	Accurate performance in operant delayed non-matching to position [7]
	Accurate performance in operant delayed non-matching to position [7]
OFC	Motivation in progressive ratio test [8]
	Inhibiting ongoing motor responses [9]
	Withholding responses in Go/NoGo tests (conflicting results) [9]
	Impulsive choice in delay discounting [9,10]

Figure 4. The rat basal ganglia and frontal cortex, part II.

A displays a sketch indicating where cortical projection neurons from different regions of the prefrontal cortex snapse on striatal neurons. Note that the sketch displays the rostral striatum, comparable to the middle sketch in Figure 3B [1]. **B** displays a short list of suggested functions for different regions of the prefrontal cortex that are of interest for the current thesis. Abbreviations (in chronological order): M1/2 – Motor cortex 1/2, ACg – Anterior cingulate cortex (Includes Cg1 and Cg2), dPrL – dorsal Prelimbic cortex, vPrL – ventral Prelimbic cortex, IL – Infralimbic cortex, mPFC – medial Prefrontal cortex, OFC – Orbitofrontal cortex.

A



B

Figure 5. Skinner box system used in the current thesis

A shows a sketch of one of the Skinner boxes used for the various behavioral tests described in Publication I-III, and Appendix II and III. The noted components are a reward pellet feeder (1), a pellet receptacle (2), a large cue light (3), a retractable lever (4), a house light (5) and a water bottle (6). Note that the sidewalls and door of the Skinner box have been omitted from the sketch. **B** shows a photo from inside the Skinner box, with a rat interacting with one of the response levers.

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This thesis represents some form of end, or at least turning point, for all the years I have spent reading books and articles at different universities. As such, there are several people that have in different ways been important for the initiation and completion of it. In order to give fair mention to all (or at least the most important ones) I will go through them in a roughly chronological order. Titles are included where it felt appropriate. Although most people should by now be referred to as Dr. so and so, that was not necessarily how I got to know them, and therefore not how I chose to address them here.

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Figure 1

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Figure 2

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Figure 3

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Publication I

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Reduced Motivation in the BACHD Rat Model of Huntington Disease Is Dependent on the Choice of Food Deprivation Strategy

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Abstract

Huntington disease (HD) is an inherited neurodegenerative disease characterized by motor, cognitive, psychiatric and metabolic symptoms. Animal models of HD show phenotypes that can be divided into similar categories, with the metabolic phenotype of certain models being characterized by obesity. Although interesting in terms of modeling metabolic symptoms of HD, the obesity phenotype can be problematic as it might confound the results of certain behavioral tests. This concerns the assessment of cognitive function in particular, as tests for such phenotypes are often based on food depriving the animals and having them perform tasks for food rewards. The BACHD rat is a recently established animal model of HD, and in order to ensure that behavioral characterization of these rats is done in a reliable way, a basic understanding of their physiology is needed. Here, we show that BACHD rats are obese and suffer from discrete developmental deficits. When assessing the motivation to lever push for a food reward, BACHD rats were found to be less motivated than wild type rats, although this phenotype was dependent on the food deprivation strategy. Specifically, the phenotype was present when rats of both genotypes were deprived to 85% of their respective free-feeding body weight, but not when deprivation levels were adjusted in order to match the rats' apparent hunger levels. The study emphasizes the importance of considering metabolic abnormalities as a confounding factor when performing behavioral characterization of HD animal models.

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Introduction

Huntington disease (HD) is an autosomal dominantly inherited neurodegenerative disease with a prevalence of 6 per 100,000 in Europe and North America [1]. Development of HD is dependent on a single mutation that results in the extension of the CAG repeat sequence present in the gene for the Huntingtin protein [2]. HD patients display a range of symptoms that can be grouped into motor, psychiatric, cognitive and metabolic symptoms. Symptoms gradually worsen as the disease progresses, and due to the lack of disease modifying treatments HD is invariably fatal.

There are numerous transgenic animal models of HD [3], and as with any disease model, a major focus of working with these is to assess how well their phenotypes mirror symptoms found in HD patients. This is complicated due to the multitude of phenotypes that are often present, and the potential risk of some phenotypes confounding the assessment of others. The metabolic phenotypes are especially interesting in this regard. While HD patients typically lose weight [4,5,6,7,8,9], the body weight and body composition phenotypes of transgenic animal models of HD vary [3]. Animals that express the full-length mutant huntingtin gene typically show an increased body weight, due to increased fat mass [10,11]. Although this is interesting in terms of modeling the metabolic symptoms of HD, an increase in body weight has been suggested to result in reduced performance on the rotarod [12,13], a common test of motor capacity and limb coordination.

Metabolic phenotypes are also of interest when considering tests of cognitive function, as these are often based on having food deprived animals perform certain tasks to retrieve food rewards [14]. Ideally, animals should be equally hungry and interested in food rewards when performing such tests, as studies where motivational differences are present can give misleading results [15]. Changes in body composition, such as the ones seen in HD models, are likely to either be caused by or lead to a change in *ad libitum* food consumption. Unless careful adjustments are made, such phenotypes might persist even after food deprivation. One

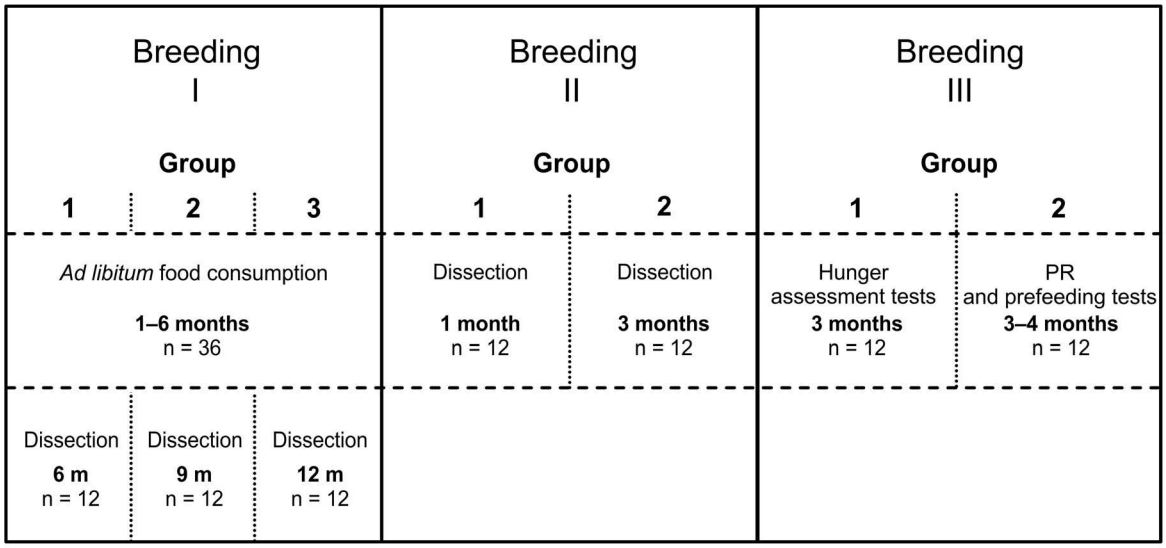

Figure 1. Overview of study groups. A total of seven groups of rats were used in the current study. These were derived from different breeding events and used in different tests, as shown in the figure. The "n" indicates the number of animals used from each genotype. Note that a total of two animals were excluded during analysis, as explained in detail under "Statistical analysis".
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proposed method to avoid this when working with HD models is to adjust food deprivation levels until animals show similar consumption rates in tests where they are given brief access to food [16,17]. Similar tests are occasionally used to assess hunger and food interest, [18,19,20,21] although in HD research one should also consider that a slowed consumption rate could be caused by motor impairments. Thus, detailed knowledge about body composition and feeding behavior of an animal model, both when deprived and *ad libitum* fed, is important for planning and interpreting a variety of behavioral tests.

The BACHD rat is a recently established animal model for HD. These rats carry a large construct containing the full-length gene for human mutant Huntingtin, with its endogenous regulatory sequences [22]. Previous studies have shown that BACHD rats have motor impairments and neuropathological phenotypes reminiscent of symptoms seen among HD patients [22]. In addition, BACHD rats appear to be impaired in some cognitive tests [23]. Previous studies have indicated that BACHD rats eat less than WT rats [22], although the setup used for that particular study demanded social isolation, and its validity for assessing natural behavior has been questioned [24]. Further, although it has been pointed out that BACHD rats appear obese [22], there has not been any study on their body composition. Therefore, we performed a longitudinal study where food intake was measured in a social homecage setup, and body composition was assessed through detailed dissections. As further behavioral characterization of the BACHD rats will be dependent on tests that require food deprivation, we also sought to evaluate an optimal food deprivation strategy for BACHD rats. For this, consumption rate of reward pellets and regular food, as well as performance in a progressive ratio test with prefeedings was assessed at different levels of food deprivation.

Materials and Methods

Animals

A total of 168 male rats were used for the study. These were acquired from three separate in-house breeding events, with heterozygous BACHD males from the TG5 line [22] paired with WT females. All animals were on Sprague Dawley background. Animals were genotyped according to previously published protocols [22] and housed in type IV cages (38×55 cm), with high lids (24.5 cm from cage floor), and free access to water. Food availability and social conditions differed between the experimental groups. Rats used for *ad libitum* food intake and body composition measurements were housed in genotype-matched pairs, and had free access to food (SNIFF V1534-000 standard chow) during the entire length of their respective test. Importantly, food was provided on the cage floor and not on the cage top. Body weight was measured weekly to assess general health, and cages were changed twice per week. Rats used for hunger assessment and PR tests were housed in genotype-matched groups of three rats per cage. They had free access to food from the cage top until the age of ten weeks. At that point, the rats were food deprived as described below. Body weight was measured daily in order to assess food deprivation levels, and cages were changed weekly. The animal facility kept 21–23°C, 55–10% humidity, and was set to a partially inverted light/dark cycle with lights on/off at 02:00/14:00 during summer, and 01:00/13:00 during winter.

The seven groups of animals were used in different tests, as described below. An overview of the animal groups, and the tests, is shown in Figure 1. All experiments were approved by the local ethics committee (Regierungspraesidium Tuebingen) and carried out in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU).

Ad libitum food consumption in a social homecage environment

Ad libitum food consumption was measured using a total of 72 rats, acquired from one breeding event. At the age of five weeks, all rats were arranged into genotype-matched pairs, and housed as described above. This gave a total of 36 cages, 18 cages per genotype. Cages with WT and BACHD rats were evenly distributed over two racks, which were placed next to each other in the same housing room. Food and water intake was assessed

twice weekly, when cages were changed. Cages were changed on Mondays and Thursdays during the last two hours of the light phase. At each cage-changing event, a known amount of food was placed inside each new cage, and the fresh water bottles were weighed. The weights of the old water bottles as well as the weight of the food left in each old cage were then measured to assess the amount of food and water consumed since the last cage change. The food was manually collected from the bedding of the old cages. After removing large food pieces, the bedding was sifted in a homemade sieve with a 1 mm mesh in order to collect small food pieces generated by food grinding. The animals' food and water consumption was followed in this way until the age of 26 weeks. Sifting of bedding materials started when animals were 15 weeks old.

Dissection for body composition assessment

A detailed dissection was performed in order to study the body composition of BACHD rats. Five different rat groups were sacrificed at 1, 3, 6, 9, and 12 months of age respectively, with each group being composed of 12 WT and 12 BACHD rats. The rat groups used for dissection at 6, 9, and 12 months of age were the same rats that were followed during the *ad libitum* food consumption test. The rat groups used for dissection at 1 and 3 months of age were acquired from a separate breeding. Housing conditions were identical for all animals, and according to the description above. Aside from the weekly food and water consumption assessment made during the *ad libitum* food intake test, food and water consumption were measured monthly as animals aged. When rats reached an age of interest, a dissection group was arranged based on the animals' food consumption, water intake, and body weights, so that the dissected group well represented the full group.

Rats were sacrificed in a carbon dioxide chamber two to four hours before dark-phase onset. Blood samples were collected after sacrifice, through retro-orbital bleeding. Body lengths and body weights were measured on the intact animals, with body length measured from nose tip to tail tip. Additional measurements of head, trunk, and tail lengths were measured from nose tip to back of the head, back of the head to anus, and anus to tail tip, respectively. After these external measurements, skin and subcutaneous adipose tissue deposits were removed and weighed. Then, internal organs and adipose deposits located in the abdomen and chest cavities were removed and weighed. The remaining carcass was weighed before removal of the brain. By later subtracting the brain weight, a measurement of bone and muscle weight (denoted bone/muscle) was acquired for each rat. Dissection of a given age group was carried out during four to six days, with rats of both genotypes being assessed on each day.

Hunger assessment tests

Two tests were used to assess hunger levels in WT and BACHD rats at three different food deprivation levels. A group of 24 animals with equal numbers of WT and BACHD rats was used for both tests. This group was acquired from a breeding separate from the ones used for the *ad libitum* food consumption and body composition measurements. As mentioned above, food deprivation started when the rats were ten weeks old. Body weights were compared to control data from age- and genotype-matched free-feeding animals, on a weekly basis, in order to acquire measurements of food deprivation levels (relative body weight). It should be noted that the control data was not gathered in the current study, but in previous tests. Rats were given small daily amounts of food inside their social homecages, approximately four hours after dark phase onset, to maintain food deprivation. During the first week of food deprivation, animals were habituated to the reward pellets (Bio-Serv, Dustless Precision Pellets® F0021, purchased through Bilaney Consultants, Duesseldorf, Germany) by daily giving each cage a spoon-full of reward pellets together with the daily amount of food. Behavior assessment started one hour after dark phase onset, and was performed in the animals' housing room, using soft red light. Rats were 13 weeks old when behavioral assessment started.

Rats were assessed in both tests on each given testing occasion. The first test assessed the rats' interest in consuming 100 reward pellets. The test used a glass cage (28.5×29×29.5 cm) with mirrors, which allowed a good view of the feeding animals. At the start of each trial, a rat was placed inside the cage, and was allowed two explore it freely during two minutes. Afterwards, a glass Petri dish containing 100 reward pellets was placed inside the cage, in one of the corners that faced the experimenter. The rats were then given a total of five minutes to consume the reward pellets, while the experimenter scored their behavior. The experimenter used two timers to separately record the total time taken to consume the reward pellets, and the time each rat actually spent eating. Thus, one timer was started when the rat first discovered the pellets, and stopped either when all pellets were consumed or when five minutes had passed. The second timer was also started when the rat first discovered the pellets, but was stopped whenever the rat stopped eating, and explored the test arena. Roughly three hours were needed to assess all 24 rats. The test schedule was arranged so that entire cages of BACHD and WT rats were assessed in an alternating manner. Thus, three rats of a given genotype were assessed in sequence, followed by three rats of the other genotype. The experimenter was blinded to the animals' genotypes.

The second test assessed the rats' interest in regular food. In this test, rats were given free access to a large amount of food in their homecages. Food was made available to the rats when four hours remained of the dark phase. Identical amounts of food were placed in the cage tops, with one-minute spacing between cages, alternating between BACHD and WT cages. The remaining food was then measured each half hour, until the end of the dark phase. A final measurement was made at the end of the subsequent light phase. At each measurement, the cages were briefly inspected for larger pieces of food, as they occasionally dropped between the bars of the cage lids.

The rats were assessed in these two tests on three separate occasions. On the first, both WT and BACHD rats were deprived to 85% of their respective free-feeding body weights. In an attempt to reverse the phenotypes that were found, the food deprivation levels were then adjusted so WT and BACHD rats were at 95 and 80% of their respective free-feeding body weights. On the final trial, the previous deprivation levels were switched, so that WT and BACHD rats were at 80 and 95% of their respective free-feeding body weights. Each test occasion was separated by a week of food deprivation, to allow gradual adjustment of deprivation levels.

Progressive ratio test

A progressive ratio (PR) test was run to assess the rats' motivation to work for a food reward at two different food deprivation settings. A group of 24 animals with equal numbers of WT and BACHD rats was used for the test. This group was acquired from the same breeding as the group used for the hunger tests described above. Food deprivation was initiated and maintained as described above. Behavioral assessment started 30 minutes after dark phase onset, in a room separate from the

animals' housing room, using soft red light. Rats were 11 weeks old when behavioral assessment started.

A bank of six operant conditioning chambers (Coulbourn Instruments, H10-11R-TC with H10-24 isolation boxes, purchased through Bilaney Consultants, Duesseldorf, Germany) was used to run the test. Each chamber was equipped with two retractable levers, placed 6 cm above the chamber floor, protruding 2 cm from the wall. The levers were placed on either side of a central pellet receptacle trough, which was placed 2 cm above the chamber floor. The pellet receptacle trough contained a yellow light, which was used to signal the delivery of a reward pellet in all protocols described below. The chambers also contained a red house light, on the wall opposite from the levers and pellet receptacle trough, which shined during the full duration of the training sessions. A water bottle was also available on this wall, to ensure *ad libitum* access to water during testing. All protocols were designed and run with Graphic State 4.1.04. Rats were given single daily sessions, meaning that a total of four daily runs with all six operant chambers were needed to assess the whole group. Each run assessed three WT and three BACHD rats in a determined order, so that a given rat was trained on the same time of day through the entire test. Each rat was assigned to a specific operant chamber, although this was arranged so that each operant chamber was used to assess equal numbers of WT and BACHD rats. Rats received their daily regimen of regular food four hours after the completion of the last run of the day.

During initial training, rats of both genotypes were deprived to 85% of their respective free-feeding body weights. Afterwards, all rats received two habituation sessions in the conditioning chambers. During these, both levers were retracted and a single reward pellet was delivered to the pellet trough at 10, 15, 20, 25, or 30-second intervals. The pellet delivery interval varied in a pseudo-randomized fashion so that each set of five deliveries used each given interval once. Pellet retrieval, or failure to retrieve the pellet within five seconds after delivery, lead to the start of the next pellet delivery interval. After the habituation sessions, rats were trained to lever push for a pellet reward. During these sessions, both levers were extended into the chamber, but only one was reinforced. Rats were either trained to push the right or the left lever, with the reinforced lever position being counter-balanced within the genotype groups. During training, the experimenter would reward rats for approaching, sniffing and touching the reinforced lever, until rats started to reliably push the lever on their own. During this, each lever push was rewarded with one pellet. Training continued until rats completed 100 lever pushes within a 30-minute session, without any help from the experimenter. The rats were then trained on an FR3 protocol, where they had to push the reinforced lever three times before being rewarded with a pellet. When a rat completed 100 ratios within a 30-minute session, it progressed to an FR5 protocol. Rats now had to push the reinforced lever five times before being rewarded with a pellet. Training on the FR5 protocol continued until rats completed 100 ratios within a 30-minute session, on three consecutive sessions. Afterwards, rats were trained on a PR protocol adapted from [16]. In the current protocol, the ten first ratios were of FR5 type. Afterwards, the required number of lever pushes increased after each completed ratio. During this progression, the required number of lever pushes increased in an arithmetic fashion within each block of ten ratios, but also changed between the blocks, to give an overall exponential progression. Thus, during the first, second and third block of ten ratios, the ratio requirement increased with one, three and five pushes per completed ratio, respectively. The PR sessions lasted 80 minutes. The main behavioral parameter of interest was a set of break points, defined as the first ratio where a rat made no responses on the reinforced lever during 10, 25, 50, 100, 300 or 600 seconds. Rats were trained until both genotype groups reached a stable performance, which in this case required 18 sessions. Performance during the six last sessions was defined as baseline performance.

Once stable PR performance had been reached, the rats were challenged in a set of four prefeeding tests. During these tests, the rats were fed specific amounts of reward pellets or regular food, just prior to their daily PR session. Rats were prefed by placing them in individual cages that contained the specified amount of food. Each prefeeding condition was assessed once, in the following order: 100 reward pellets, 250 reward pellets, 4.5 g of regular food, 11.25 g of regular food. Each prefeeding test was separated by two regular PR sessions to ensure that rats returned to their baseline performance.

After completion of the first round of prefeeding tests, the food deprivation level of WT rats was adjusted until they consumed food at the same rate as BACHD rats. Consumption rate was assessed daily by measuring the amount of food consumed during 15 minutes of free access to regular food, placed in the cage tops of the rats' homecages. The rats were still given daily PR sessions during food deprivation adjustments. The food consumption tests were run four hours after completion of the last PR run, i.e. at the time when the rats were usually given their daily food ration. When WT rats had reached a consumption rate equal to that of BACHD rats, six additional PR sessions were run to establish a new baseline. The prefeeding tests were then repeated in the same manner as described above. Rats were 20 weeks old at the end of the test.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism v.6.01 (GraphPad Software, San Diego California USA, http://www.graphpad.com).

Food consumption in the *ad libitum* food consumption test was analyzed both in terms of the absolute amount of food consumed and the amount of food consumed relative to the animals' body weight. The main analysis of food consumption was based on the weight of large food pieces, as the food debris gathered through sifting of the bedding material also contained hair and bedding pieces. A separate analysis where food consumption was corrected for the amount of food debris was still performed. For this, the mean amount of food debris was calculated for each cage, based on their longitudinal data. This was then added to the weight of the large food pieces measured at each cage changing. For the relative food consumption, rats in a given cage were assumed to eat equal amounts of food. The approximate amount of food consumed by one of the rats was subsequently related to the mean body weight of the two rats. Two-way repeated measures ANVOAs were used to analyze body weight as well as absolute and relative food consumption. Age was used as within-subject factor, and genotype as between-subject factor.

For data gathered in the dissection study, body weight, absolute weight of adipose and bone/muscle tissues, as well as bone/muscle weight relative to body length were analyzed using regular two-way ANOVAs. The factors of interest were still age and genotype. The weights of adipose tissue, bone/muscle tissue and internal organs relative to body weight were analyzed in individual t-tests, or Mann-Whitney tests, between genotypes, within each age group. As the observed phenotypes did not vary between different adipose tissue deposits, only the combined weight of all deposits will be addressed here. One BACHD rat meant for the dissection of six months old animals died before the dissection, making that particular age group 12 WT and 11 BACHD rats.

Results from the two hunger tests were analyzed both within and between each testing occasion. For each test occasion of the reward pellet consumption test, the time needed to consume the pellets was analyzed with t-tests to compare the two genotypes. The time spent exploring the test arena was only analyzed on the first test occasion, using t-test, as rats showed essentially no interest in exploring the arena on later trials. One BACHD rat was excluded from the analysis of the last trial, as he failed to consume all reward pellets within the maximum trial time. The amount of food consumed during the food consumption test was on each test occasion analyzed with two-way repeated measures ANOVA, using time as within-subject factor, and genotype as between-subject factor. To better understand the effect of repeated testing and food deprivation levels, the time needed to consume 100 reward pellets, and the amount of food consumed during the first 30 minutes of the food consumption test were analyzed in additional detail. Thus, data from all three test-occasions were analyzed in two-way repeated measures ANOVAs, using genotype as between-subject factor, and either session number or food deprivation level as within-subject factor. Analysis of baseline performance during the PR test was also made with repeated measures two-way ANOVAs, with break point as within-subject factor, and genotype as between-subject factor. Drops in motivation during prefeeding sessions were analyzed for the 600-seconds break point, as a percentage of the ratio reached during the two preceding PR sessions. Once again, repeated two-way ANOVAs were used to analyze the results, using prefeeding condition as within-subject factor, and genotype as between-subject factor. Separate analyses were performed for prefeeding with reward pellets, and regular food. Bonferroni *post-hoc* test was used to follow up any significant effects of genotype, or interaction effects found in the two-way ANOVAs. Alpha for all analyses was set to 0.05.

Results

Ad libitum food consumption

To assess BACHD rats' growth and food consumption in a low-stress and social environment, we housed genotype-matched rats in pairs (Figure 2A), and measured their weekly body weight and food consumption. Rats of both genotypes grew steadily during the test, as indicated by the significant effect of age on body weight ($p<0.0001$, $F_{(21,1449)} = 2766$) (Figure 2B). BACHD and WT rats grew at a similar rate, and showed similar body weights through the entire test, with no significant genotype effect or age x genotype interaction. The rats' food consumption also changed with age ($p<0.0001$, $F_{(20,680)} = 110.5$) (Figure 2C). In general, food consumption increased gradually until the age of nine weeks, and then slowly dropped. Importantly, WT and BACHD rats consumed equal amounts of food between six and eight weeks of age, but there were a number of differences seen at older ages. At nine and ten weeks of age, BACHD rats appeared to consume more food that WT rats, although this did not reach statistical significance. Directly following this, food consumption dropped steadily among BACHD rats, while WT rats remained arguably stable until the age of 16 weeks. Due to this, BACHD rats eventually ate less than WT rats, as indicated by the significant results from the *post-hoc* analysis at 17 weeks of age and onwards ($p<0.05$–0.01). The difference in how food consumption changed with age among BACHD and WT rats was also evident in a significant age x genotype interaction ($p<0.0001$, $F_{(20,680)} = 19.06$). Relating food consumption to the rats' body weight gave largely the same results, with a significant age effect ($p<0.0001$, $F_{(60,680)} = 1930$) and age x genotype interaction ($p<0.0001$, $F_{(20,680)} = 12.99$) (Figure 2D). However, this analysis made the increased food intake among young BACHD rats more apparent, with the *post-hoc* test indicating significant differences between BACHD and WT at seven to ten weeks of age ($p<0.01$–0.0001). In contrast, the decreased food consumption among old BACHD rats was less apparent, with the *post-hoc* test only indicating a few significant data points at 18 to 21 weeks of age ($p<0.05$–0.01). It should be noted that BACHD rats produced less food debris compared to WT rats (Figure S1A and B). Correcting for this did not dramatically affect the food consumption phenotype, although the genotype differences became less apparent (Figure S1C). Finally, BACHD rats consumed dramatically less water compared to WT rats (Figure S1D).

Body composition of BACHD rats

In order to assess BACHD rats' body composition, we dissected BACHD and WT rats at five different ages. As expected, older rats weighed more, leading to a significant age effect on body weight ($p<0.0001$, $F_{(4,109)} = 444.1$) (Figure 3A). In line with previous data, there were no differences in body weight between the genotypes in any age group, and also no significant difference in apparent growth. The body composition of BACHD rats was however different from that of WT rats. BACHD rats had significantly lower percentage of bone and muscle ($p<0.001$, all ages), and higher percentage of adipose tissue ($p<0.05$–0.001) in all age groups (Figure 3B). These differences were also apparent when analyzing the absolute weights of the respective tissues. Both WT and BACHD rats gained adipose tissue with age, as indicated by a significant age effect on the weight of total adipose tissue ($p<0.0001$, $F_{(4,109)} = 142$) (Figure 3C). However, BACHD rats carried an excess amount of adipose tissue, as indicated by both a significant genotype effect ($p<0.0001$, $F_{(1,109)} = 81.25$), and significant results from the *post-hoc* analysis of all groups, except the one-month old rats ($p<0.05$–0.0001). There was also a significant age x genotype interaction ($p<0.0001$, $F_{(4,109)} = 7.686$) that was dependent on data from the one and three months old groups. The bone/muscle weight also increased with age for both genotypes ($p<0.0001$, $F_{(4,109)} = 555.4$) (Figure 3D). However, BACHD rats were found to have significantly less bone/muscle tissue compared to WT rats in all but the one-month old age groups. This was indicated both by a significant genotype effect ($p<0.0001$, $F_{(1,109)} = 70.69$), and significant results from the *post-hoc* analysis ($p<0.01$–0.0001). A significant age x genotype interaction ($p<0.001$, $F_{(4,109)} = 4.18$) also indicated that there was a difference in the rats' growth. Importantly, this effect was dependent on the data of the one–month old group.

The rats' body length also increased with age for both genotypes ($p<0.0001$, $F_{(4,109)} = 1517$), although a significant genotype effect ($p<0.0001$, $F_{(1,109)} = 86.46$) and *post-hoc* tests ($p<0.01$–0.0001) revealed that BACHD rats were smaller than WT (Figure 3E). This was apparent in all age groups except the one-month old animals. It should, however, be noted that one-month old BACHD rats were shorter than WT rats when analyzing litter-matched groups (data not shown). The reduced body length among BACHD rats was mainly due to them having shorter tails and heads compared to WT rats (Figure S2).

BACHD rats also showed a lower amount of bone/muscle tissues in relation to their body length (Figure 3F). Rats of both genotypes gained relative amounts of bone and muscle with age ($p<0.0001$, $F_{(4,109)} = 570.6$). However, BACHD rats had lower relative amounts of bone and muscle from three months of age, as evident from a significant genotype effect ($p<0.0001$, $F_{(1,109)} = 47.32$) and *post-hoc* analysis ($p<0.05$–0.0001).

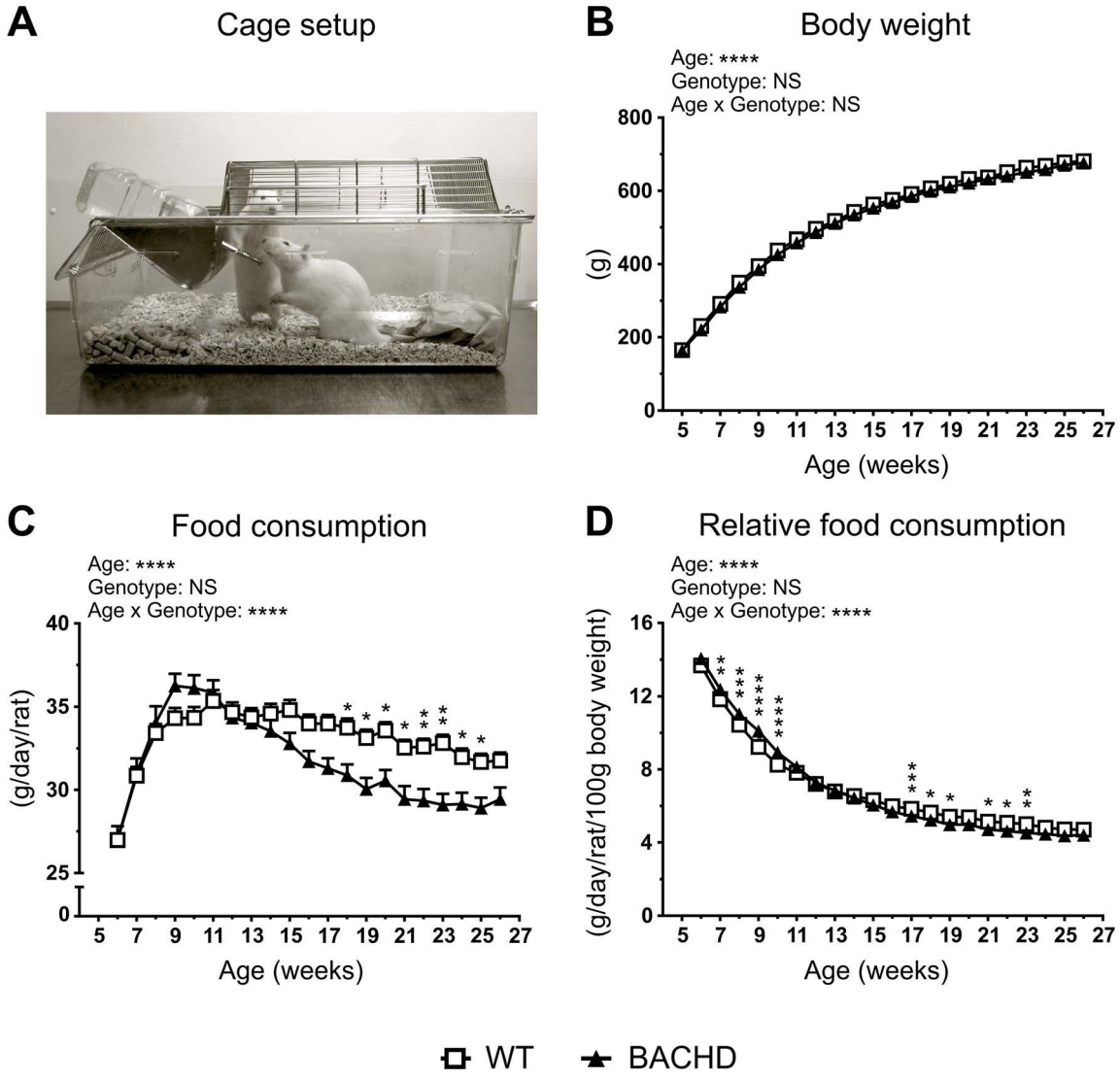

Figure 2. Body weight and food consumption. (**A**) Housing conditions during the *ad libitum* food consumption test. (**B**) Body weight of rats plotted against their age. (**C**) Approximate daily food consumption per rat (calculated from weekly food consumption per cage), plotted against the age of the animals. (**D**) Relative daily food consumption per rat (calculated from weekly food consumption and average body weight per cage), plotted against the age of the animals. The graphs show group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and significant results from *post-hoc* analysis are displayed for individual data points. Genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
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Assessment of hunger during food deprivation of BACHD rats

Two tests based on voluntary consumption of reward pellets and regular food, were run to assess BACHD rats' hunger level at different levels of food deprivation (Figure 4A). When both WT and BACHD rats were deprived to 85% of their respective free-feeding body weights, BACHD rats were found to consume both reward pellets and regular food at a slower rate than WT rats (Figure 4B). In the pellet consumption test, BACHD rats needed longer time to eat the reward pellets ($p<0.01$), but did not spend more time exploring the arena, compared to WT rats. The slower feeding speed led to a significant increase in trial time for BACHD rats (data not shown). In the food consumption test, BACHD rats were found to have eaten less than WT rats at almost all investigated intervals, as evident from the significant genotype effect ($p<0.01$, $F_{(1,6)} = 14.62$), and the significant results from the *post-hoc* analysis ($p<0.05$–0.01). It should be noted that a difference in actual consumption rate was only seen during the first 30 minutes, resulting in an initial difference in the amount of food consumed, which then persisted through the remaining part of the test. This difference in behavior gave a significant time x genotype interaction ($p<0.01$, $F_{(9,54)} = 2.840$) in the amount of food consumed by the rats.

In an attempt to reverse the phenotypes described above, the food deprivation levels were adjusted so that BACHD and WT rats were at 80 and 95% of their respective free-feeding body weights (Figure 4C). In the pellet consumption tests, BACHD rats now needed a similar amount of time to consume the reward pellets, although there was a borderline significant trend towards BACHD rats needing more time ($p = 0.0535$). With the exception of one WT rat, all rats spent the entire trial eating, and showed minimal interest in exploring the test arena. In the food

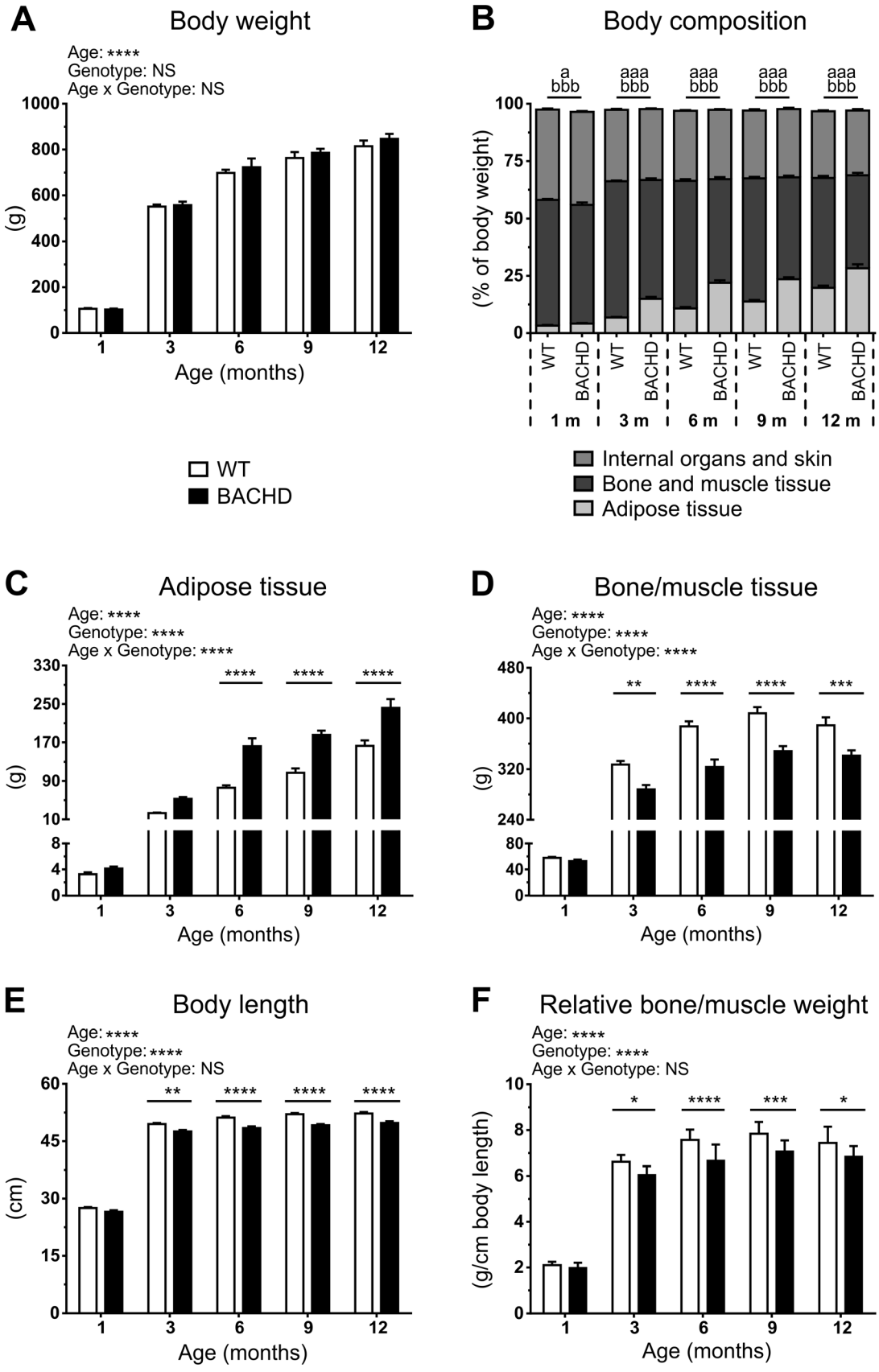
A
Body weight
Age: ****
Genotype: NS
Age x Genotype: NS
(g)
Age (months)
WT
BACHD
B
Body composition
a
bbb
aaa
bbb
(% of body weight)
WT
BACHD
1 m
3 m
6 m
9 m
12 m
Internal organs and skin
Bone and muscle tissue
Adipose tissue
C
Adipose tissue
Age: ****
Genotype: ****
Age x Genotype: ****
D
Bone/muscle tissue
Age: ****
Genotype: ****
Age x Genotype: ****
E
Body length
Age: ****
Genotype: ****
Age x Genotype: NS
(cm)
F
Relative bone/muscle weight
Age: ****
Genotype: ****
Age x Genotype: NS
(g/cm body length)

Figure 3. Body composition assessed through dissection. (**A–F**) Data from the dissection groups as stated in the graph titles. The graphs show group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and significant results from *post-hoc* analysis are displayed inside each graph. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****. For (**B**), ANOVA was not performed, and the indicated differences concern single comparisons between WT and BACHD rats within the age groups. Significant differences are indicated with "a" and "b" for differences in the relative amount of adipose and bone/muscle tissue respectively, written according to the same grading as above.
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consumption tests, BACHD and WT rats consumed food at the same rate during the first 150 minutes. During the remaining part of the test, WT rats ate more, eventually leading to a significant difference in the total amount of food consumed during the test ($p<0.01$). The behavioral differences led to a significant time x genotype interaction effect ($p<0.0001$, $F_{(9,54)} = 8.642$).

In a final test, the food deprivation levels were adjusted so that BACHD and WT rats were at 95 and 80% of their respective free-feeding body weights (Figure 4D). At this point, BACHD rats consumed the reward pellets at the same rate as WT rats, as the aforementioned trend was no longer present. With the exception of two BACHD rats, all rats spent the entire trial eating, and showed minimal interest in exploring the test arena. One BACHD rat did not consume all reward pellets within five minutes. In the food consumption test, BACHD rats were once again found to have consumed less food than WT at all investigated intervals, resulting in a significant genotype effect ($p<0.001$, $F_{(1,6)} = 42.52$), and significant results from the *post-hoc* analysis ($p<0.05$–0.0001). BACHD rats ate at a slower rate during the first hour. The consumption rate gradually declined among WT rats, while it gradually increased among BACHD rats, ending up at similar levels after 150 minutes. This difference in behavior gave a significant time x genotype interaction ($p<0.0001$, $F_{(9,54)} = 8.47$) in the amount of food consumed by the rats.

A more detailed analysis of the results was performed with the aim of better assessing the impact of food deprivation levels on the consumption rate in the two tests. Separate two-way ANOVA analysis of the time needed to consume 100 reward pellets, using genotype as between-subject factor, and either food deprivation level or the number of test sessions as within-subject factor, revealed similar statistical results (Figure 5A). In either case, there was a significant genotype effect ($p<0.05$, $F_{(1,21)} = 5.476$), and performance on the first session, where both genotypes were deprived to 85%, differed significantly between genotype groups ($p<0.05$). Both analyses also revealed a significant effect of their respective within-subject parameter ($p<0.01$, $F_{(2,42)} = 7.861$ and 6.6333 for session and deprivation level, respectively). However, inspection of the graphed data indicated that the time needed to consume the reward pellets did not clearly decrease with increasing food deprivation levels, but did so with increased numbers of test sessions. Performing the same analyses on the amount of food consumed during the first 30 minutes of the food consumption test revealed different results (Figure 5B). Both analyses once again revealed a significant genotype effect ($p<0.01$, $F_{(1,6)} = 15.59$), and significant effects of their respective within-subject parameters ($p<0.01$, $F_{(2,12)} = 8.220$ and 17.04 for session and deprivation level, respectively). *Post-hoc* analysis of data analyzed in terms of food deprivation level revealed a significant difference in consumption rate when rats of both genotypes were deprived to 85% of their free-feeding body weight. This was also found when analyzing the data in terms of the number of test sessions given to the rats, although that analysis also revealed a significant difference in consumption rate during the third session. In contrast to the results from the pellet consumption test, the consumption rate in the food consumption test appeared to gradually increase with an increased food deprivation level, while not showing any gradual change during repeated testing.

Progressive ratio performance during different levels of food deprivation

To better assess differences in the motivational state among the rats, a progressive ratio test was run with two different food deprivation settings. All rats learned to push the lever in order to obtain a reward pellet, although there were some discrete behavioral differences between WT and BACHD rats during the initial training steps. During habituation, BACHD rats made fewer entries into the pellet receptacle (Figure S3A, B) and were initially slower at retrieving the pellets (Figure S3C). During CRF, FR3 and FR5 training, BACHD rats were generally slower at both retrieving the pellets, and returning to the reinforced lever (Figure S4 and S5).

During the fixed ratio part of the PR protocol, BACHD rats were still slower at retrieving the reward pellets, but they no longer showed an increase in lever return latencies (Figure S6). These results were largely unaffected when food deprivation levels were adjusted. WT rats tended to take longer time to complete the FR5 ratios, although this became significant only after adjustment of their deprivation level (Figure S6). Importantly, there were no overt differences between genotypes in the overall response frequency on the rewarded lever during the fixed ratios (Figure S6). The same was true for the mean number of lever pushes made on the non-reinforced lever during the entire PR session (Figure S7).

Analysis of how the rats reached a series of break points, when all were deprived to 85% of their free-feeding body weight, revealed both a significant genotype effect ($p<0.01$, $F_{(1,22)} = 10.66$) and differences in the three highest break points ($p<0.01$), with BACHD rats reaching lower ratios (Figure 6A). These differences were not present when the food deprivation level of WT rats had been adjusted so that their food consumption rate matched that of BACHD rats. Similarly, when all rats were deprived to 85% of their free-feeding body weight, BACHD rats responded with more pronounced drops in motivation during prefeeding of both reward pellets and regular food, as indicated by significant genotype effects ($p<0.01$, $F_{(1,22)} = 9.461$ and $p<0.01$, $F_{(1,21)} = 8.343$ for reward pellet and regular food prefeeding, respectively) and prefeeding x genotype interactions ($p<0.001$, $F_{(2,44)} = 11.19$ and $p<0.05$, $F_{(1,21)} = 8.341$ for reward pellet and regular food prefeeding, respectively) (Figure 6B). Once again, these phenotypes were not present when the food deprivation level of WT rats had been adjusted, leading to identical responses in the prefeeding tests. It should be noted that only the last break point, break point 600, was suitable for prefeeding analysis. Prefeeding induced a strong interest in water among WT rats, which dramatically affected their early break points (data not shown). It should also be noted that there was a significant difference in body weight once the food deprivation levels had been adjusted, with WT rats being significantly heavier than BACHD rats (data not shown). The WT rats weighed roughly 50 g more than BACHD rats, resulting in them being at 95% of their free-feeding body weight.

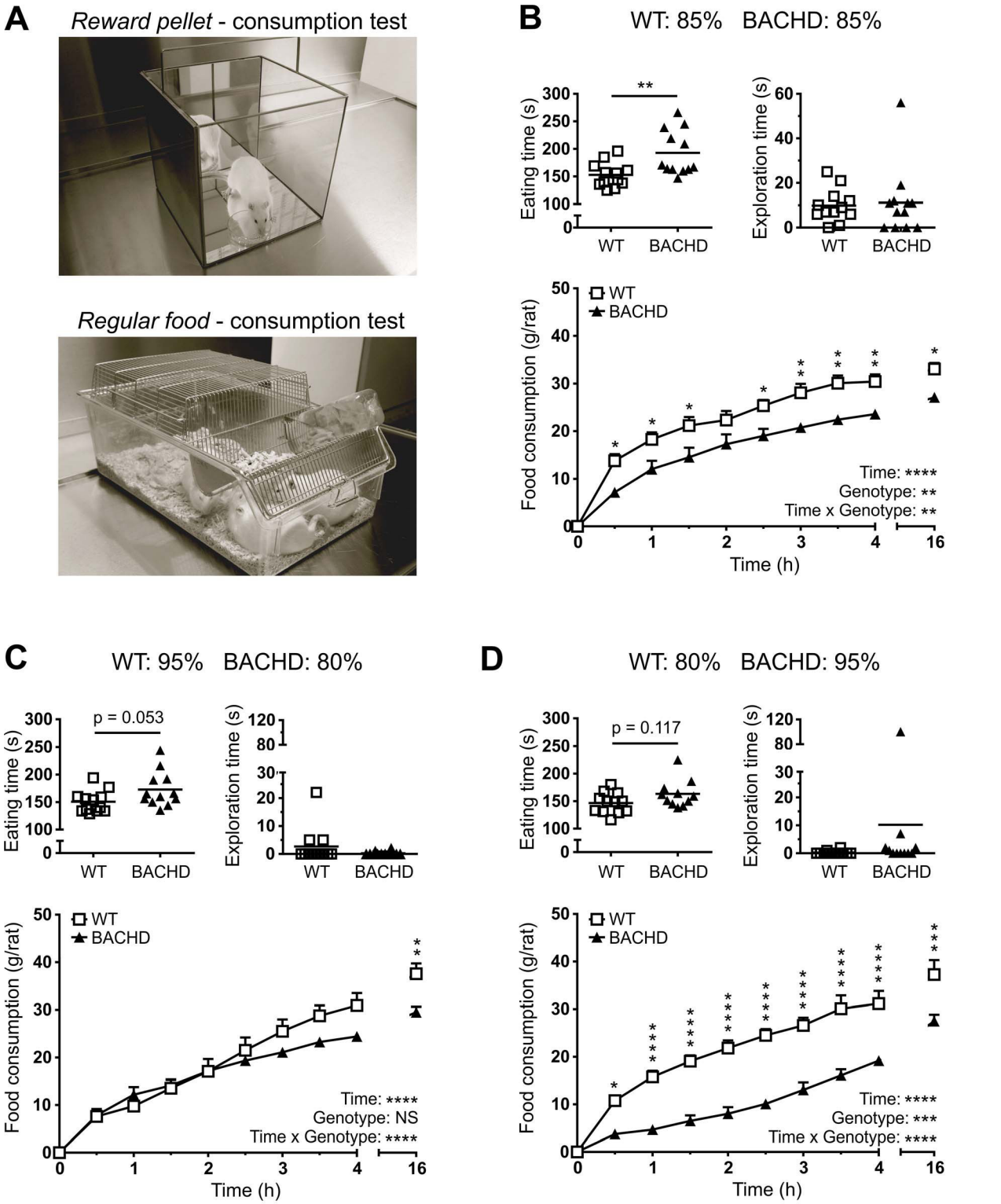

Figure 4. Hunger and food interest assessment. Setups (**A**) and performance in the two consumption tests during the first (**B**), second (**C**) and third test session (**D**), with the different food deprivation levels stated in the title of each figure panel. The time needed to eat 100 reward pellets and the time spent exploring in the reward pellet consumption setup, are displayed in the top left and right graphs of each panel, respectively. The bottom graph of each panel shows the cumulative food consumed per rat during the regular food consumption test. Scatter plots for reward pellet consumption test results indicate individual values and group mean. Line graphs for regular food consumption indicate group mean plus standard error of the mean. Statistical test results are given inside the graphs. For the regular food consumption test, two-way ANOVA results are displayed in the bottom right corner, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
doi:10.1371/journal.pone.0105662.g004

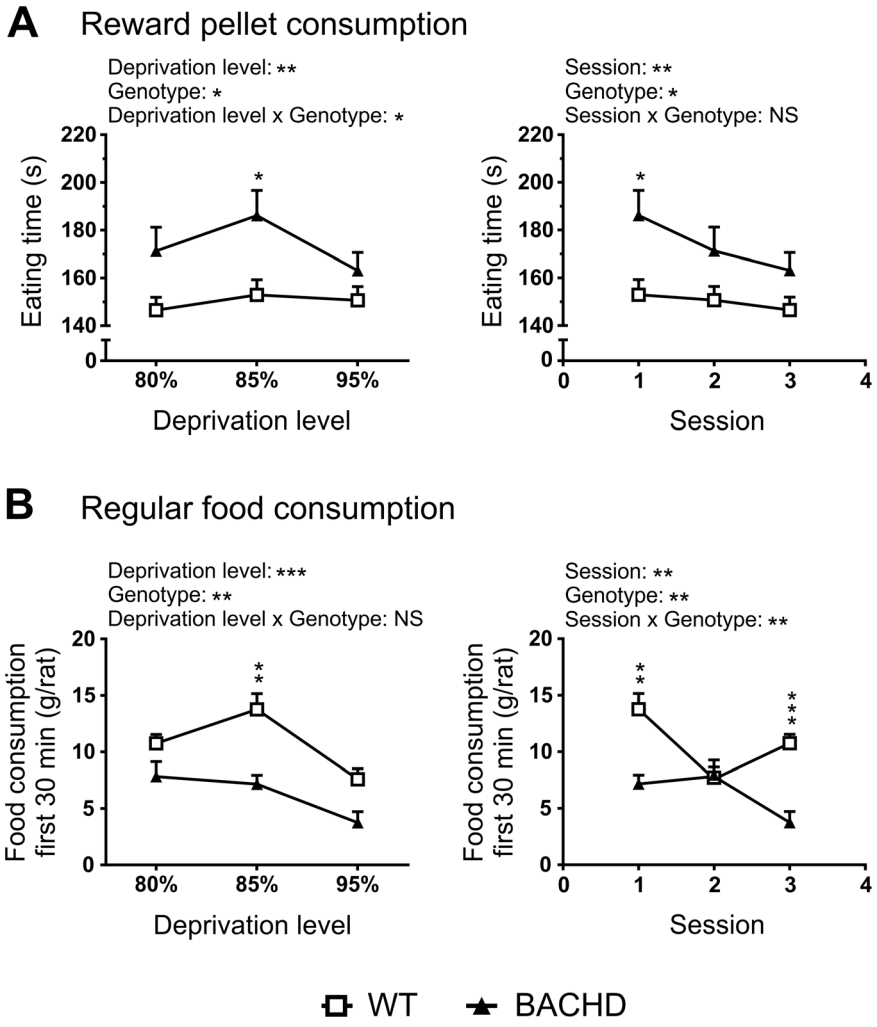

Figure 5. Impact of repeated testing and food deprivation on consumption tests. (**A**) The time needed to consume 100 reward pellets is plotted against the deprivation level (left graph) and session number (right graph). (**B**) The food consumed during the first 30 minutes of the regular food consumption test is plotted against the deprivation level (left graph) and session number (right graph). The graphs show mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
doi:10.1371/journal.pone.0105662.g005

Discussion

Body composition and food intake of BACHD rats

Many transgenic animal models of HD show an altered body weight compared to their WT littermates. Animals that express a fragment of the disease-causing gene typically have a reduced body weight [25,26,27], while the ones that express the full-length gene typically have an increased body weight [10,11]. We show here, that although BACHD rats did not differ from WT rats in terms of body weight, they displayed several changes in body composition. Strikingly, BACHD rats carried an excess amount of adipose tissue. This is in line with phenotypes of other full-length models of HD, as the increased body weight of BACHD and YAC128 mice has been shown to at least in part be due to an increase in adipose tissue mass [28,29]. It should be pointed out that R6/2 and N171-82Q mice, which only express a fragment of the disease-causing gene, also carry excess amounts of adipose tissue [25,30]. R6/2 mice have further been shown to maintain this increased fat mass even when they start to lose weight [25]. Thus, the increase in adipose tissue seems to be a common phenotype of transgenic HD models, although it does not always result in obesity.

Increased amounts of adipose tissue could theoretically be the result of increased food intake, decreased home cage activity, metabolic disturbances, or a combination of the three. While BACHD mice have been shown to eat more than their WT littermates [28], R6/2 and YAC128 mice have been found to have unchanged food intake [25,29]. A previous study on BACHD rats, in which food intake was followed from three to eighteen months of age, indicated that the transgenic rats ate less than their WT littermates [22]. These results were well reproduced here, despite the different housing conditions. The current study also assessed food intake at ages younger than three months, where BACHD rats appeared to consume more food compared to WT rats. It should be noted, however, that the appearance of the food consumption phenotypes was to some degree dependent on whether or not the weight of the consumed food was normalized to the animals' body weight. The aim of this normalization was to relate the rats' food intake to a measurement of their body size, and through this investigate if the reduced food intake among

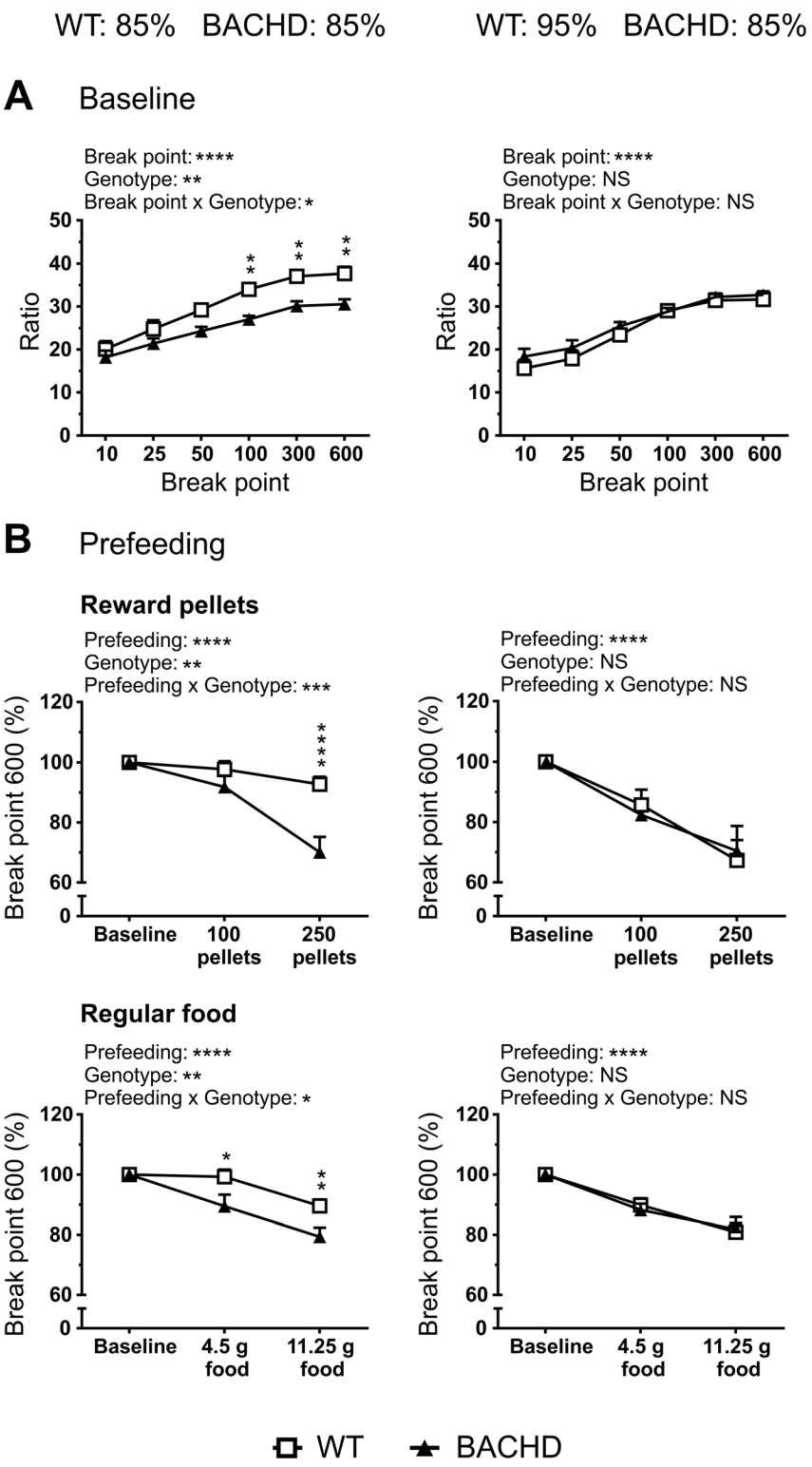

Figure 6. Progressive ratio test performance. Performance in the PR test is shown for when animals of both genotypes were deprived to 85% of their free-feeding body weight (graphs to the left in each figure panel) and when the deprivation level of WT rats had been adjusted to achieve equal food consumption rates between genotypes (graphs on the right of each figure panel). (**A**) Baseline performance during six consecutive PR sessions preceding the prefeeding tests. The ratio, where a given break point was reached, is indicated. (**B**) Performance during prefeeding with reward pellets (top panel) and regular food (bottom panel). The drop in motivation is displayed as percentage of baseline performance for break point 600. The graphs show group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.

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BACHD rats could be due to them being smaller than WT rats. Using body weight as an approximation of body size is, however, probably only suitable at young ages, as the body weight of older BACHD rats is distorted due to obesity. Thus, further studies are needed to reach conclusions on this matter. In addition, as food intake phenotypes are unlikely to explain the increase in adipose tissue, metabolic parameters of BACHD rats need to be further characterized. In this regard, it is important to note that the obesity phenotype of BACHD mice was abolished when the expression of mutant Huntingtin was silenced in the hypothalamus [28]. Interestingly, hypothalamic lesions can induce obesity that is not always associated with increased food intake, but can persist despite unchanged or even reduced food intake [31,32,33,34,35]. The differential effects appear to depend on which specific neuronal population is damaged [35,36], which might relate to the common phenotype of increased fat mass, but varied food intake seen across HD animal models.

In the current study, BACHD rats were shown to have a smaller body size and disproportionately lower amount of bone/muscle tissue compared to WT rats. Information about similar parameters is scarce for other HD models, although YAC128 mice have been shown to have unchanged lean body mass [29], while R6/2 mice show a progressive reduction in lean body mass as they age [25]. These are both in contrast to the bone/muscle phenotype seen in BACHD rats, as the lower amount of bone/muscle tissue seen in the current study did not seem to progress with age. Instead, the body size and bone/muscle phenotypes seen in the BACHD rats appeared to be caused by discrete developmental deficits and stunted growth. It is unlikely that these phenotypes were the result of malnutrition during testing, as food was available *ad libitum* on the cage floor. It is possible, however, that BACHD pups might have had difficulties when competing for mothers' milk, leading to malnutrition at early ages. Such factors have been shown to affect the growth of animals from large litters [37]. Alternatively, the growth of BACHD rats might be disturbed on a molecular level, as Huntingtin has been shown to be important during fetal development [38]. The fact that BACHD rats had smaller heads compared to WT rats is particularly interesting, as similar symptoms have been seen in HD gene-carriers [39]. Thus, the discrete developmental deficits found in the BACHD rats might be closely connected to developmental deficits of human patients.

Food deprivation and motivation of BACHD rats

Behavioral assessment of HD animal models through the use of operant conditioning tests is of interest, as cognitive symptoms are common in HD patients and might become valuable to clinically track disease progression and treatment effects [40,41,42]. Many conditioning protocols require food deprivation in order to both efficiently train the animals to perform a given task and to maintain high performance. However, food deprivation of HD models requires extra care as they can be expected to have changes in body composition. To better understand how to optimally food deprive BACHD rats, we assessed their interest in food in a total of three different tests.

Free intake of reward pellets and regular food is sometimes used to assess an animal's hunger level and interest in food [18,19,20,21]. In the current study, WT and BACHD rats deprived to 85% of their free-feeding body weight did not seem to differ in their interest in consuming 100 reward pellets, although BACHD rats needed more time to eat all pellets. Food deprivation levels were then adjusted in an attempt to reverse the phenotypes, however, this did not seem to affect the rats' behavior. Instead, both the time spent exploring the arena and the time needed to consume all pellets decreased with repeated testing. The training effect on the consumption rate eventually led to BACHD rats consuming the reward pellets at an equal rate compared to WT rats. There were indications that rats deprived to 95% of their free-feeding body weight spent more time exploring the arena compared to rats deprived to 80%, but this generally concerned one or two rats of an entire group of twelve. As the current protocol did not appear to be sensitive even to large changes in food deprivation levels, it is unlikely to be a suitable test for assessing discrete differences in food interest. It is also clear that the apparent training effect could be misinterpreted as a food deprivation effect, if one assessed a given group of animals repeatedly with the aim of gradually adjusting their food deprivation level. The slowed consumption speed seen among BACHD rats in the pellet consumption test is, however, an interesting phenotype on its own. While eating, rats typically stood on all four paws and used their tongue to pick up the pellets. Thus, the slower feeding rate among BACHD rats is likely due to impairments in quite basic processes that are needed for eating. These could include impaired chewing, swallowing or tongue movements as well as reduced saliva production. It is tempting to hypothesize that the slower feeding speed among BACHD rats could be due to phenotypes similar to the tongue protrusion symptoms that are often seen among HD patients [43,44]. Interestingly, there are protocols for measuring tongue protrusion [45] in rats, although these tests must be performed carefully, as the smaller head size of BACHD rats likely means that they have shorter tongues as well.

In the regular food consumption test, BACHD rats consumed less food than WT rats when both groups were deprived to 85% of their respective free-feeding body weight. Consumption rate during the first 30 minutes of the test changed in a predictable way when deprivation levels were adjusted, with more deprived rats eating at a faster rate. This suggests that the protocol was well suited for the assessment of food interest and hunger levels. Our results further showed that when BACHD and WT rats were deprived to 80 and 95% of their respective free-feeding body weights, they consumed food at an identical rate for the initial 150 minutes, indicating that the rats were equally hungry. As the test session continued, BACHD rats once again ate less than WT rats, which likely reflected differences in the rats' satiety levels. It should be noted that the feeding behavior of either genotype did not significantly differ when comparing their 80 and 85% food deprivation test sessions. Thus, although the test seems suitable to assess food interest, it does not appear to be very sensitive. Assessing food consumption in single animals, rather than in groups, would most likely improve the test's sensitivity. It would further allow separate scoring of the time spent eating and the time spent not eating, as it was done in the reward pellet consumption test. However, despite extensive habituation, we have found it difficult to get our rats to efficiently consume regular food in any other setup than their home cages. As the test did not allow separate scoring of the time the rats spent feeding and doing other activities, it was not possible to conclude if the difference in consumption rate was strictly due to a difference in hunger and food interest. This idea is especially difficult to support when considering the results of the pellet consumption test. In an attempt to reach a conclusion on the matter, we ran a PR test with prefeedings.

When both WT and BACHD rats were deprived to 85% of their respective free-feeding body weight, BACHD rats were clearly less motivated to work for food rewards in the PR test. Similar phenotypes have been found in other HD models [16,46] and they are typically discussed in terms of apathy, which is a common symptom among HD patients [47,48]. However,

BACHD rats also responded with more pronounced drops in motivation during the prefeeding tests, which would typically be interpreted as BACHD rats being less hungry compared to WT rats [49,50,51]. This would also support the idea that the BACHD rats' lower consumption rate in the first session of the food consumption test was to some degree caused by lower hunger and food interest. When the food deprivation level of WT rats was adjusted to achieve equal food consumption rates to those of the BACHD rats, all genotype differences that were previously seen in the PR test disappeared. As WT and BACHD rats did not differ during prefeeding tests, it is reasonable to assume that they were equally hungry and that the food consumption test was suitable for establishing food deprivation levels that ensured this. As they also no longer differed in baseline performance, the motivational deficit seen in the first PR test was likely dependent on a difference in hunger levels, rather than an apathy-related phenotype. It is interesting to note that after the food deprivation levels had been adjusted, BACHD rats weighed approximately 50 g less than WT rats. This difference was similar to the one found in bone/muscle tissue, suggesting that WT and BACHD rats carried a similar amount of adipose tissue. Secretion of leptin, which affects satiety and food intake [52,53], is proportional to adipose tissue mass [54], and it is possible that the food deprivation adjustment led to equal hunger and food interest due to equal levels of leptin. Importantly, higher leptin levels have been shown to reduce motivation in PR tests [55], which gives a possible explanation for the initial motivational difference.

Most of the conclusions above are based on the idea that prefeeding responses depend exclusively on hunger levels and not on other aspects of motivation. One could argue that animals that suffer from motivational deficits not related to hunger, might also respond stronger on the prefeeding tests. Thus, seeking a situation where animals respond equally to prefeeding could in itself lead to the lack of differences in PR performance. It is therefore important to note that other studies have found motivational differences despite identical responses on prefeeding tests [51], and that motivational deficits have been found in BACHD mice after adjusting deprivation levels until animals consumed food at the same rate [16]. It should also be noted that the true nature of the motivational phenotype seen here is mainly of importance when such phenotypes are being characterized. If one simply wishes to minimize motivational differences when working with BACHD rats, regardless if these are due to hunger levels or other aspects of motivation, adjusting deprivation levels so that WT and BACHD rats consume regular food at a comparable rate should suffice. Still, the current study only considered quite young animals. It is possible that older BACHD rats suffer from motor impairments that could affect the validity of the food consumption test. Also, motivational phenotypes not related to hunger might become apparent among older BACHD rats. We aim at addressing these ideas in a longitudinal study of PR performance.

Summary

In the current study, BACHD rats were found to have metabolic disturbances, which is in line with other animal models of HD. We further found that unless these phenotypes were taken into consideration during food deprivation, BACHD rats were less motivated than WT rats in a progressive ratio test. Thus, metabolic phenotypes are important to consider as possible confounding factors when assessing apathy-related phenotypes of BACHD rats. The same is likely true for other HD animal models with metabolic abnormalities.

Our results further indicated that basing the animals' food deprivation levels on their consumption rates of regular food was a convenient way to avoid motivational differences between BACHD and WT rats. Thus, previous studies that applied this method when studying apathy in HD animal models [16] likely avoided hunger-based motivational differences, and our results support the future use of this method. It is also important to consider its use in behavioral tests where the main readout is not directly related to apathy or motivation, such as [17], as motivational differences have been shown to affect animals' behavior in such tests too [15].

Supporting Information

Figure S1 Food debris and water consumption during the *ad libitum* food consumption test. (**A**) The approximate daily amount of food debris produced per cage (calculated from a three- to four-day average), plotted against the age of the rats. (**B**) The approximate amount of food debris per cage relative to the average food consumption per cage, plotted against the age of the rats. (**C**) The approximate daily food consumption per rat (calculated from the weekly food consumption per cage) after accounting for food debris left in the cages, plotted against the age of the rats. (**D**) The approximate daily water consumption per rat (calculated from the weekly water consumption per cage), plotted against the age of the rats. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****. For (**D**), WT and BACHD rats differed highly significant (****) for all data points between 11 and 26 weeks of age.
(TIF)

Figure S2 Body length measurements. (**A–D**) Data from length measurement as stated in the graph titles. The graphs show group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and significant results from *post-hoc* analysis are displayed inside each graph. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

Figure S3 Habituation to the operant conditioning boxes. (**A**) The total number of head entries made into the pellet receptacle during habituation sessions. (**B**) The total time spent with the head inside of the pellet receptacle during habituation sessions as a measurement of the duration of receptacle visits. (**C**) The mean latency to enter the pellet receptacle after the delivery of a reward pellet. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

Figure S4 Performance on the CRF protocol. Results from the final session of CRF training are shown as indicated by graph titles. Session duration measured the time the rats needed to complete 100 ratios. Retrieval latency measured the time between the release of the reinforced lever and the entry into the pellet receptacle. Lever return latency was defined as the interval between the first receptacle entry following reward delivery and the lever push that followed. Graphs indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Significant genotype

differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

Figure S5 Performance on fixed ratio protocols. Results for several basic parameters of FR3 and FR5 protocols are shown as indicated by the graph titles. Session duration measured the time the rats needed to complete 100 ratios. Ratio duration measured the time between the first and last lever push of each ratio. Ratio interval was defined as the time between the last lever push of one ratio and the first lever push of the ratio that followed. Retrieval latency measured the time between the release of the reinforced lever and the entry into the pellet receptacle. Lever return was defined as the interval between the first receptacle entry following reward delivery and the first lever push of the ratio that followed. Scatter plots of FR3 results indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Only results from the final session, where rats performed at criterion, are displayed. Line graphs of FR5 results indicate group mean plus standard error of the mean, plotted against the training session. Only the three final sessions, where rats performed at criterion, are included. Two-way ANOVA results are displayed at the top right corner of each FR5 graph, and significant results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

Figure S6 Performance on the fixed ratio part of the progressive ratio protocol. Results for the basic parameters of the ten FR5 ratios run at the start of each PR session. (**A**) Data from sessions where BACHD and WT rats were both deprived to 85% of their respective free-feeding body weights. (**B**) Data from sessions where food deprivation was adjusted to match the food consumption rate of BACHD and WT rats. Details for each parameter are described in the figure legend of Figure S4 and S5. Lever push frequency was calculated based on the pushes made on the reinforced lever during the full length of a ratio, i.e. the ratio duration plus interval to subsequent ratio. Results displayed were obtained from the sessions used for baseline curves in Figure 6A. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed at the top right corner of each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

Figure S7 Mean number of errors for the fixed ratio part of the progressive ratio protocol. Errors made by the rats during the ten FR5 ratios run at the start of each PR session. (**A**) Data from sessions where BACHD and WT rats were both deprived to 85% of their respective free-feeding body weights. (**B**) Data from sessions where food deprivation was adjusted to match the food consumption rate of BACHD and WT rats. Results were obtained from the sessions used for baseline curves in Figure 6A. Graphs indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Significant genotype differences are indicated by ($p<0.05$) *, ($p<0.01$) **, ($p<0.001$) *** and ($p<0.0001$) ****.
(TIF)

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Author Contributions

Conceived and designed the experiments: EKHJ LEC OR HPN. Performed the experiments: EKHJ LEC. Analyzed the data: EKHJ LEC. Contributed to the writing of the manuscript: EKHJ LEC HPN.

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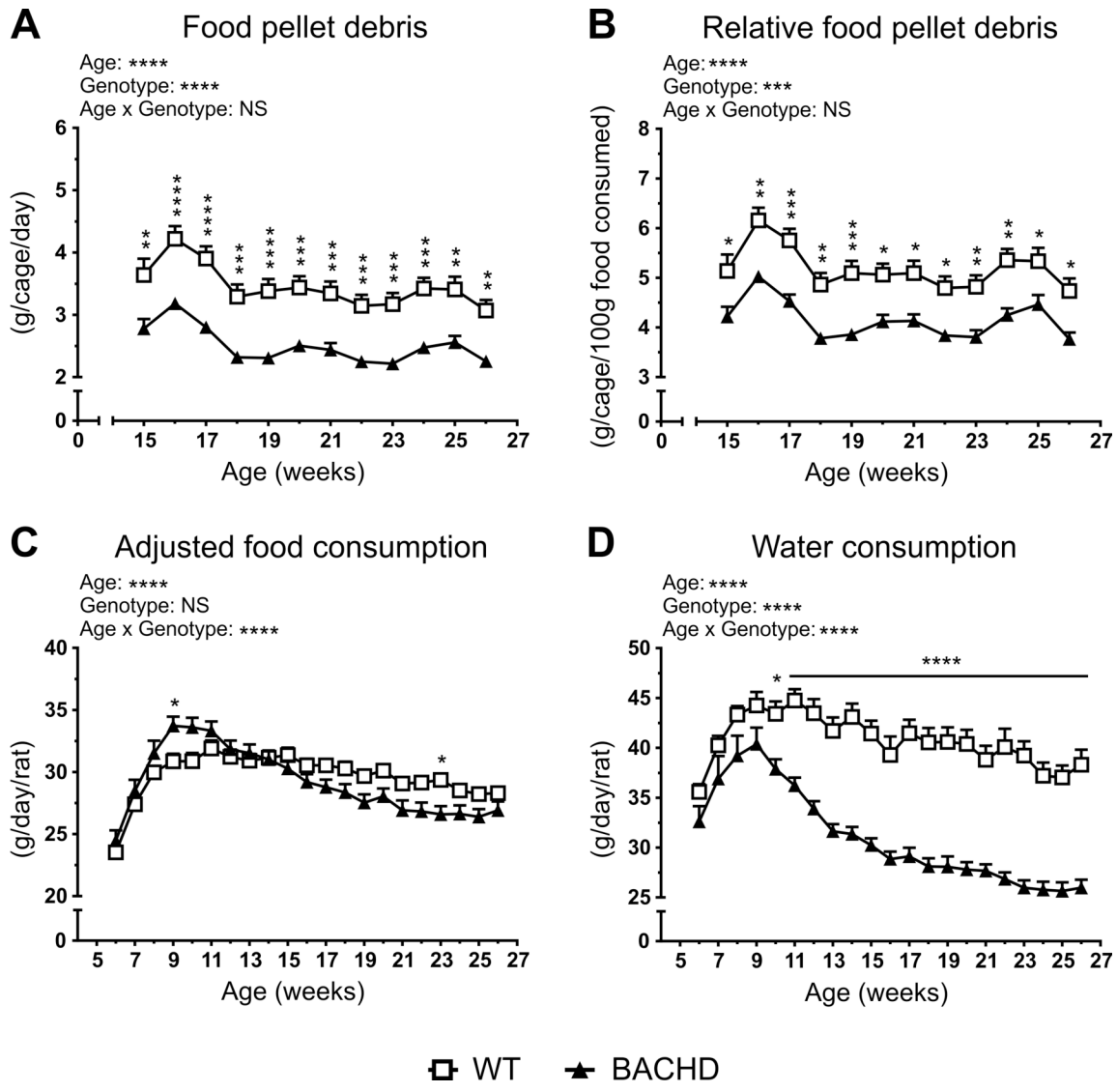
A
Food pellet debris
Age: ****
Genotype: ****
Age x Genotype: NS
(g/cage/day)
Age (weeks)
B
Relative food pellet debris
Age: ****
Genotype: ***
Age x Genotype: NS
(g/cage/100g food consumed)
Age (weeks)
C
Adjusted food consumption
Age: ****
Genotype: NS
Age x Genotype: ****
(g/day/rat)
Age (weeks)
D
Water consumption
Age: ****
Genotype: ****
Age x Genotype: ****
(g/day/rat)
Age (weeks)
WT
BACHD

Figure S1. Food debris and water consumption during the *ad libitum* food consumption test. (A) The approximate daily amount of food debris produced per cage (calculated from a three- to four-day average), plotted against the age of the rats. **(B)** The approximate amount of food debris per cage relative to the average food consumption per cage, plotted against the age of the rats. **(C)** The approximate daily food consumption per rat (calculated from the weekly food consumption per cage) after accounting for food debris left in the cages, plotted against the age of the rats. **(D)** The approximate daily water consumption per rat (calculated from the weekly water consumption per cage), plotted against the age of the rats. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****. For **(D)**, WT and BACHD rats differed highly significant (****) for all data points between 11 and 26 weeks of age.

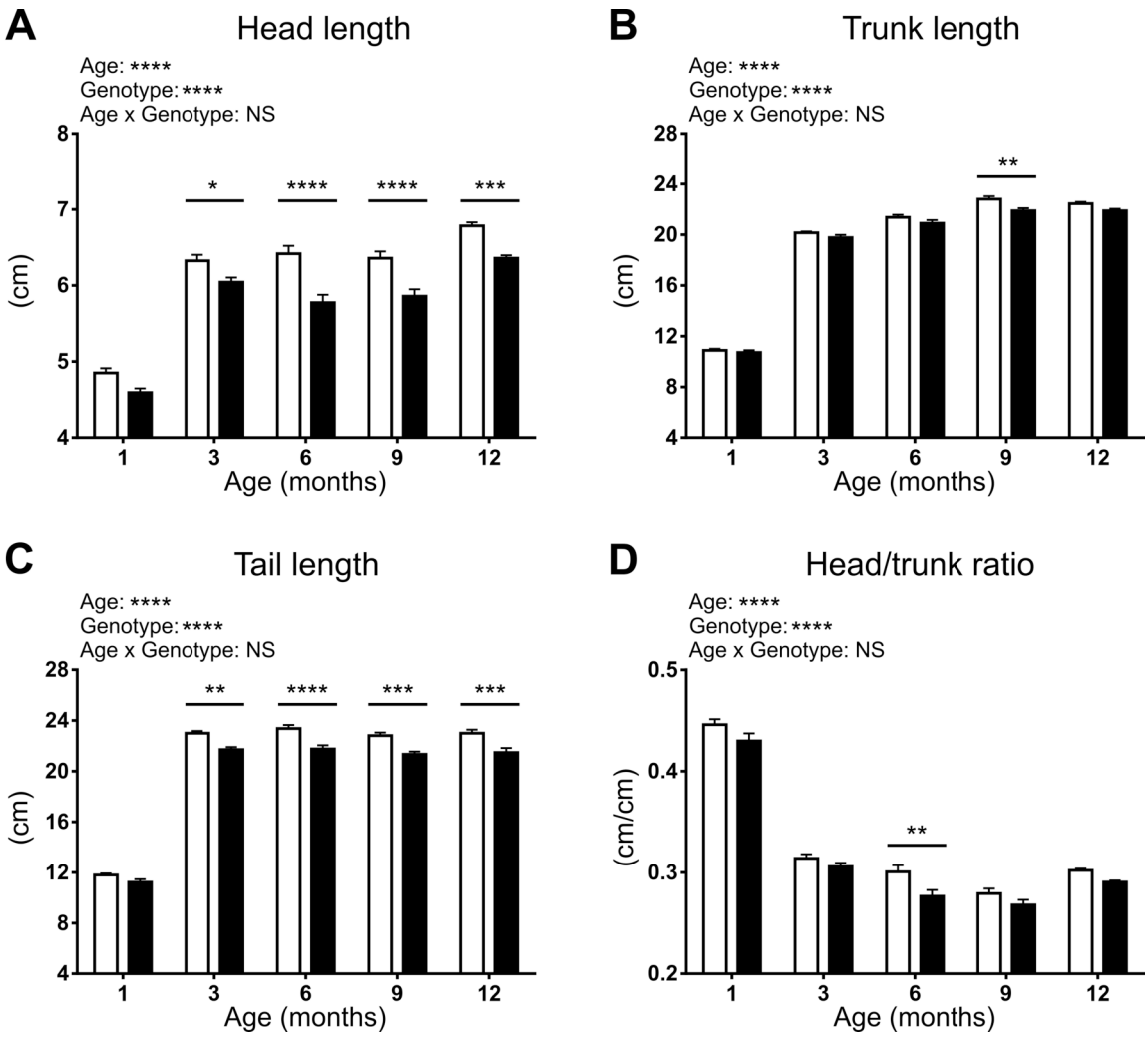
A
Head length
Age: ****
Genotype: ****
Age x Genotype: NS
(cm)
Age (months)
B
Trunk length
Age: ****
Genotype: ****
Age x Genotype: NS
(cm)
Age (months)
C
Tail length
Age: ****
Genotype: ****
Age x Genotype: NS
(cm)
Age (months)
D
Head/trunk ratio
Age: ****
Genotype: ****
Age x Genotype: NS
(cm/cm)
Age (months)

Figure S2. Body length measurements. (A-D) Data from length measurement as stated in the graph titles. The graphs show group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and significant results from *post-hoc* analysis are displayed inside each graph. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

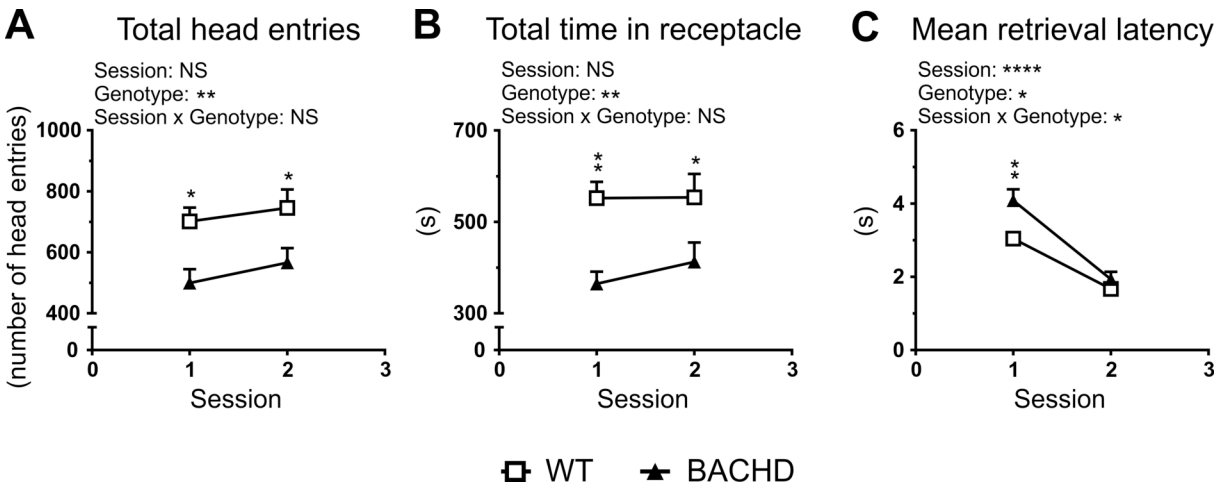
A
Total head entries
Session: NS
Genotype: **
Session x Genotype: NS
(number of head entries)
1000
800
600
400
0
0
1
2
3
Session
B
Total time in receptacle
Session: NS
Genotype: **
Session x Genotype: NS
(s)
700
500
300
0
0
1
2
3
Session
C
Mean retrieval latency
Session: ****
Genotype: *
Session x Genotype: *
(s)
6
4
2
0
0
1
2
3
Session
WT
BACHD

Figure S3. Habituation to the operant conditioning boxes. (A) The total number of head entries made into the pellet receptacle during habituation sessions. **(B)** The total time spent with the head inside of the pellet receptacle during habituation sessions as a measurement of the duration of receptacle visits. **(C)** The mean latency to enter the pellet receptacle after the delivery of a reward pellet. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

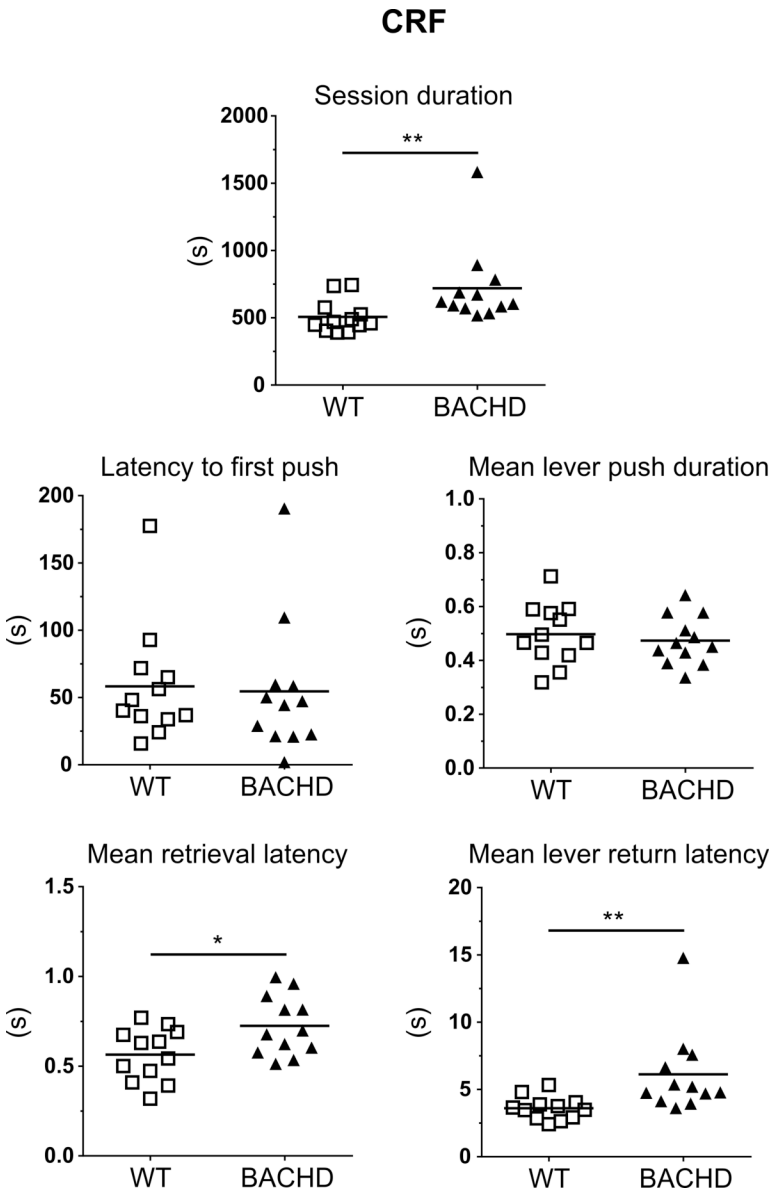
CRF
Session duration
2000
1500
1000
500
0
(s)
**
WT
BACHD
Latency to first push
200
150
100
50
0
(s)
WT
BACHD
Mean lever push duration
1.0
0.8
0.6
0.4
0.2
0.0
(s)
WT
BACHD
Mean retrieval latency
1.5
1.0
0.5
0.0
(s)
*
WT
BACHD
Mean lever return latency
20
15
10
5
0
(s)
**
WT
BACHD

Figure S4. Performance on the CRF protocol. Results from the final session of CRF training are shown as indicated by graph titles. Session duration measured the time the rats needed to complete 100 ratios. Retrieval latency measured the time between the release of the reinforced lever and the entry into the pellet receptacle. Lever return latency was defined as the interval between the first receptacle entry following reward delivery and the lever push that followed. Graphs indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

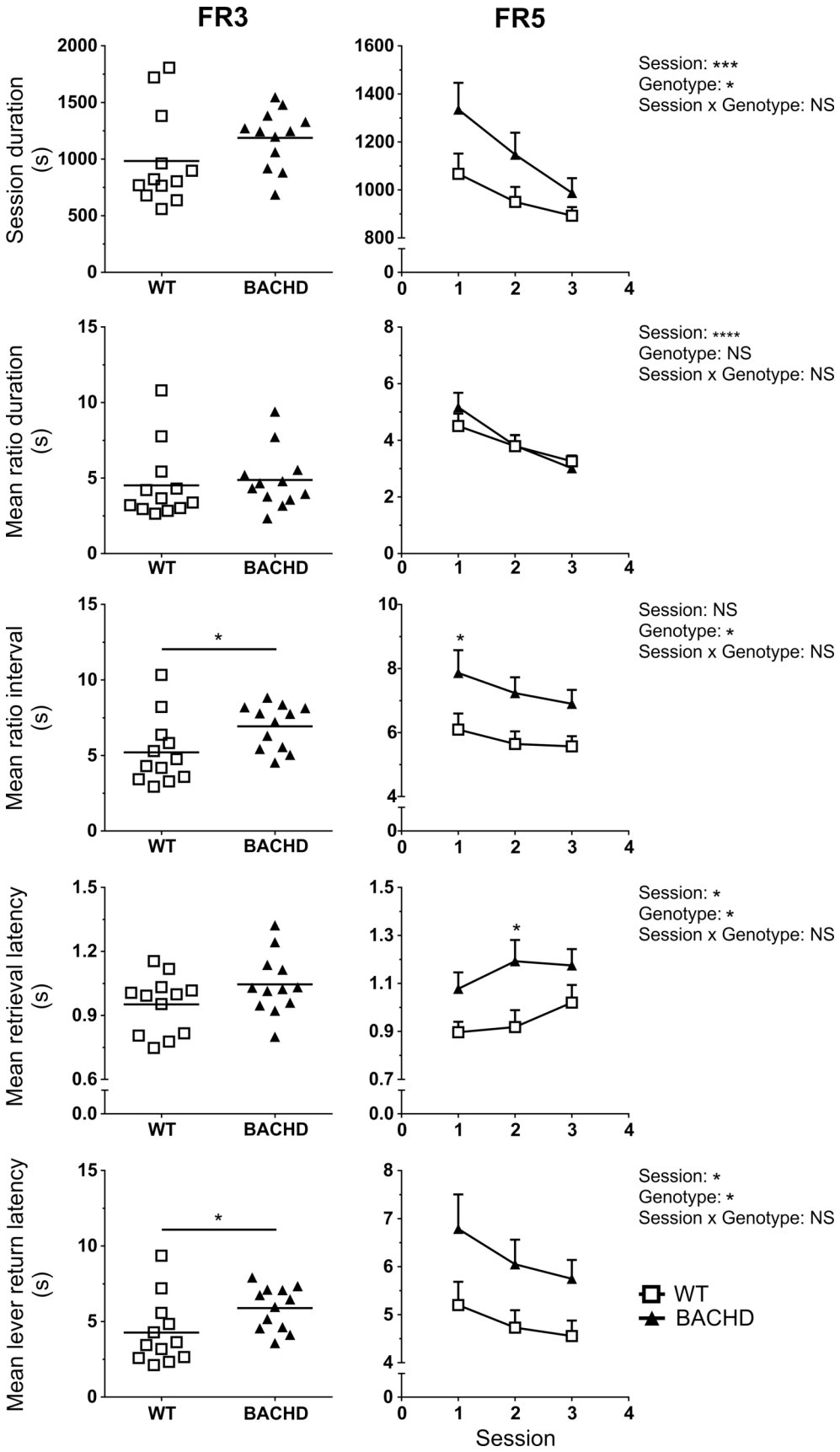
FR3
FR5
Session duration (s)
Mean ratio duration (s)
Mean ratio interval (s)
Mean retrieval latency (s)
Mean lever return latency (s)
WT
BACHD
Session
Session: ***
Genotype: *
Session x Genotype: NS
Session: ****
Genotype: NS
Session x Genotype: NS
Session: NS
Genotype: *
Session x Genotype: NS
Session: *
Genotype: *
Session x Genotype: NS
Session: *
Genotype: *
Session x Genotype: NS
WT
BACHD

Figure S5. Performance on fixed ratio protocols. Results for several basic parameters of FR3 and FR5 protocols are shown as indicated by the graph titles. Session duration measured the time the rats needed to complete 100 ratios. Ratio duration measured the time between the first and last lever push of each ratio. Ratio interval was defined as the time between the last lever push of one ratio and the first lever push of the ratio that followed. Retrieval latency measured the time between the release of the reinforced lever and the entry into the pellet receptacle. Lever return was defined as the interval between the first receptacle entry following reward delivery and the first lever push of the ratio that followed. Scatter plots of FR3 results indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Only results from the final session, where rats performed at criterion, are displayed. Line graphs of FR5 results indicate group mean plus standard error of the mean, plotted against the training session. Only the three final sessions, where rats performed at criterion, are included. Two-way ANOVA results are displayed at the top right corner of each FR5 graph, and significant results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

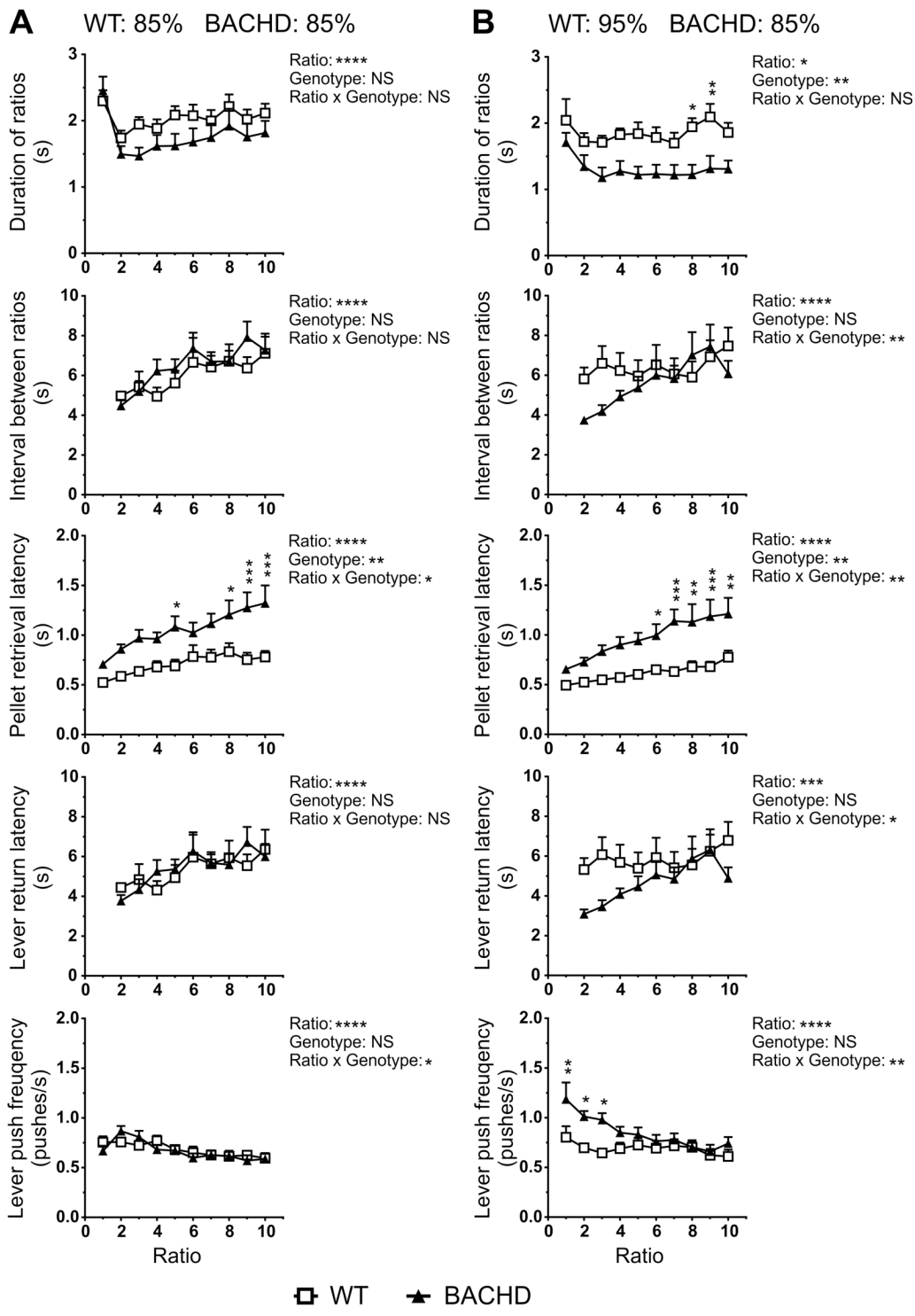
A
WT: 85% BACHD: 85%
B
WT: 95% BACHD: 85%
Duration of ratios (s)
Ratio: ****
Genotype: NS
Ratio x Genotype: NS
Ratio: *
Genotype: **
Ratio x Genotype: NS
Interval between ratios (s)
Ratio: ****
Genotype: NS
Ratio x Genotype: NS
Ratio: ****
Genotype: NS
Ratio x Genotype: **
Pellet retrieval latency (s)
Ratio: ****
Genotype: **
Ratio x Genotype: *
Ratio: ****
Genotype: **
Ratio x Genotype: **
Lever return latency (s)
Ratio: ****
Genotype: NS
Ratio x Genotype: NS
Ratio: ***
Genotype: NS
Ratio x Genotype: *
Lever push freuqency (pushes/s)
Ratio: ****
Genotype: NS
Ratio x Genotype: *
Ratio: ****
Genotype: NS
Ratio x Genotype: **
Ratio
Ratio
WT
BACHD

Figure S6. Performance on the fixed ratio part of the progressive ratio protocol. Results for the basic parameters of the ten FR5 ratios run at the start of each PR session. **(A)** Data from sessions where BACHD and WT rats were both deprived to 85% of their respective free-feeding body weights. **(B)** Data from sessions where food deprivation was adjusted to match the food consumption rate of BACHD and WT rats. Details for each parameter are described in the figure legend of Figure S4 and S5. Lever push frequency was calculated based on the pushes made on the reinforced lever during the full length of a ratio, i.e. the ratio duration plus interval to subsequent ratio. Results displayed were obtained from the sessions used for baseline curves in Figure 6A. The graphs indicate group mean plus standard error of the mean. Two-way ANOVA results are displayed at the top right corner of each graph, and results from *post-hoc* analysis are shown for individual data points. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

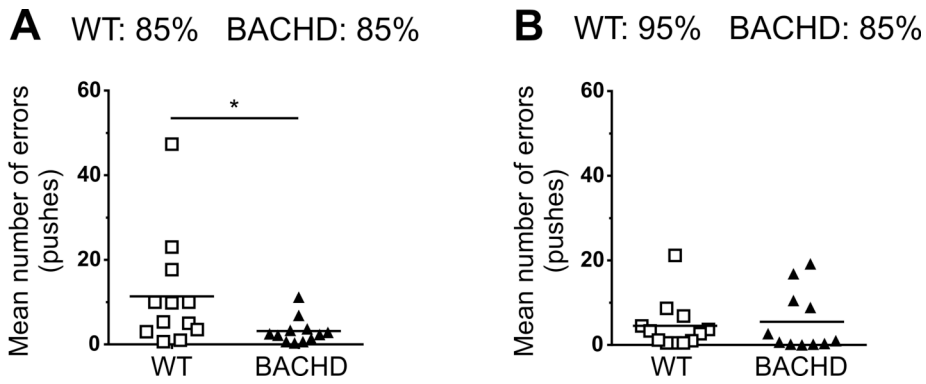
A
WT: 85%
BACHD: 85%
Mean number of errors (pushes)
60
40
20
0
*
WT
BACHD
B
WT: 95%
BACHD: 85%
Mean number of errors (pushes)
60
40
20
0
WT
BACHD

Figure S7. Mean number of errors for the fixed ratio part of the progressive ratio protocol. Errors made by the rats during the ten FR5 ratios run at the start of each PR session. **(A)** Data from sessions where BACHD and WT rats were both deprived to 85% of their respective free-feeding body weights. **(B)** Data from sessions where food deprivation was adjusted to match the food consumption rate of BACHD and WT rats. Results were obtained from the sessions used for baseline curves in Figure 6A. Graphs indicate the performance of individual rats and group mean. Results from t-tests or Mann-Whitney tests are indicated in the graphs. Significant genotype differences are indicated by ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) *** and ($p < 0.0001$) ****.

Publication II

PLOS ONE

RESEARCH ARTICLE

Further investigation of phenotypes and confounding factors of progressive ratio performance and feeding behavior in the BACHD rat model of Huntington disease

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Abstract

Huntington disease is an inherited neurodegenerative disorder characterized by motor, cognitive, psychiatric and metabolic symptoms. We recently published a study describing that the BACHD rat model of HD shows an obesity phenotype, which might affect their motivation to perform food-based behavioral tests. Further, we argued that using a food restriction protocol based on matching BACHD and wild type rats' food consumption rates might resolve these motivational differences. In the current study, we followed up on these ideas in a longitudinal study of the rats' performance in a progressive ratio test. We also investigated the phenotype of reduced food consumption rate, which is typically seen in food-restricted BACHD rats, in greater detail. In line with our previous study, the BACHD rats were less motivated to perform the progressive ratio test compared to their wild type littermates, although the phenotype was no longer present when the rats' food consumption rates had been matched. However, video analysis of food consumption tests suggested that the reduced consumption rate found in the BACHD rats was not entirely based on differences in hunger, but likely involved motoric impairments. Thus, restriction protocols based on food consumption rates are not appropriate when working with BACHD rats. As an alternative, we suggest that studies where BACHD rats are used should investigate how the readouts of interest are affected by motivational differences, and use appropriate control tests to avoid misleading results. In addition, we show that BACHD rats display distinct behavioral changes in their progressive ratio performance, which might be indicative of striatal dysfunction.

Introduction

Huntington disease (HD) is an autosomal dominantly inherited neurodegenerative disorder, which is caused by a specific mutation in the gene for the huntingtin protein [1,2]. The

decision to publish, or preparation of the manuscript.

Competing interests: Authors BF and LEC are employees of QPS Austria. There are no patents, products in development, or marketed products to declare. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

mutation concerns an expansion of the CAG repeat sequence present in the gene's first exon, which results in an elongated stretch of glutamine in the translated protein. Patients who carry an allele with more than 40 CAG repeats invariably develop HD [3,4]. During the disease process there is extensive neuronal loss, starting in the caudate nucleus of the striatum, but eventually encompassing most brain regions [5–7]. This results in a wide range of clinical signs that are commonly grouped into motor, psychiatric, cognitive and metabolic symptoms [8]. There are currently no disease-modifying treatments available for HD, and the disease is invariably fatal [2,8,9].

Several different transgenic animal models of HD have been generated [2, 10–14]. Thus, a large amount of work in HD research concerns the characterization of these animal models to better understand which aspects of the disease are well represented in a given model, which ones are not present, and which aspects might be unique to the model itself. When considering behavioral characterization studies, one also has to consider that as the models are likely to show a range of different phenotypes (disease-related or not), some might confound the readouts of others. As an example, metabolic phenotypes have been found to confound tests that assess motoric function [15,16].

Our group primarily works with the BACHD rat model of HD. These rats carry a transgenic construct that contains the full-length disease-causing human gene with 97 CAG/CAA repeats [17]. We recently published a study where we concluded that male BACHD rats, similar to other HD models that carry the full-length disease-causing gene, show a strong obesity phenotype [18]. Interestingly, we found that although the rats were obese, their body weight was still similar to that of their wild type (WT) littermates due to developmental deficits (reduced body size, disproportionally low muscle weight). In addition, the obesity phenotype persisted despite the fact that the BACHD rats generally consumed less food compared to WT rats [18].

One of the reasons for us favoring a rat model over any of the mouse models was the wider range of cognitive tests that are available for rats. However, the apparent metabolic phenotypes of the male BACHD rats raised some concerns. Specifically, we were concerned that these phenotypes might result in BACHD rats being less motivated than WT rats when performing various tests of cognitive function, as many of these are based on working for food rewards [19]. Motivational differences have been shown to affect both apparent cognitive abilities and choice of strategy in the Barnes maze [20]. For most cognitive tests, it is not known how a motivational difference affects the animals' performance. Thus, interpretations of behavioral phenotypes found in an animal model that might show reduced motivation should be done carefully.

In our initial study we therefore ran a progressive ratio test to assess male BACHD rats' motivation to perform lever pushes for a food reward [18]. Specifically, we assessed the performance during both a standard and an alternative food restriction protocol. The standard food restriction protocol was based on common practice, where all animals are food restricted until they reach a specified body weight, typically 85% of their free-feeding weight [18,19]. Using this protocol, we found that BACHD rats were less motivated than their WT littermates to perform the test. This was an interesting phenotype on its own, as it might be related to apathy symptoms that are frequently found in HD patients [21,22]. However, as the BACHD rats are obese without showing an increased body weight it would also mean that they likely carried more adipose tissue compared to WT rats during this restriction protocol. This would in turn mean that they likely had an increased serum concentration of leptin, a protein that is secreted from adipose tissue and regulates energy metabolism [23]. Importantly, changes in leptin signaling within the central nervous system have been shown to affect motivation in the progressive ratio test [24–26]. Specifically, increased leptin levels are able to reduce motivation [24,25], while knock-down of leptin receptors can increase motivation [26]. Thus, the reduced

motivation among male BACHD rats might have been a result of their metabolic phenotypes. The alternative food restriction protocol aimed to elucidate this. Rather than being based on reaching a specific relative body weight, this protocol was based on adjusting the rats' food restriction level so that their apparent hunger and food interest was similar [18]. The rats' apparent hunger was assessed by measuring their food consumption rates in a test where they were given free access to food during 15 minutes. When maintained on the standard food restriction protocol, male BACHD rats consumed food at a lower rate compared to WT rats, although this could be resolved by giving WT rats an increased daily amount of food. When BACHD and WT rats showed comparable food consumption rates, there was no longer any difference in motivation to perform the progressive ratio test. Thus, we suggested that motivational differences between BACHD and WT rats can be expected when using standard food restriction protocols, that these phenotypes are likely caused by metabolic phenotypes rather than psychiatric phenotypes, and that the alternative food restriction protocol might be more suitable to use during tests of cognitive characterization [18].

The study itself still had certain shortcomings, which we have sought to cover in the follow-up study presented here. Briefly, our first study only considered rats of relatively young ages (2–4 months of age) and we here aimed to further investigate to what extent the findings were reproduced at older ages. Further, we have investigated the rats' body composition during the alternative food restriction protocols as well as how the leptin levels among BACHD and WT rats changed during different parts of our tests (i.e. during the different food restriction protocols). Additional control tests have been performed in order to exclude fatigue and satiation as confounding factors in the progressive ratio results. Finally, more detailed evaluation of the food consumption test used for assessing the rats' apparent hunger, and a separate test allowing assessment of individual animals' feeding behavior, have been performed in order to better understand the nature of the reduced food consumption rate seen among male BACHD rats.

Material and methods

Animals

A total of 48 male rats were used for the study. These were acquired from two separate in-house breeding events, with hemizygous BACHD males from the TG5 line [17] paired with WT females (Crl:CD(SD), Charles River, Germany). All animals were on Sprague-Dawley background. Animals were genotyped according to previously published protocols [17] and housed in genotype-matched groups of three in type IV cages (38 × 55cm), with high lids (24.5cm from cage floor). Rats had free access to water through the entire study. During experiments, body weight was measured daily to track the rats' relative food restriction level and assess basic health. Between experiments, body weight was measured weekly. During experiments, rats were food restricted according to two protocols described in detail below and in [18]. During both protocols, each cage was given a specific daily amount of food (SNIFF V1534-000 standard chow) to maintain appropriate restriction levels. Rats had free access to food between the experiments.

The animal facility kept 21–23˚C, 55–10% humidity, and was set to a partially inverted light/dark cycle with lights on/off at 02:00/14:00 during summer, and 01:00/13:00 during winter.

The 48 rats were split into two groups of 24 rats, both composed of 12 WT and 12 BACHD rats. The first group was used for a longitudinal progressive ratio test, leptin measurements and endpoint dissection to investigate body composition. This group will be referred to as Group I. The second group was used for a longitudinal pasta-handling test, although the results from this are not considered here (unpublished data) (see [27] for protocol). They were

also used for the detailed study of BACHD rats' food consumption phenotypes, which is presented here. This group will be referred to as Group II. Group I was tested at 2, 7, 12 and 17 months of age in the progressive ratio test, while the leptin measurements were only performed at the last age. The results from the test at 2 months were presented in our previous publication [18] and will only be referred to in this publication. Group II was assessed in the pasta-handling test at 2, 7 and 12 months of age. The detailed study of BACHD rats' food consumption presented here was performed at the end of their 12 months experiment. Fig 1 presents an overview of the tests performed with the two different animal groups.

All experiments were approved by the local ethics committee (Regierungspraesidium Tuebingen) and carried out in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU).

Food restriction protocols

As noted above, two different food restriction protocols were used throughout the study. The first one focused on restricting the animals to a specific relative body weight. During this, both BACHD and WT rats were restricted until they reached 85% of their respective free-feeding body weight. This relative body weight, or food restriction level, was calculated using previously gathered data from growth curves of BACHD and WT rats. Thus, the calculations could be made with gender, age and genotype-matched values and took normal growth into account. This protocol was used as the start point for all tests described below, and will be referred to as the standard food restriction protocol.

Once data from performance on the standard food restriction protocol had been gathered, the restriction was changed to the alternative protocol. As noted above, this restriction was based on the rats' food consumption rates (assessed in a test described in [18] and below), rather than their relative body weight. During this, the amount of food given to the WT rats was increased, while the amount given to BACHD rats was kept more or less constant, until WT and BACHD rats showed similar food consumption rates. At that point, data for a second baseline was gathered.

It should be noted that it was rarely possible to give the exact same amount of food during extended periods of time to either of the genotypes, as both the standard and alternative restriction still had to take natural growth into account.

Progressive ratio

As mentioned above, Group I was used for a longitudinal experiment using the progressive ratio test. This was the same group of animals that had been used for our initial study [18], and only the results from their test runs at 7, 12 and 17 months of age will be presented here. A detailed description of the protocol and setup is available elsewhere [18], and is only described briefly in the current publication.

Behavioral assessment started 30 minutes after dark phase onset, in a room separate from the animals' housing room, using soft red light. A bank of six operant conditioning chambers (Coulbourn Instruments, H10-11R-TC) was used to run the test. Each chamber was equipped with two retractable levers, one on either side of a central pellet receptacle trough equipped with a yellow light. This light was used to signal the delivery of a reward pellet. The chambers contained a red house light on the wall opposite from the levers and pellet receptacle trough, which shone during the full duration of the training sessions. A water bottle was also available on this wall, to ensure *ad libitum* access to water during testing. The progressive ratio protocol was designed and run with Graphic State 4.1.04. Rats were given single daily sessions, meaning

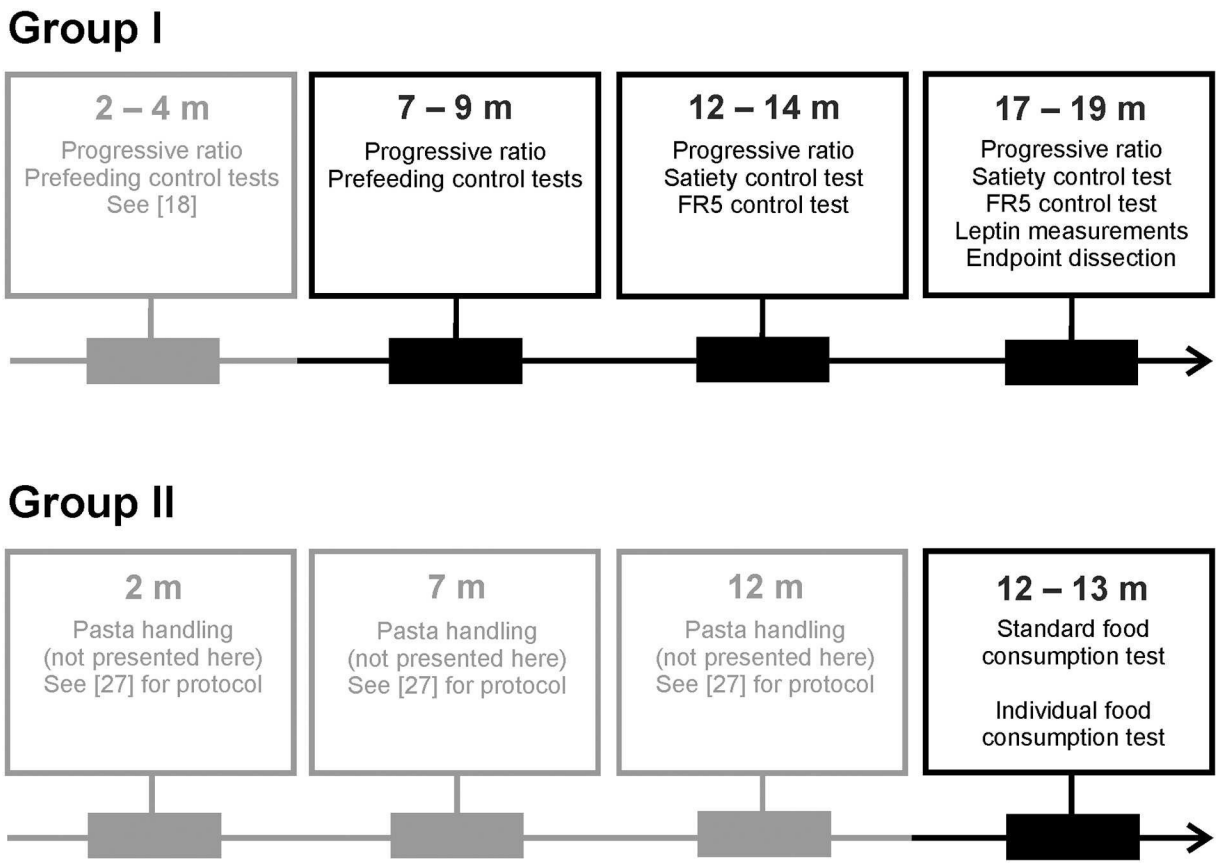

Fig 1. Study overview. The study used two groups of rats that were assessed in different behavioral tests, as indicated in the figure. The horizontal arrows indicate the time frame during which the work was performed, with the different tests ages indicated in text boxes. Gray-colored boxes and text indicate tests that are presented elsewhere, but constitute important information about the rats' behavioral testing experience. Group I was used in a longitudinal progressive ratio test with a total of four test ages. Different control tests were used at different ages, as detailed in the Material and Methods section. The results from the first age are presented elsewhere [18]. Group II was used for the detailed analysis of the reduced food consumption rate seen among BACHD rats. This analysis was only performed at a single test age. The group had previous experience in a pasta-handling test, the results of which will be published elsewhere.

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that a total of four daily runs with all six operant chambers were needed to assess the whole group. Each run assessed three WT and three BACHD rats in a determined order, so that a given rat was trained on the same time of day through all tests. Each rat was assigned to a specific operant chamber, although this was arranged so that each operant chamber was used to assess equal numbers of WT and BACHD rats. Rats received their daily amount of regular food four hours after the completion of the last run of the day.

At each test age the rats were first put on food restriction for approximately 14 days. This aimed at restricting both WT and BACHD rats to 85% of their respective free-feeding body weights, as described above. At the first test age, all rats were then habituated to the operant conditioning boxes and subjected to initial lever-training protocols before finally being trained on the progressive ratio protocol. These steps are described in detail elsewhere [18]. For all subsequent ages (i.e. the results presented in the current publication), rats were directly trained on the final progressive ratio protocol, as no other retraining appeared to be necessary.

The main aim of the progressive ratio test is to assess how many lever pushes a rat is willing to perform in order to get a reward pellet (Bio-serv, Dustless Precision Pellets® F0021,

purchased through Bilaney consultants, Duesseldorf, Germany). At the start of each test session, both levers were extended into the conditioning chamber, allowing rats to interact with them. The levers remained in this position for the full duration of the test session. One lever was reinforced, while the other one was non-reinforced. The exact position (i.e. left or right lever) of the reinforced and non-reinforced lever was counter-balanced for the two genotypes and remained constant for individual rats through all experiments. Pushing the reinforced lever resulted in reward pellets being delivered. At the start of each session, the rats needed to push five times in order to receive a reward pellet. After ten completed ratios, i.e. ten pellets received, the number of required pushes increased after each completed ratio. The increase was made in an arithmetic fashion within each block of ten ratios, but also changed between the blocks, to give an overall exponential progression. Thus, during the first, second and third block of ten ratios, the ratio requirement increased with one, three and five pushes per completed ratio, respectively. The sessions lasted 80 minutes. The main behavioral parameter of interest was a set of break points, defined as the first ratio where a rat made no responses on the reinforced lever during 10, 25, 50, 100, 300 or 600 seconds. Rats were trained until both genotype groups had reached a stable performance. A baseline was then constructed from the last few sessions as detailed below.

Once a baseline had been achieved using the standard food restriction protocol, the alternative food restriction protocol was initiated. During this, the rats were still given daily progressive ratio sessions, but in addition, a food consumption test was run each day at the time when the rats would normally receive their daily amount of food. As noted above, WT rats were then given an increased amount of food until they showed a comparable food consumption rate to BACHD rats. At that point, data for a stable baseline of progressive ratio performance was once again gathered. When a second baseline had been obtained, the rats were put back on free feeding and the test ended.

Although the exact number of sessions used for the different progressive ratio baselines presented in this publication differed, none used fewer than six consecutive sessions. It should also be noted that the feeding test was run on a weekly basis during training on the standard food restriction protocol. As mentioned in [18], the training took a substantial amount of time at each age, and despite the intention of assessing the rats' behavior at 2, 7, 12 and 17 months of age, the more exact ages for the baselines presented in [18] and here are 2–4, 7–9, 12–14 and 17–19 months of age.

Several parameters were analyzed in addition to the set of break points described above. These included the total number of completed ratios (i.e. rewards obtained), the total number of pushes performed on the reinforced lever, the total number of pushes performed on the non-reinforced lever and several parameters regarding the latency to retrieve the reward pellets. For this, there was first the full retrieval latency, calculated from the delivery of the pellet to the point where the rat entered the pellet trough to retrieve it. This parameter was then split. This produced the latency to leave the reinforced lever, which measured the time from delivery of the reward pellet to the OFF-signal of the last lever push the rat performed on the reinforced lever. The latency to move from the lever to the pellet trough was then calculated separately, measuring the time from the OFF-signal of the last lever push to the point when the rat entered the pellet trough. Two additional parameters were added to describe the latency to leave the reinforced lever in greater detail. The first one calculated the number of excessive pushes (i.e. additional pushes performed after the delivery of the reward pellet) that the rats performed on the reinforced lever before retrieving the pellet. The result of this parameter was expressed as the mean number of excessive pushes performed per completed ratio. The other parameter calculated the latency to leave the lever specifically on ratios where no excessive pushes were performed, and was called the latency to release the reinforced lever.

Separate analysis for the first ten FR5 ratios was also performed, including a set of slightly different parameters. These constituted the latency to perform the first lever push, the time needed to complete a given ratio, the latency to return to the reinforced lever after retrieving the reward pellet and the pellet retrieval latency (calculated as the full retrieval latency explained above).

Progressive ratio control tests

In our initial study [18], a set of prefeeding tests was used to further evaluate the motivational difference between WT and BACHD rats. On each test occasion, the rats were fed a fixed amount of either regular food or reward pellets prior to performing the progressive ratio test. The resulting drop in motivation was then analyzed and discussed. In total, the rats were assessed in four different test sessions, which were presented on alternating days with normal progressive ratio tests. These prefeeding tests were repeated at the 7–9 months test age. However, on that occasion both WT and BACHD rats failed to return to their baseline performance during sessions that separated the prefeeding tests. Instead, the rats gradually became less motivated with each prefeeding test being run. Because of this, the results were excluded from the current manuscript. In addition, the prefeeding tests were not rerun at the subsequent test ages.

During the 12–14 and 17–19 months test ages, the rats' progressive ratio performance was also assessed at satiety, before food restriction according to the standard protocol was initiated. We hypothesized that the results would be similar to the ones obtained when using the alternative food restriction protocol, as WT and BACHD rats should in both cases be equally hungry and/or satiated. These tests used the same basic progressive ratio protocol, but the sessions were only 45 minutes long. In addition, the test sessions were started two hours after the dark-phase onset, to give both WT and BACHD rats ample time to finish their main feeding bout of the dark phase.

Another control test was added during the 12–14 and 17–19 months test ages. In this protocol, there was no progression, and the required number of lever pushes was kept at five pushes through the entire session (FR5 protocol). Single sessions of this protocol were run after establishing the satiety baseline at 12–14 months, and all three baselines at 17–19 months of age (i.e. satiety, standard food restriction and alternative food restriction). The sessions were run on the same time schedule as the standard progressive ratio protocol, had the same maximum duration, but sessions also ended once a rat had acquired 200 pellets. This protocol was run in order to investigate if the motivational differences in progressive ratio performance might have been caused by BACHD rats becoming fatigued or satiated during the sessions.

Leptin measurements

During the 17–19 months test age of Group I, blood samples were collected after establishing each progressive ratio baseline (i.e satiety, standard food restriction and alternative food restriction). At each stage, the blood samples were collected the day after the FR5 control test had been run. In addition, a fourth set of blood samples was collected at the endpoint of the experiment, when rats were sacrificed and dissected as described below. Samples were collected during the same time of day on all occasions. The first three sets of samples were collected from the rats' tail vein. This was done by inserting a needle of 0.6 mm diameter into the vein and collecting roughly 1 ml of whole blood into a microcentrifuge tube. No anesthesia or specific fixation method were required for this procedure, as the rats had been extensively handled by the experimenters during the study. After collection, the samples were allowed to clot while being kept on ice, and were then centrifuged at 5˚C with 1000*g*, for 30 minutes. The

resulting blood serum was collected and stored at -80˚C until ELISA analysis was performed approximately 10 months later.

Leptin concentrations were measured at QPS Austria GmbH (Grambach, Austria) using a Quantikine ELISA kit (Mouse/Rat leptin Quantikine ELISA kit, R&D systems, Austria, Vienna). Serum samples from animals at satiety were diluted 1:10 and 1:20 for WT and BACHD rats, respectively. For all other samples, dilution series of 1:2.5, 1:5 and 1:10 were prepared. The final sample preparation resulted in an additional 1:2 dilution, according to the kit's accompanying protocol. Concentration measurement was based on the supplied leptin standard. Duplicate samples were analyzed for satiety samples. For other samples, a mean concentration was calculated based on 1–3 samples, depending on how many samples from the dilution series were within the range of the standard curve. For most samples, this resulted in duplicate measurements.

Body composition analysis

After completing the set of tests run at 17–19 months of age, the rats of Group I were sacrificed while they were still maintained on the alternative food restriction protocol. Briefly, the rats were sacrificed in a carbon dioxide chamber two to four hours before dark-phase onset. Body lengths and body weights were then measured on the intact animals, with body length measured from nose tip to tail tip. Additional measurements of head, trunk and tail length were taken from nose tip to back of the head, back of the head to anus and anus to tail tip, respectively. Afterwards, blood samples were collected transcardially and processed as described above. The rats were then subjected to a detailed dissection aimed at investigating their body composition. First, skin and subcutaneous adipose tissue deposits were removed and weighed. Then, internal organs and adipose deposits located in the abdomen and chest cavity were removed and weighed. The remaining carcass was weighed to obtain a measurement of bone and muscle weight (denoted bone/muscle). The dissection of Group I was performed during four consecutive days.

Standard food consumption test

The standard food consumption test was used at several points during the study to assess the rats' food consumption rates and formed the basis of the alternative food restriction protocol. The protocol for this test has been described in our initial study of the BACHD rats' food consumption rates [18], and similar protocols have been described by others [28–33]. The aim of the test is to acquire a basic measurement of the rats' apparent interest in food, i.e. hunger levels. For this, a small amount of food was placed in the cage tops of the rats' homecages (approximately 50 g, the exact weight differed between cages (+/- 5 g), but was carefully noted, down to two decimals). The food was then left there for 15 minutes. Afterwards, the remaining food in each cage was measured.

As noted above, the food consumption tests were run in connection to the actual time of feeding for the rats. After calculating how much food the rats consumed during the test, this amount was subtracted from the cages' daily food amount.

For Group I, this test was run weekly during the progressive ratio training when rats were maintained on the standard food restriction protocol, and daily during the progressive ratio training when rats were maintained on the alternative food restriction protocol. For Group II, where characterizing the food consumption rate phenotype was the primary aim, the test was run daily during both food restriction protocols. Specifically, the rats' behavior during the standard food restriction protocol was first assessed during eight consecutive days to establish a baseline of their performance. Afterwards, they were run in the individual food consumption

test as described below. Once that had been completed, the rats were run on the standard food consumption test for an additional three sessions. During these three days, videos of the rats' performance were recorded. Afterwards, a single session was run where the food was placed on the cage floors instead of the cage tops. When all of that was done, the rats were put on the alternative food restriction, and the standard food consumption test was once again run daily, until BACHD and WT rats showed similar food consumption rates. At that point, the rats were again run in the individual food consumption test. After this, the rats were assessed in the standard food consumption test during three consecutive days in order to gather videos of their performance. The video scoring of the tests is described in detail below.

Individual food consumption test

The fact that the standard food consumption test is run in groups, leads to some drawbacks. As an example, detailed scoring of the number of bites and duration of chewing episodes cannot reliably be scored from videos of the test. Because of this, we also sought to evaluate the consumption rates and feeding behavior of individual animals, in Group II. Through their pasta-handling test (data not shown), the rats had been extensively habituated to a roughly cube-shaped glass cage (28.5 × 29 × 29.5 cm, also described in [18]). Because of this, they readily consumed regular food inside the same setup, which made them suitable for the current study. In addition, the setup allowed for good quality close-up videos of the rats' behavior.

As noted above, the rats were assessed in this test after stable baselines of their performance in the standard food consumption test had been established (during both food restriction protocols). Each animal was given single daily sessions where they were placed inside the glass cage and given a single food piece. The trial then continued until the rats had consumed the food piece. The entire trial was video-recorded to allow for subsequent video scoring (see below). The food pieces had been filed down to approximately 2.4 g (+/- 0.1 g) (the exact weight of each food piece was noted, down to two decimals) to achieve consistent weight and blunt edges for all trials. During both the standard and alternative food restriction, several sessions were run in order to establish stable baseline performance. At the end of the test, the rats' head length, from nose tip to the back of the head, was measured.

Video analysis

As noted, video recordings of both the standard and the individual food consumption tests were made to better investigate the nature of the phenotypes that had been found. During scoring, experimenters were blinded to the rats' genotypes, while this was not the case when the videos were gathered. All video scoring was performed using the Observer XT software (v.12.5.927, Noldus, The Netherlands, Wageningen). The following behaviors were scored for the standard food consumption test:

Food-oriented behaviors. This included all behaviors that could be argued to be food-oriented. In addition to the more specific behaviors noted below, this primarily considered occasions when the rats appeared to be searching through the bedding material for food pieces, but in general included most behaviors performed at or around the food crib. In contrast, behaviors where the rats investigated smells and sounds from outside the cage, or general activity in the part of the cage that was not situated below the food crib, was not considered food-oriented.

Food crib attention. Episodes of food-crib attention were scored when the rats clearly investigated the food inside the food crib. Naturally, this included the time they actively spent biting on food pieces, but also occasions where they only sniffed the food or clearly angled their heads towards it while being in its direct vicinity.

Biting episode. This was specifically scored when the rats where actively biting or trying to bite the food pieces in the food crib.

Consuming a separate food piece. On occasion, rats would bite off a larger food piece, or find a food piece in the bedding material below the food crib. They would then frequently take the piece in their mouth, walk away from the food crib and sit still in another part of the cage. Although it was rarely directly visible, it was assumed that they were then actively consuming the food piece, which was scored as a separate behavior. The behavior was clearly distinguishable from both grooming and resting, as the rats sat very still in a hunched position, rather than performing typical grooming movements or lying down.

Through the tests sessions, these behaviors occurred in episodes of different durations. For each behavior, the total number of episodes, the mean episode duration and the total time spent doing a specific type of behavior was calculated. From this, the total time spent on two other behavioral parameters were calculated. General food crib attention was calculated by subtracting the total time of biting episodes from the total time spent paying attention to the food crib. The parameter thus described the total time the rats spent on more cursory investigations of the food crib. Other food-oriented behaviors was calculated by subtracting the total time spent paying attention to the food crib and the total time spent consuming a separate food piece from the total time spent on arguably food-oriented behaviors. Finally, the latency to initiate biting was calculated for food crib attention episodes where biting occurred.

For both the standard and alternative food restriction protocols, only one video per cage was analyzed. The videos were chosen so that the rats' food consumption rate on the analyzed session was a good approximation of their baseline performance. For a given cage, scoring was made on each individual rat, although the tail and ear markings that were used for identifying them were not visible on the videos. Thus, the rats were given arbitrary names based on their position inside the cage at the session start, to keep them apart during scoring.

The scoring of the individual food consumption test focused on the detailed behavior of how the rats consumed single food pieces. In general, the rats spent essentially no time doing general exploration of the setup, so a separate scoring of this was not necessary. Thus, the following parameters were scored:

Time needed to consume the food piece. Rats were considered to be feeding when clearly biting and gnawing on the food piece. In addition, making clear chewing motions when either holding the food piece or standing in its direct vicinity and remaining focused on it was considered active feeding. Rats were not considered to be actively feeding if they were walking around investigating the setup or were clearly not focusing on the food pellet, even if these behaviors often included some chewing motions. In addition, eating food dust from the cage floor was excluded from the active feeding time. Still, it should be noted that these behaviors were rare.

Number, duration and frequency distribution of biting episodes. A biting episode was considered any phase where the rats were actively biting or gnawing pieces off of the main food piece. The start of these episodes was clearly identifiable with the rat using its forepaws to lift the food piece upwards, and simultaneously lowering its head, in order to position the food piece into its open mouth. The specific nature of the biting episode could then be quite varying, although the rat typically either bit a single piece off or performed several gnawing motions with its lower jaw. The end of the biting episode, and the start of the chewing episode, was then scored when the rat lifted its head from the food piece and started chewing. In addition to calculating the total number and mean duration of biting episodes, the frequency distribution of biting episodes with different durations was analyzed. This analysis used 15 bins of 0.2 seconds, and a final bin containing biting episodes that were longer than three second.

Number, duration and frequency distribution of chewing episodes. Once the rat had managed to bite a piece off from the main food piece, it typically spent some time chewing before returning to bite another piece off. The chewing episodes were considered to end when the rat initiated another biting episode. Through this, the bouts of active feeding were split into several alternating biting and chewing episodes. In addition to calculating the total number and mean duration of chewing episodes, the frequency distribution of chewing episodes of different durations was analyzed. This analysis used 25 bins of 0.2 seconds, and five bins of three seconds for longer chewing episodes.

On some occasions the rats bit off pieces that were too large to eat in a single bite. The rats would then drop the main food piece and hold on to the piece that was bitten off, in order to bite smaller pieces off from it. These events were scored as a single biting episode, as no chewing was initiated. On other occasions, the rats would bite a piece off and then spend some time using small mouth movements to get the whole piece into their mouths before actually starting to chew it. On these occasions, the chewing episode was considered to start from the point that the rats had bitten the piece off in order to include also the small mouth movements. Thus, the biting episodes included behaviors that aimed at getting a comfortable food piece off of the food pellet while the chewing episodes included behaviors that focused on managing to chew and swallow those food pieces.

In addition to the parameters above, the theoretical bite size for each rat was calculated based on the number of biting episodes the rats had made and the measured weight of the food pellet. Further, the food consumption rate was calculated based on the food pellet's weight and the time needed to consume it.

Statistical analysis

Analysis of baseline performance during the progressive ratio test comprised of several different graphing and analysis methods. Single comparisons of BACHD and WT performance were subjected to *t*-test, *t*-test with Welch correction or Mann-Whitney test depending on the data's apparent distribution. Parameters presented in curves were analyzed with two-way repeated measures ANOVAs using in most cases genotype as between-subject factor and break point, age, food restriction protocol or behavioral protocol as within-subject factor. Sidak's multiple comparison *post-hoc* test was used to follow up on any significant effects of genotype, or on interaction effects found in the two-way ANOVAs. Analysis of performance during the FR5 part of the progressive ratio protocol (i.e. performance during the first ten ratios) was performed in the same manner, but with ratio being used as within-subject factor. During the study, some rats became ill and had to be sacrificed. Thus the n of the analyses changed as follows: 7–9 months data (WT: 12, BACHD: 11), 12–14 months data (WT: 12, BACHD: 11) and 17–19 months data (WT: 12, BACHD: 9 for data from standard food restriction, WT: 11, BACHD: 9 for data from alternative food restriction). Analysis of age progression excluded animals for which data was not available at all ages. No other exclusion criteria were used.

To gain further information of the rats' progressive ratio performance, data from the final break point (break point 600) from all baselines established during standard and alternative food restriction was analyzed in a three-way ANOVA. The analysis used genotype as between-subject factor and age and food restriction protocol as within-subject factors. Significant two-way interactions were graphed and pairwise analyses were made using Sidak's multiple comparison *post-hoc* test. As the analysis included age, data from rats that had been sacrificed before the end of the study were excluded. This put the n for the analysis at 11 for WT and 9 for BACHD rats.

Parameters investigated in connection to leptin level analysis were analyzed through a series of single comparisons between BACHD and WT rats, using *t*-test, *t*-test with Welch correction or Mann-Whitney test depending on the data's apparent distribution. Curves and ANOVAs were avoided due to the strong non-normal distribution in WT rats' leptin levels, which was found to influence statistical readouts and obscure the findings concerning the alternative food restriction protocol. The current approach was chosen to avoid excluding experimentally sound data. Analysis was performed on the 11 WT and 9 BACHD rats for which progressive ratio data and blood samples were available at all three baselines (satiety, standard food restriction and alternative food restriction). WT rats were, in addition, subjected to paired analysis of body weight, leptin levels and BP600 for the two different food restriction protocols.

Parameters from dissection results were also analyzed in a series of single comparisons between BACHD and WT rats, using *t*-test, *t*-test with Welch correction or Mann-Whitney test depending on the data's apparent distribution.

Curves comparing mean baseline food consumption rates during standard and alternative food restriction protocols, for both the standard and individual food consumption tests, were analyzed with two-way repeated measures ANOVA. As above, these used genotype as between-subject factor and restriction protocol as within-subject factor. Sidak's multiple comparison *post-hoc* test was used to follow up on any significant effects of genotype, or interaction effects. In addition, performance of WT rats was subjected to paired analysis, comparing the performance on both restriction settings. Additional curves showing food consumption rate on all test sessions are included in the figures for descriptive purpose. The standard food consumption test was based on mean consumption rates for cages, resulting in an n of 4 for both WT and BACHD rats. The individual food consumption test was based on individual performances. Group II consisted of a total of 12 WT and 12 BACHD rats. However, 2 WT rats had to be excluded from the analysis, as they did not reliably consume the food piece during the alternative food restriction protocol, leaving an n of 10 WT and 12 BACHD rats.

The video analysis of the standard food consumption test focused on a series of individual comparisons between WT and BACHD rat performance, using *t*-test, *t*-test with Welch correction or Mann-Whitney test depending on the data's apparent distribution. No specific analysis of behavioral changes due to the change of food restriction protocol was performed, although additional graphs depicting the change, but using the statistics of the individual comparisons, were made. This was because the rats' actual identity was not visible in the videos, and thus repeated measures analysis could not be performed. Scoring within each baseline performance was done on an individual basis, giving an n of 12 for both WT and BACHD rats.

Video analysis of behavior during the individual food consumption test was only performed for the alternative food restriction protocol, as the restriction protocol did not appear to have any effect on food consumption rate in this test. Analysis consisted of a series of individual comparisons between WT and BACHD rat performance, using *t*-test, *t*-test with Welch correction or Mann-Whitney test depending on the data's apparent distribution. In addition, the distribution of biting and chewing episodes of different durations were analyzed with two-way repeated measures ANOVA using genotype as between-subject factor and episode duration as within-subject factor. The analysis was performed on both absolute numbers of episodes and data related to the total number of episodes performed. No *post-hoc* analysis was performed. As noted above, the analysis used 10 WT and 12 BACHD rats. An additional distribution analysis with fewer episode duration bins was also performed. This analysis used a series of individual comparisons between BACHD and WT rats, applying tests describe above, rather than a two-way ANOVA.

Alpha for all analyses was set to 0.05. The three-way ANOVA was performed with SPSS statistics v.20.0.0 (IBM Corporation, Armonk, New York, USA, http://www.ibm.com). All other

statistical analyses were conducted using GraphPad Prism v.6.01 (GraphPad Software, San Diego California USA, http://www.graphpad.com).

Results

Survival

Most rats remained healthy through the entire duration of the study, and only a few rats (three BACHD and one WT rat from Group I) were sacrificed due to illness. In all cases, the illnesses concerned tumors. Although the higher incidence of sacrifice among BACHD rats in this study might suggest that BACHD rats show a generally shorter life span than WT rats, we have not seen any consistent indications of this when considering all studies performed at our institute.

Progressive ratio

The results from Group I's performance on the progressive ratio test at four months of age [18] were well reproduced when the rats were retested at older ages in the current study (Figs 2 and 3). Specifically, BACHD rats performed fewer pushes on the reinforced lever, completed fewer ratios and reached lower breakpoints compared to WT rats when the standard food restriction protocol was used (Fig 2A). Rats of both genotypes appeared to be gradually less motivated to perform the test as they aged (Fig 2B), although the motivational differences between the genotypes remained largely unchanged. Still, *post-hoc* analysis revealed that a subtle progression effect might be present. When using the alternative food restriction protocol, the genotype differences were no longer present and BACHD and WT rats consistently showed similar levels of motivation in the progressive ratio test (Fig 3A). This was primarily due to a clear drop in motivation among WT rats, although performance also dropped slightly among BACHD rats. Performance on the alternative food restriction protocol showed no statistically significant change with age, although weak trends indicated that the motivation dropped slightly (Fig 3B). Pushes on the non-reinforced lever were rare for both genotypes at all ages and on both food restriction protocols, with no indication of genotype or interaction effects (S1 Fig). Rats of both genotypes performed their highest number of non-reinforced lever pushes during the 7–9 months test period when the standard food restriction protocol was used. At all following baselines, the number of non-reinforced pushes appeared to remain stable.

The results from the three-way ANOVA analysis of break point 600 supported the results described above and added certain analysis elements (Fig 4). The ANOVA did not reveal any overall effect of genotype, while both the restriction protocol and age had a general impact on break point 600 (Fig 4A). Further, each of the reported two-way interactions (Genotype x Restriction protocol, Genotype x Age, and Restriction protocol x Age) were significant, although the Genotype x Age interaction was considerably weaker than the others (Fig 4A). The three-way interaction (Restriction protocol x Age x Genotype) was, in contrast, not significant (Fig 4A). The significant two-way interactions were subjected to further analysis (Fig 4B). From this, it was once again noted that although both WT and BACHD rats dropped in motivation between the two baselines, the effect was stronger among WT rats. This effect likely caused the significant Genotype x Restriction protocol interaction. The analysis further indicated that as rats grew older, their performance appeared to drop at a faster rate among BACHD rats compared to WT rats. This likely caused the significant Genotype x Age interaction effect. Finally, the performance difference between rats maintained on the standard and alternative food restriction protocols was particularly strong during the 7–9 months test age. This likely caused the significant Restriction protocol x Age interaction effect.

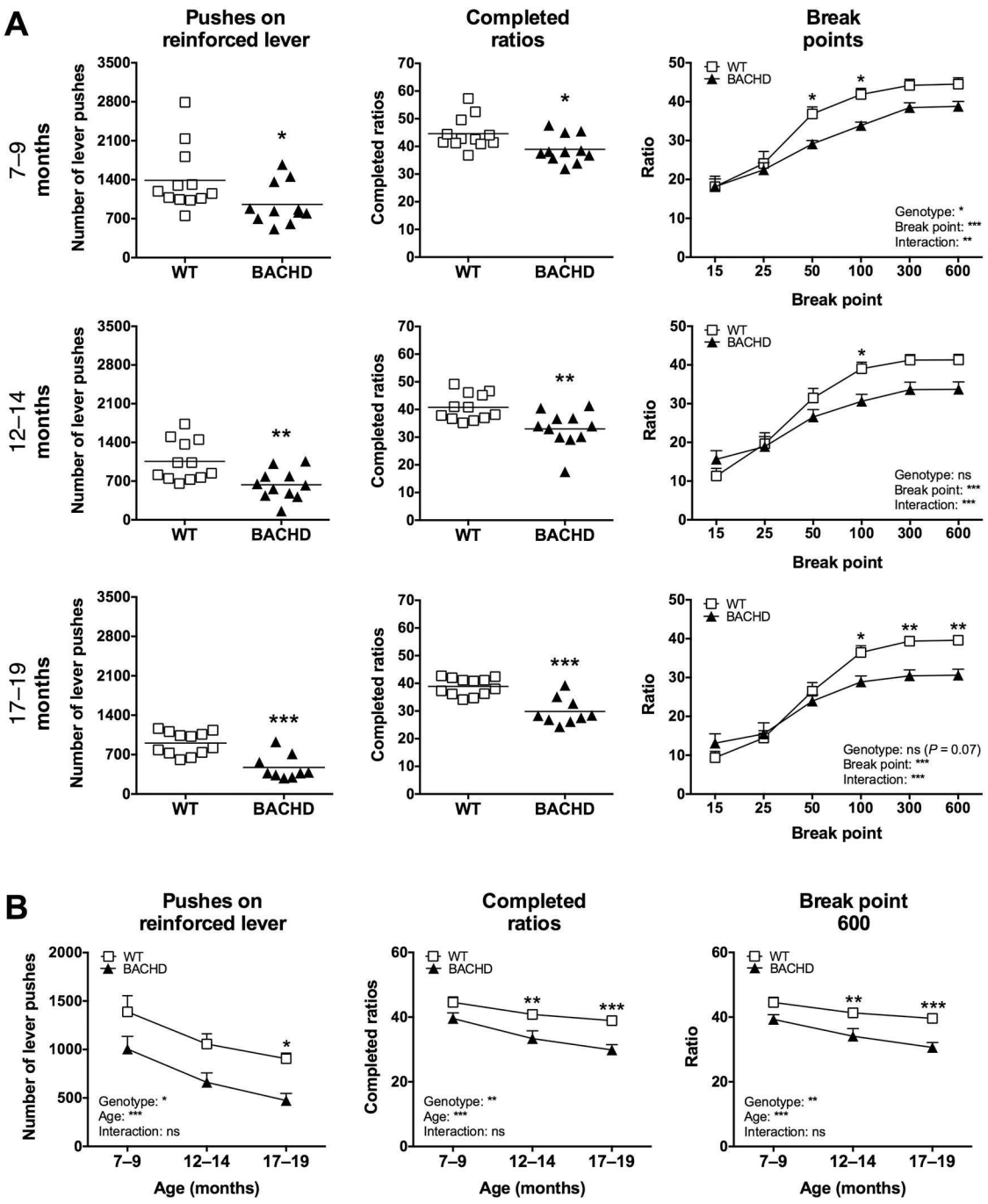

Fig 2. Primary readouts of progressive ratio performance during standard food restriction. The graphs show the performance of Group I in the progressive ratio test, when rats were maintained on the standard food restriction protocol. (A) displays the baseline performance at the three older ages. The mean number of pushes performed on the reinforced lever and mean number of completed ratios are displayed in scatter plots, where each data point represents an individual animal's performance. The groups' mean values are also indicated. The graphs for break point analysis display the ratio where a given break point was reached, with group mean plus standard error being shown. (B) displays the age progression of the main readouts. The graphs indicate group mean plus standard error. For the scatter plots, significant results from *t*-test or Mann-Whitney test are shown inside the graphs. For (B) as well as for all break point graphs, repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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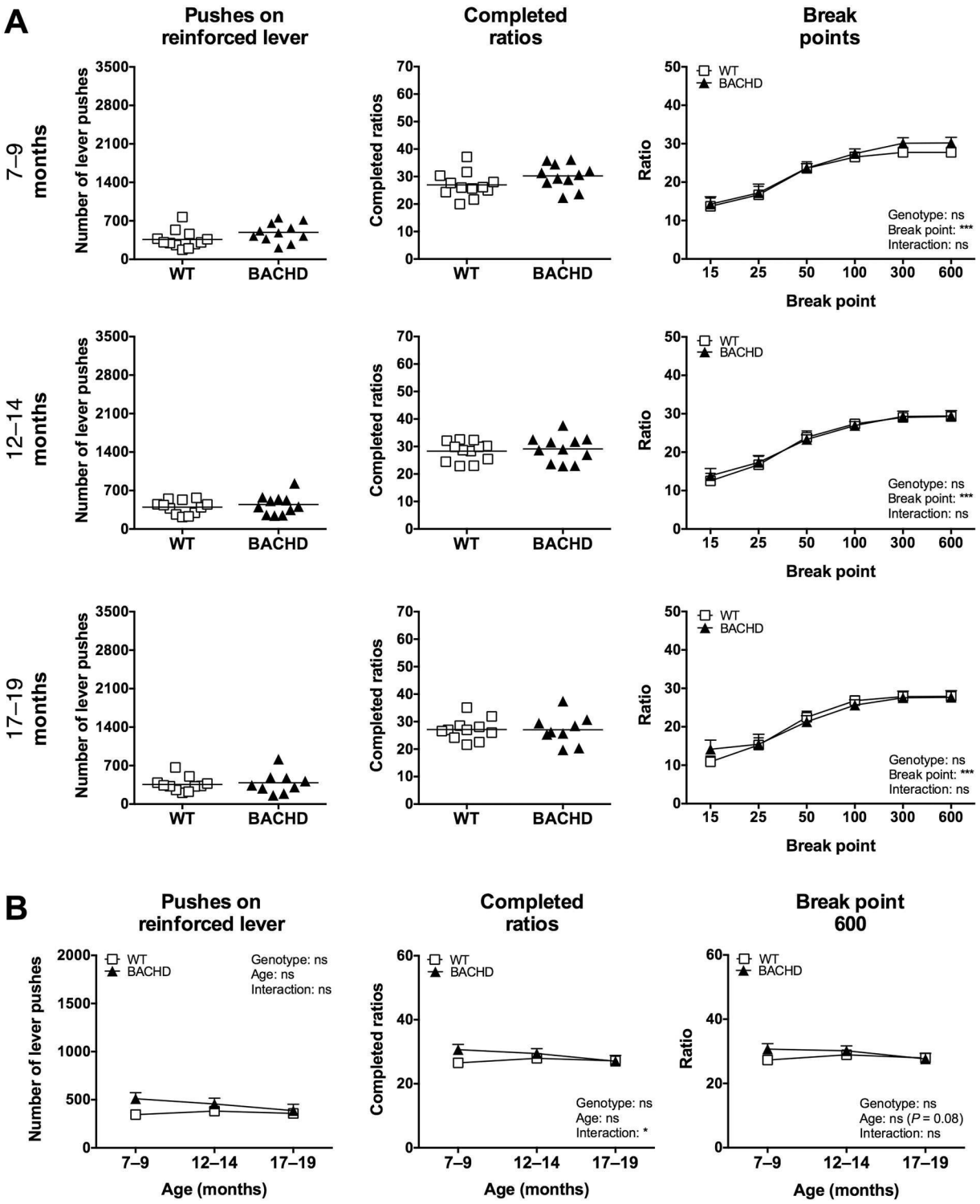

Fig 3. Primary readouts of progressive ratio performance during alternative food restriction. The graphs show the performance of Group I in the progressive ratio test, when animals were food restricted so that their food consumption rates were matched. (A) displays the baseline performance at the three older ages. The mean number of pushes performed on the reinforced lever and mean number of completed ratios are displayed in scatter plots, where each data point represents an individual animal's performance. The groups' mean values are also indicated. The graphs for break point analysis display the ratio where a given break point was reached, with group mean plus standard error being shown. (B) displays the age progression of the main readouts. The graphs indicate group mean plus standard error. For the scatter plots, significant results from *t*-test or Mann-Whitney test are shown

inside the graphs. For (B) as well as for all break point graphs, repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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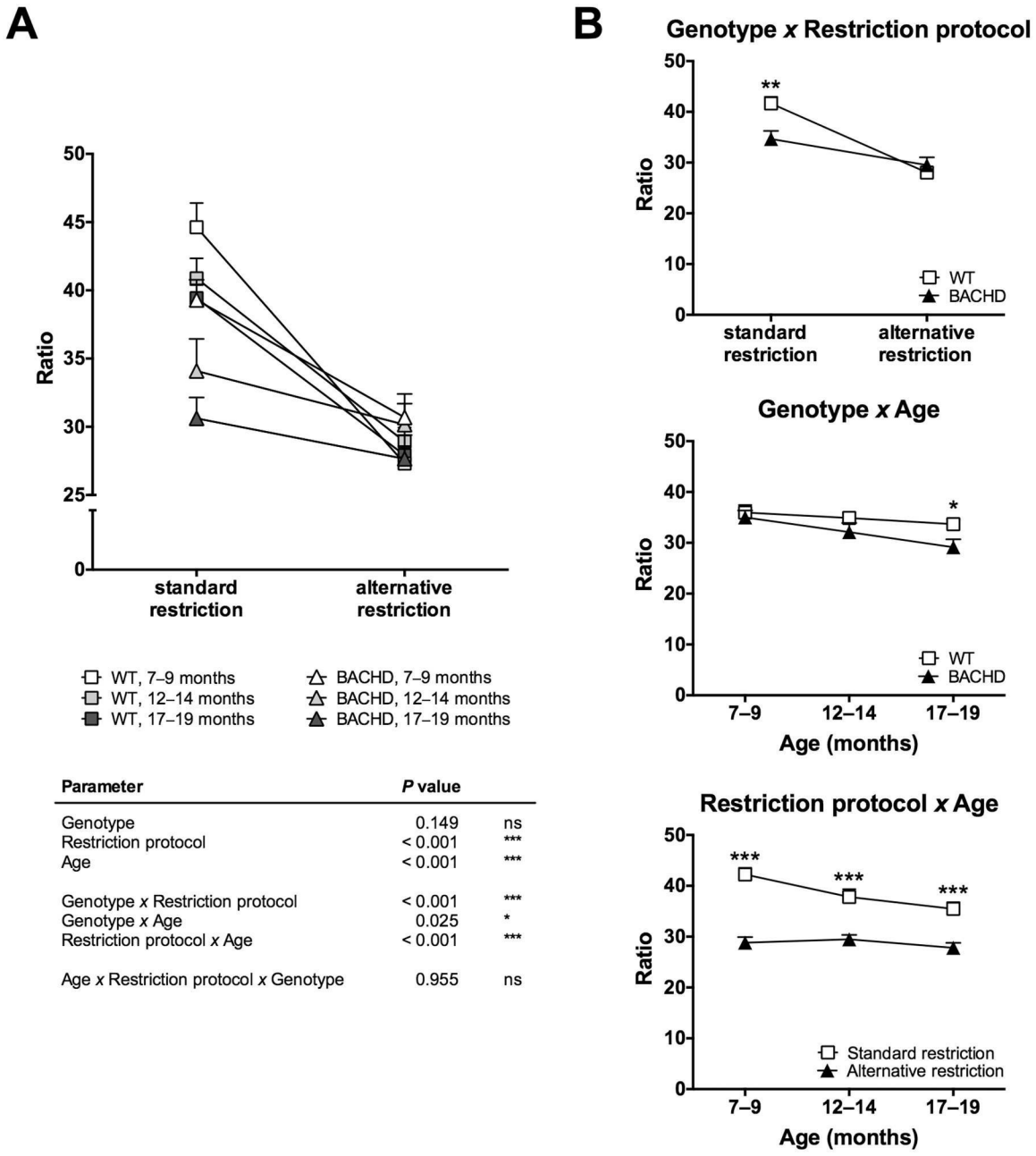

Parameter	*P* value	
Genotype	0.149	ns
Restriction protocol	< 0.001	***
Age	< 0.001	***
Genotype *x* Restriction protocol	< 0.001	***
Genotype *x* Age	0.025	*
Restriction protocol *x* Age	< 0.001	***
Age *x* Restriction protocol *x* Genotype	0.955	ns

Fig 4. Three-way ANOVA analysis of break point 600. The graphs show the results from a three-way ANOVA analysis of break point 600 for the performance baselines displayed in Figs 2 and 3. (A) displays all included data points and a summary table of the statistics. (B) displays plots for the significant two-way interaction effects. All graphs display group mean plus standard error. In (B), results from pairwise comparisons with Sidak's multiple comparison *post-hoc* test are displayed for data points that differed significantly from each other. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

doi:10.1371/journal.pone.0173232.g004

BACHD rats were consistently found to have longer full pellet retrieval latencies compared to WT rats, regardless of which food restriction protocol was used (Fig 5A). As described in the Material and Methods section, the full pellet retrieval latency was composed of the latency to leave the reinforced lever and the time needed to move from the reinforced lever to the pellet receptacle. BACHD rats were slightly slower than WT rats in terms of leaving the reinforced lever (Fig 5B), which appeared to be caused by them making a higher number of excessive lever pushes before retrieving the pellet (Fig 5C), rather than having problems with simply releasing the lever (Fig 5D). In addition, BACHD rats were consistently found to be slower than WT rats in moving from the reinforced lever to the pellet trough (Fig 5E), which likely represented the main cause of their slowed full retrieval latency. Concerning age progression, WT rats showed stable pellet retrieval latencies, while BACHD rats appeared to become slower as they were retested (Fig 5A and 5E). The number of excessive lever pushes (Fig 5C), and other parameters (Fig 5B and 5D), remained arguably stable with increasing age.

There were no striking differences between the BACHD and WT rats' performance during the FR5 phase of the progressive ratio test (S2 Fig). Still, there was a trend indicating that BACHD rats needed longer time than WT rats to complete the very first ratio of the session (S2B Fig). In addition, BACHD rats were again found to need significantly longer time than WT rats to retrieve the reward pellets on both food restriction protocols (S2C Fig).

Progressive ratio control tests

During the test performed at 2–4 months of age, we used a prefeeding control test [18]. The aim was to control for differences in the BACHD and WT rats' hunger levels. As mentioned in the Material and Methods section, this was repeated for the 7–9 months test, but the results were excluded, as the rats did not reliably return to their baselines between the prefeeding tests. A separate set of control tests was thus added at 12–14 and 17–19 months of age. On both occasions, the rats were assessed in the progressive ratio test and in an FR5 test at satiety. During the 17–19 months test, the FR5 protocol was also run after establishing the progressive ratio baselines for the standard and alternative food restriction protocols. At satiety, BACHD rats were less motivated than WT rats to perform the progressive ratio test (Fig 6A), but were equally motivated to perform the FR5 test (Fig 6B). This was true for both test ages. Importantly, both BACHD and WT rats completed more ratios (Fig 6A and 6B) and performed more pushes on the reinforced lever (Fig 6C) during the FR5 protocol compared to the progressive ratio protocol. When comparing progressive ratio test performances during satiety and the standard food restriction protocol, rats of both genotypes showed increased motivation to lever-push for rewards on the latter. This effect appeared to be somewhat stronger among WT rats, particularly at the last test age (S3 Fig).

During the last test age, the FR5 control test was repeated when the rats were maintained on the standard and alternative food restriction protocols. During this, most of the rats reached the maximum of 200 reward pellets without making larger breaks, and thus no detailed analysis of break points could be made. Instead, the primary readouts were the number of completed ratios and the number of lever pushes performed on the reinforced lever. Similar to the FR5 test at satiety, there were no differences between BACHD and WT rats in these parameters, and both completed more ratios (Fig 7A) and performed more lever pushes (Fig 7B) compared to their progressive ratio performance.

Leptin measurements

BACHD rats showed no significant difference in body weight compared to WT rats at either of the different baselines (Fig 8A), but along with the poorer progressive ratio performance (Fig 8B),

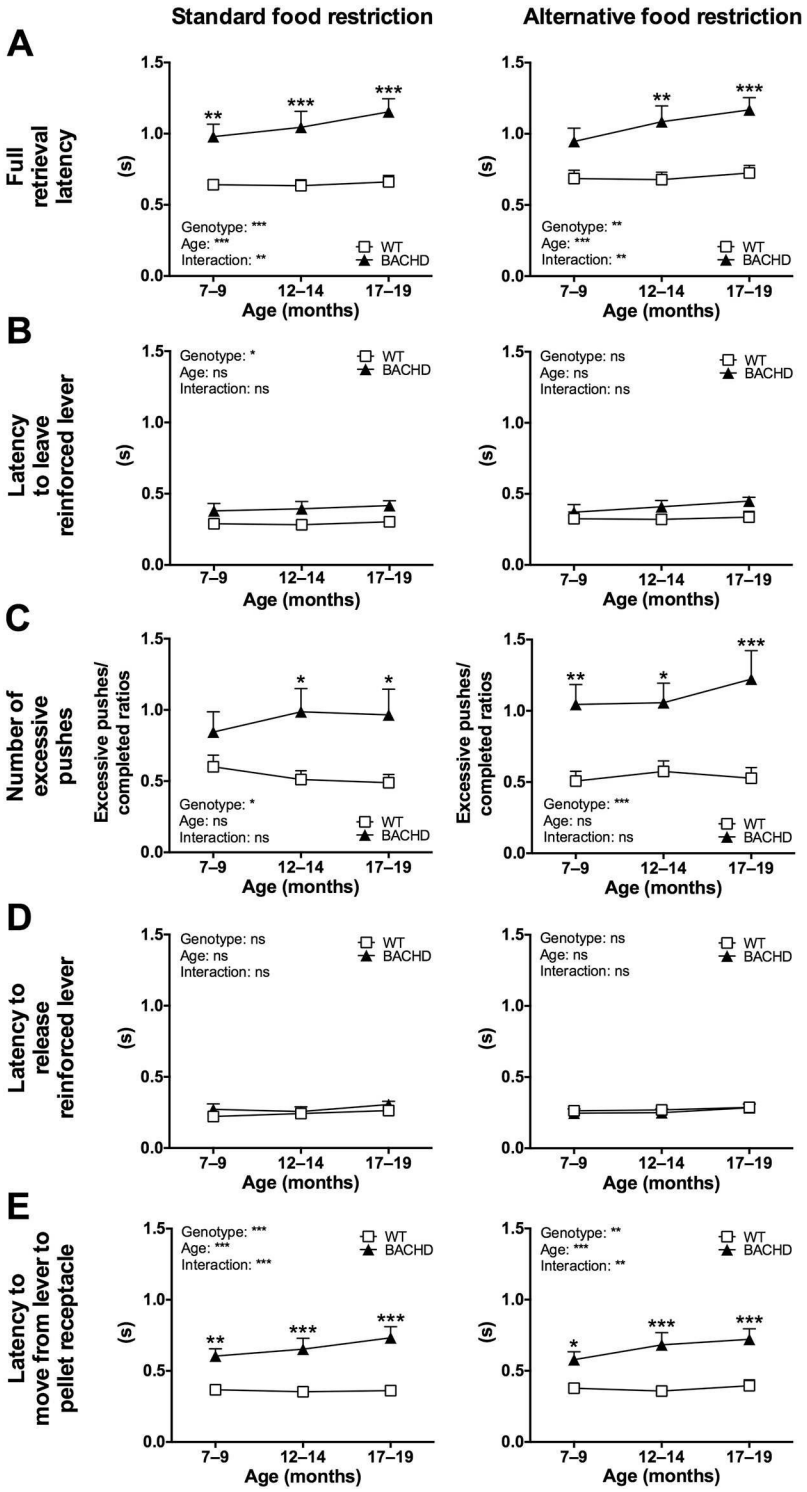

Fig 5. Detailed analysis of pellet retrieval latency during the progressive ratio test. The graphs show age progression of various parameters related to the latency to retrieve the reward pellet during progressive ratio testing of Group I. Results from both food restriction protocols are shown. (A) shows the full retrieval latency, while (B)–(E) show its individual components. Detailed information on how the different parameters were measured is described in the Material and Methods section. The graphs indicate group mean plus standard error. Repeated two-way ANOVA results are displayed inside each graph, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

doi:10.1371/journal.pone.0173232.g005

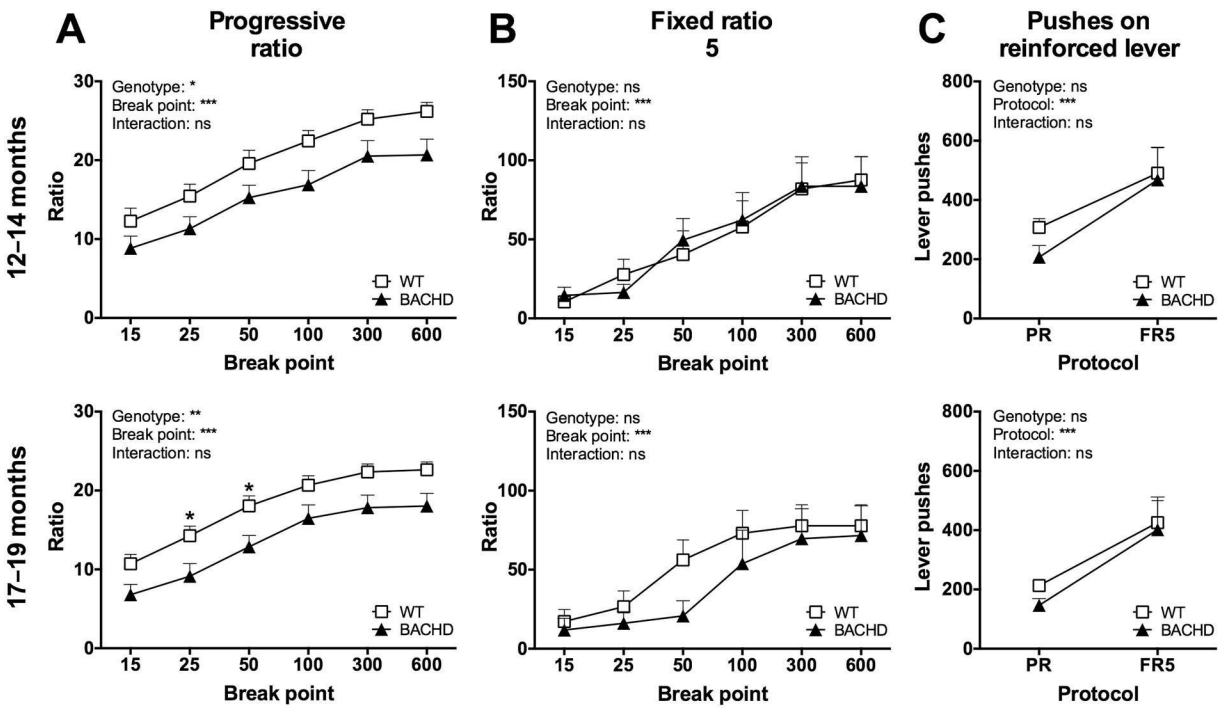

Fig 6. Progressive ratio and FR5 control test performance during satiety. The graphs show performance of Group I in the progressive ratio and FR5 control tests when rats were maintained on free-feeding conditions. (A) shows break point analyses for progressive ratio testing at 12–14 and 17–19 months of age. (B) shows break point analyses for FR5 testing at the same ages. (C) shows comparisons of the mean number of lever pushes performed on the reinforced lever during the two test protocols. The graphs display group mean plus standard error. Repeated two-way ANOVA results are displayed above each graph, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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they showed significantly higher serum concentrations of leptin (Fig 8C). The difference in leptin levels was strongest at satiety and during the standard food restriction protocol. Although the difference was milder during the alternative food restriction protocol, it was still present. Paired analyses of WT rats further showed that the switch from standard to alternative food restriction resulted in them becoming heavier (Fig 8D) and being less motivated to perform the progressive ratio test (Fig 8E), while having increased serum leptin concentrations (Fig 8F).

Body composition analysis

The detailed dissection of Group I at the study's endpoint indicated that BACHD and WT rats did not differ in body weight (Fig 9A), but in body composition (Fig 9B). Specifically, BACHD rats carried a larger amount of adipose tissue than WT rats (Fig 9C), displayed higher serum concentrations of leptin (Fig 9D) and had lower absolute and relative bone/muscle tissue mass (Fig 9E and 9F, respectively). Although BACHD rats have regularly been found to be shorter than WT rats in our institute, no significant difference in the total body length was found in this cohort. A trend was, however, present due to the BACHD rats having significantly shorter tails (data not shown).

Standard food consumption test

When the standard food restriction protocol was used, BACHD rats of Group II consistently consumed less food than WT rats in the standard food consumption test (Fig 10A). When WT

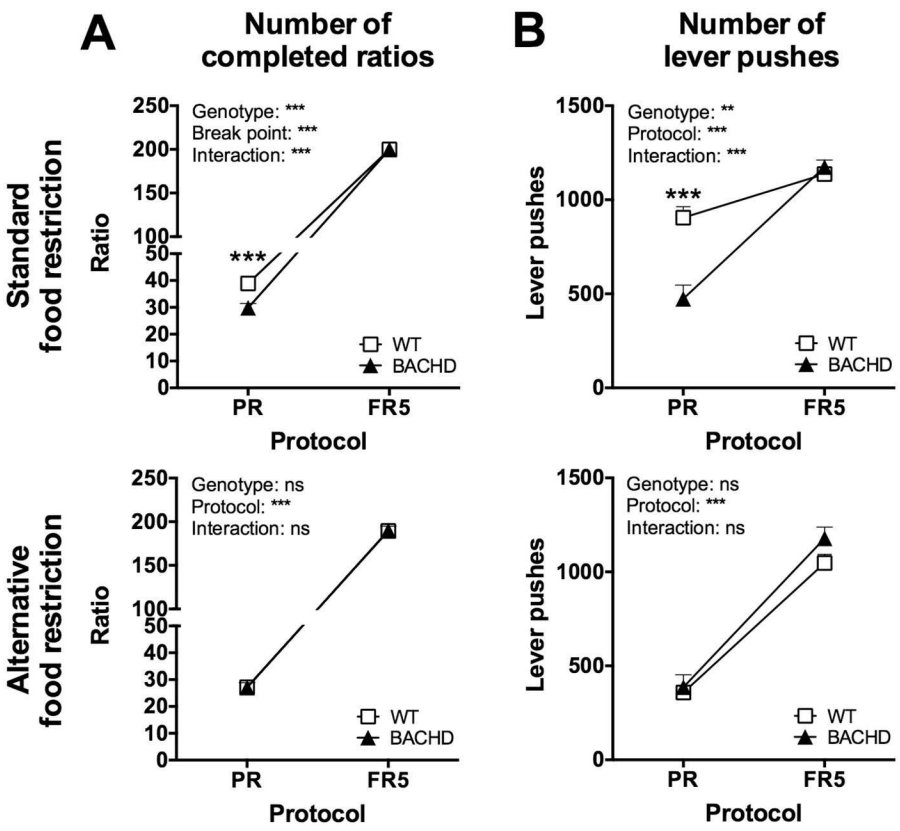

Fig 7. FR5 control test performance during standard and alternative food restriction. The graphs show comparisons of Group I's performance on the progressive ratio and FR5 control tests, when rats were maintained on the standard and alternative food restriction protocols. The number of completed ratios (A) and number of lever pushes performed on the reinforced lever (B) were analyzed, as detailed break point analysis could not be performed. The graphs display group mean plus standard error. Repeated two-way ANOVA results are displayed inside each graph, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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rats were given more food on a daily basis they responded with reduced food consumption rates (Fig 10A). Through careful adjustments of their feeding regimen it was possible to obtain a setting where they showed comparable food consumption rates to the BACHD rats (i.e. the alternative food restriction protocol) (Fig 10A). Baseline values of the rats' performance were created, using all sessions performed on the standard food restriction protocol and the last ten sessions performed on the alternative food restriction protocol. Statistical analysis of these baselines showed a clear change in food consumption rate among WT rats due to the adjustment (Fig 10B and 10C). Similar results were obtained for Group I and for several other animal groups that we have assessed (data not shown). Notably, there was no apparent change in the phenotype when the food was placed on the cage floor instead of in the food crib, although rats of both genotypes consumed generally more food in the former setting (S4 Fig).

Detailed video scoring of the rats' behavior during the standard food consumption test did not indicate any striking differences between WT and BACHD rats when they were maintained on standard food restriction (Fig 11). WT rats consumed more food during the consumption test compared to BACHD rats (Fig 11A), in line with their behavior during baseline performance (Fig 10A). Rats of both genotypes spent comparable amounts of time on arguably food-oriented behaviors, such as paying attention to and biting the food that had been placed

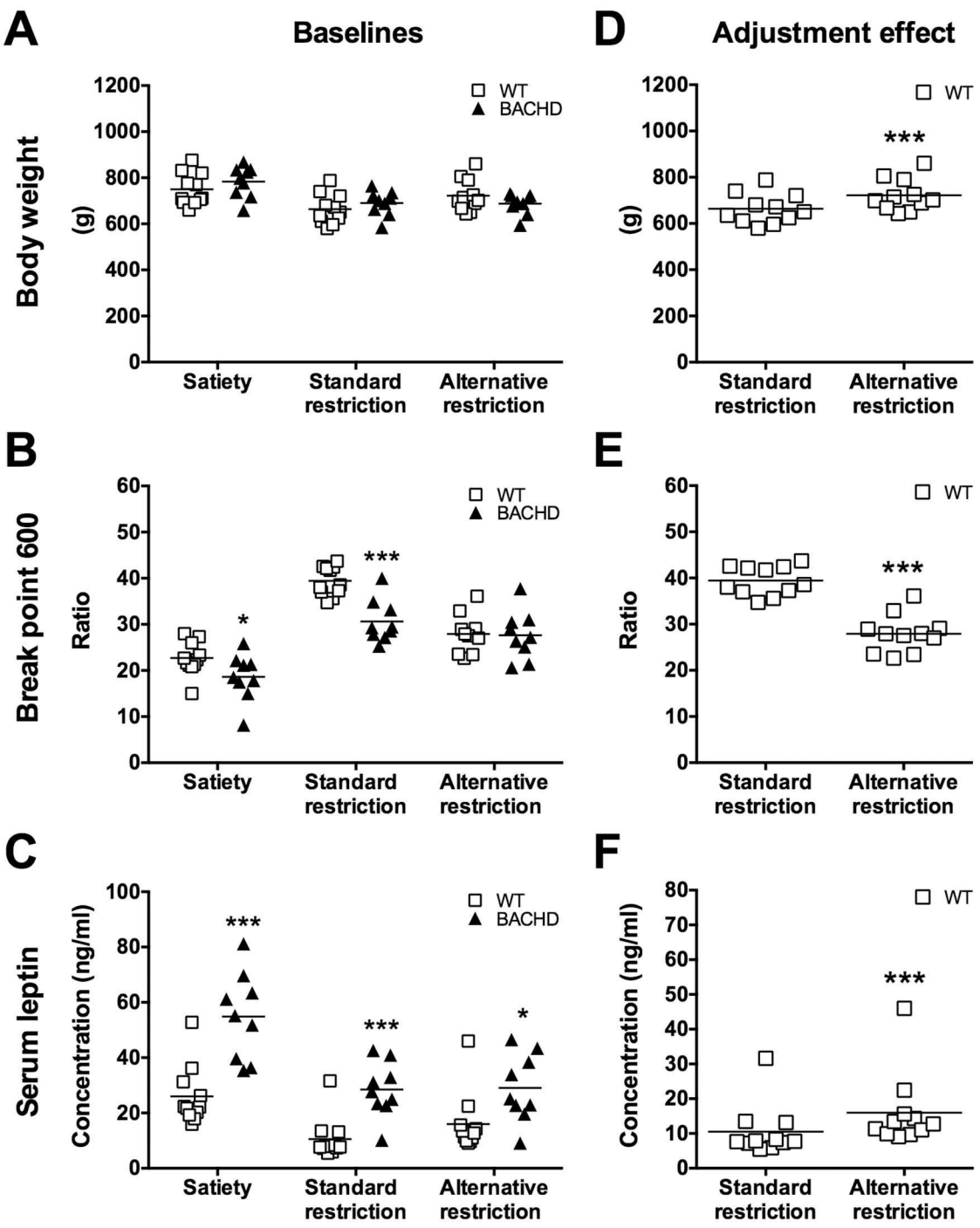

Fig 8. The effect of food restriction adjustment on body weight, progressive ratio performance and serum leptin levels. The graphs show body weight, the number of completed ratios at break point 600 and serum leptin levels of Group I during different food restriction protocols, at 17–19 months of age. (A)–(C) show comparisons between WT and BACHD rats, while (D)–(F) show the specific comparison of WT rats before and after food restriction adjustment. The graphs indicate values from individual rats and group mean. Significant results from *t*-test, Mann-Whitney test, Wilcoxon test or paired *t*-test are displayed inside each graph. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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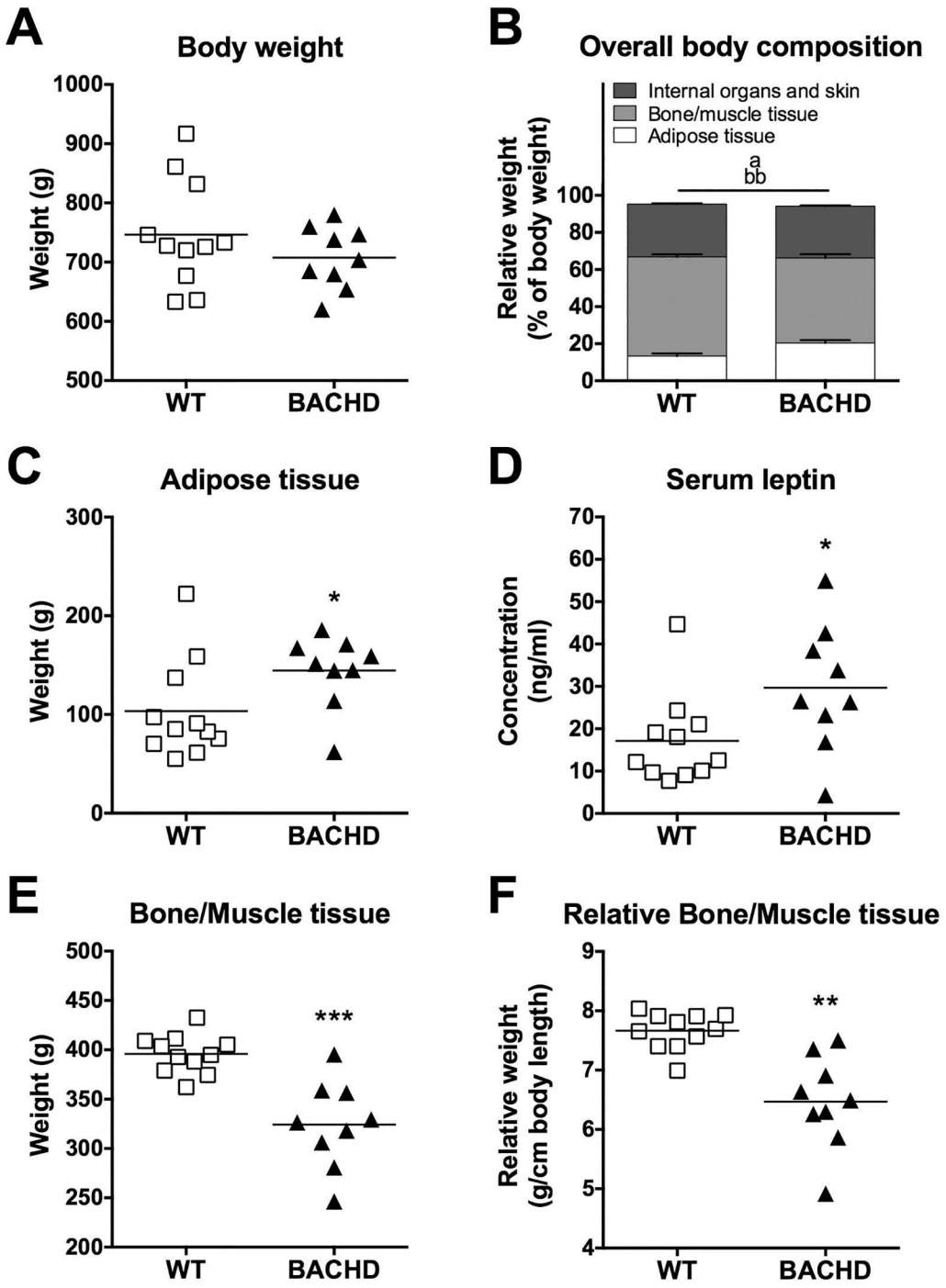

Fig 9. Body composition analysis of rats maintained on alternative food restriction. Parameters of body composition obtained from the dissection of Group I at 19 months of age. Rats were at that time maintained on the alternative food restriction. All graphs except (B) indicate values from individual rats and group mean. (B) indicates group mean plus standard error. Bone/muscle weight in (E) was related to the animals' body lengths to obtain the relative bone/muscle values presented in (F). Significant results from *t*-test or Mann-Whitney tests are displayed inside each graph. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***. For (B), "a" denotes a significant difference in adipose tissue ($P < 0.05$) and "bb" denotes a significant difference in bone/muscle tissue ($P < 0.01$).

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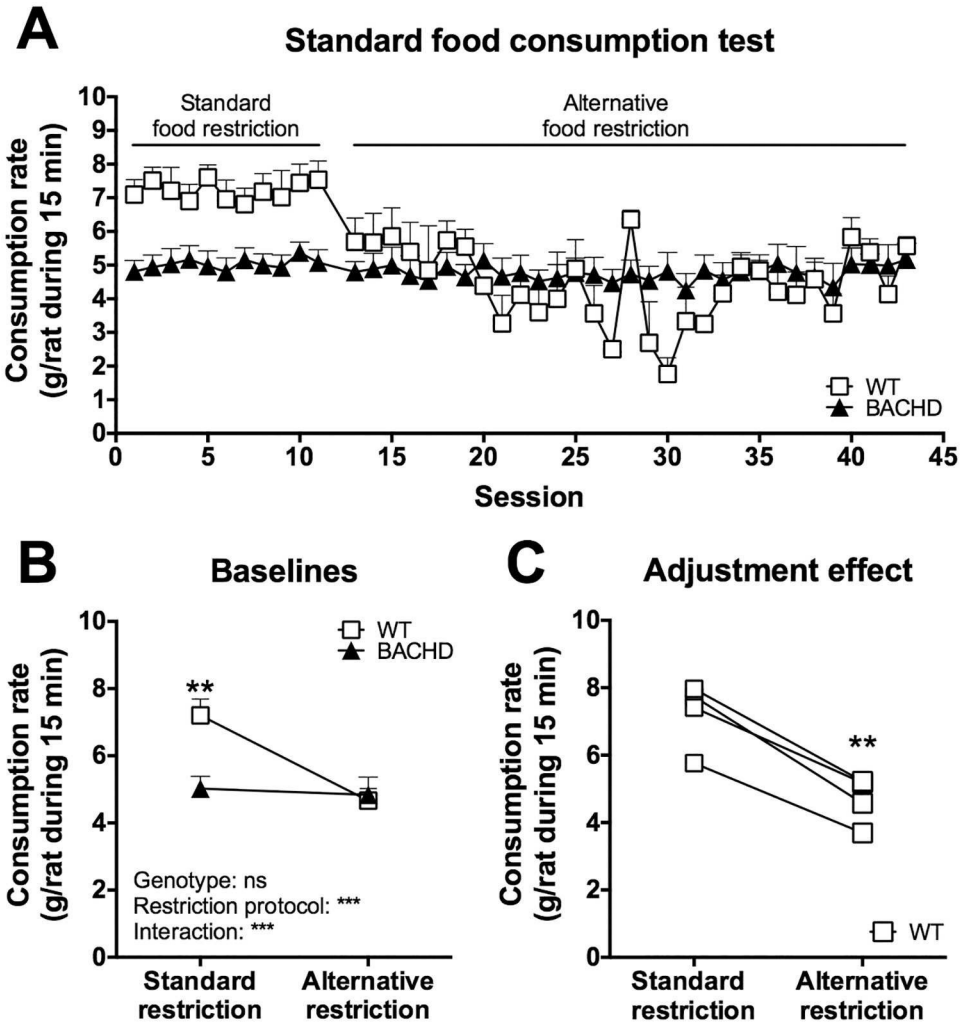

Fig 10. Food consumption rates in the standard food consumption test. Group II's performance in the standard food consumption test at 12 months of age on standard and alternative food restriction is displayed. (A) shows the performance on individual sessions, while (B) and (C) show comparisons of baseline performance during the different food restriction protocols. In (A) and (B) the symbols indicate group mean plus standard error, in (C) the symbols indicate individual WT cages. For (B), repeated two-way ANOVA results are indicated inside the graph, and results from *post-hoc* analysis are displayed in case WT and BACHD differed significantly. For (C), significant results from paired *t*-test is indicated. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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in the food crib (Fig 11B). However, further analysis revealed that BACHD rats had a higher number of both food crib attention (Fig 11C) and biting episodes (Fig 11D). These were, however, shorter compared to WT rats', resulting in the comparable total time spent on either behavior (Fig 11B). Furthermore, BACHD rats had a shorter latency to initiate biting, but there was no difference in how often a food crib attention episode developed into a biting episode (Fig 11D). There was also no difference between genotypes regarding the number of times the rats bit off larger food pieces (Fig 11E). There were, however, trends indicating that BACHD rats took less time to consume such a piece compared to WT rats and that they bit off a separate piece at a slightly lower frequency (Fig 11E). In line with this, there was a significant difference in the total time spent consuming separate food pieces, with BACHD rats spending

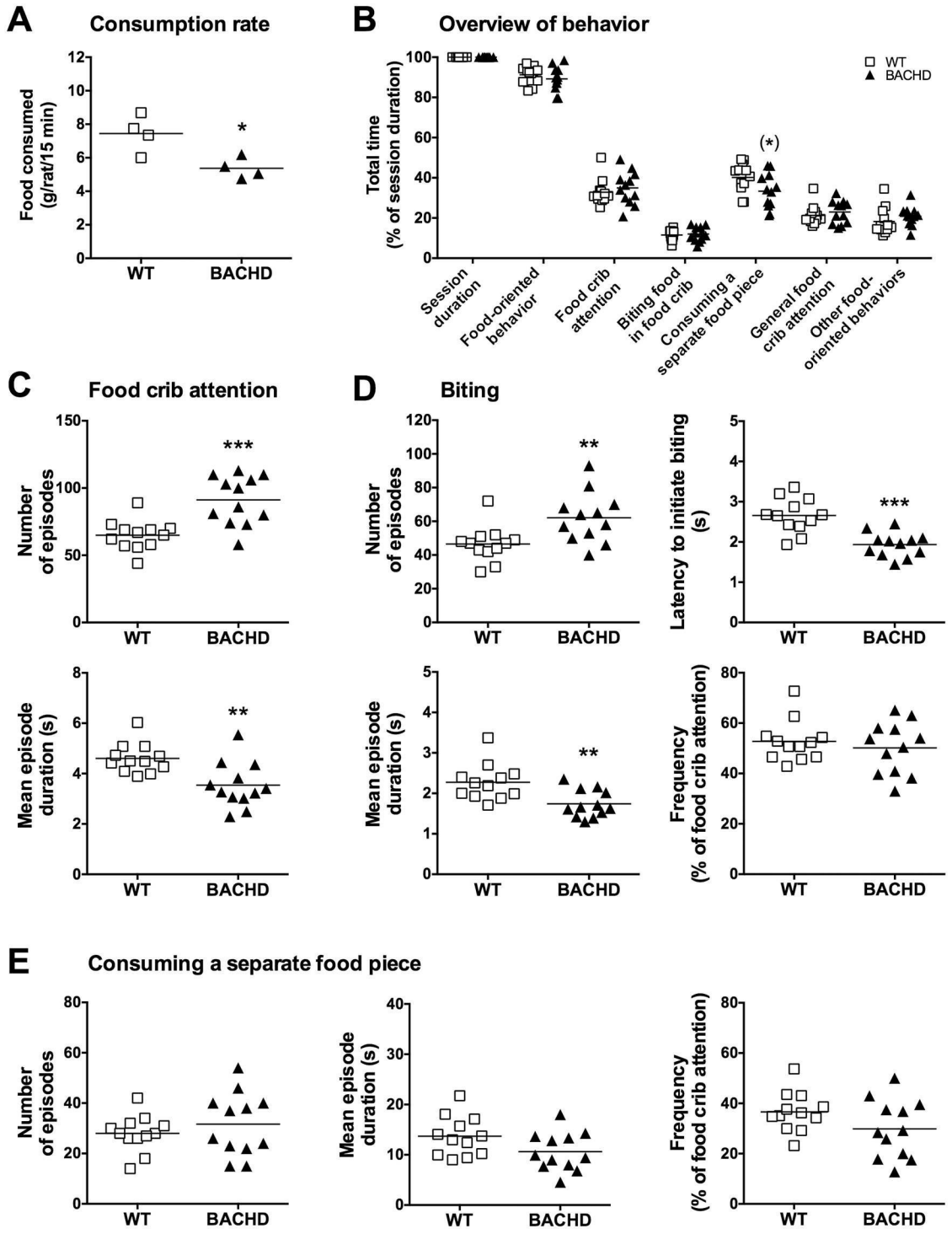

Fig 11. Video scoring of behavioral parameters from the standard food consumption test during standard food restriction. Group II's performance on the standard food consumption test during the standard food restriction protocol was subjected to detailed video analysis. (A) shows the consumption rate measured for individual cages on the video scored session. (B)–(E) display the behavior of individual rats during the same session. (B) shows the total amount of time spent on different behaviors, in relation to the duration of the test session. (C)–(E) show details concerning some of the behaviors, indicating the number of behavioral episodes, mean episode duration, frequency of behavior and the latency to initiate the behaviors. Frequency relates to the percentage of food crib attention episodes that turn into biting episodes (D) and episodes were rats consume a separate food piece (E). Significant results from *t*-test or Mann-Whitney tests are

displayed inside each graph. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***. Results in (B) were corrected for multiple comparisons using the Sidak method. Significance levels that were lost through this approach are indicated with a parenthesis.

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less time on this activity compared to WT rats (although the difference was no longer significant when multiple comparison corrections were considered) (Fig 11B).

As noted, WT and BACHD rats consumed comparable amounts of food when they were maintained on the alternative food restriction protocol (Fig 12A). Interestingly, under these conditions WT rats spent less time than BACHD rats on food-oriented behaviors (Fig 12B). This was primarily due to them spending less time than BACHD rats on general food crib attention, while the time spent actively biting the food, consuming separate food pieces and performing other food-oriented behaviors did not significantly differ between the genotypes (Fig 12B). BACHD rats still showed a higher number of food crib attention episodes compared to WT rats, although there was no longer any difference in the mean duration of individual episodes (Fig 12C). The rats' behavior during biting episodes was similar to what was found during the standard food restriction protocol, with BACHD rats showing a higher number of episodes, a shorter mean duration of individual episodes, but no difference in biting episode frequency compared to WT rats. However, in contrast to the previous results, there was no difference between WT and BACHD rats in the latency to initiate biting (Fig 12D). As noted above, BACHD rats spent in total less time than WT rats on consuming separate food pieces when the standard food restriction protocol was used (Fig 11B). An opposite trend was found during the alternative food restriction (Fig 12B and 12E). Specifically, WT rats showed fewer episodes where they consumed separate food pieces compared to BACHD rats (Fig 12E). Interestingly, there was a trend indicating that WT rats still bit off food pieces at a higher frequency (Fig 12E). As before, there was no difference between WT and BACHD rats concerning the mean duration of episodes spent consuming separate food pieces.

In addition to the analysis shown in Figs 11 and 12, a series of curves were made to better display how the WT rats' behavior changed as a result of the change in food restriction protocol (S5 and S6 Figs). As expected from the results described above, WT rats showed a specific drop in the time spent on food-oriented behavior (S5B Fig) due to a drop in the time spent on general food crib attention (S5E Fig). This in turn appeared to be due to a drop in the mean duration of individual food crib attention episodes, rather than a drop in the number of such episodes (S6A Fig). In line with this, the latency to initiate biting among WT rats was reduced when the alternative food restriction protocol was used (S6B Fig).

Individual feeding test

Most rats reliably consumed the full food piece without frequent or extensive breaks, regardless of which food restriction protocol was used. Two WT rats, however, did not reliably consume the food pellet during the alternative restriction and had to be excluded from the analysis. During both restriction protocols, WT and BACHD rats showed a relatively high consumption rate on initial sessions compared to their stable baseline performance (Fig 13A). For analyzing mean baseline consumption rates, sessions 5–15 and 5–12 were used for the standard and alternative food restriction protocols, respectively. BACHD rats showed a generally lower food consumption rate compared to WT rats during both restriction protocols, although the phenotype was somewhat stronger when the rats were maintained on the alternative food restriction (Fig 13B). The change in food restriction protocol did not appear to have a major impact on the WT rats' performance (Fig 13C), with the exception of the aforementioned two rats that generally lost interest in consuming the food pellet.

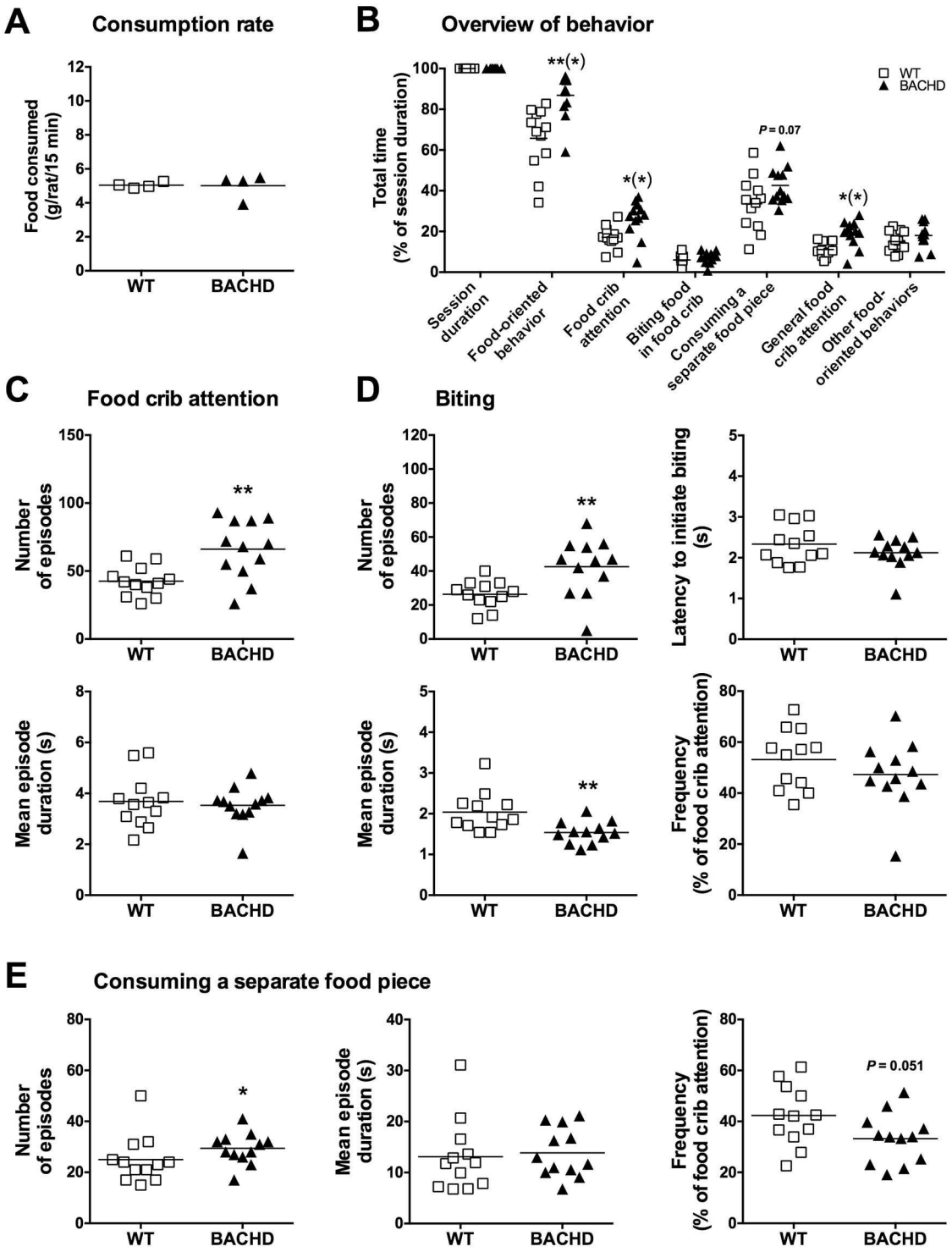

Fig 12. Video scoring of behavioral parameters from the standard food consumption test during alternative food restriction. Group II's performance on the standard food consumption test during the alternative food restriction protocol was subjected to detailed video analysis. (A) shows the consumption rate measured for individual cages on the video scored session. (B)–(E) display the behavior of individual rats during the same session. (B) shows the total amount of time spent on different behaviors, in relation to the duration of the test session. (C)–(E) show details concerning some of the behaviors, indicating the number of behavioral episodes, mean episode duration, frequency of behavior and the latency to initiate the behaviors. Frequency relates to the percentage of food crib attention episodes that turn into biting episodes (D) and episodes were rats consume a separate food piece (E). Significant results from *t*-test or Mann-Whitney tests are

displayed inside each graph. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***. Results in (B) were corrected for multiple comparisons using the Sidak method. Significance levels that were lost through this approach are indicated with a parenthesis.

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Video scoring was performed on videos gathered on the first, fifth, sixth and seventh test session of the alternative food restriction test. The first session was chosen due to the phenotype being particularly strong, while session 5–7 were thought to represent baseline performance. Individual biting and chewing episodes were easily identifiable in the videos and made up >96% of the time scored as active feeding (data not shown). The unaccounted time was most likely lost due to the manual nature of the scoring method, which resulted in slight breaks

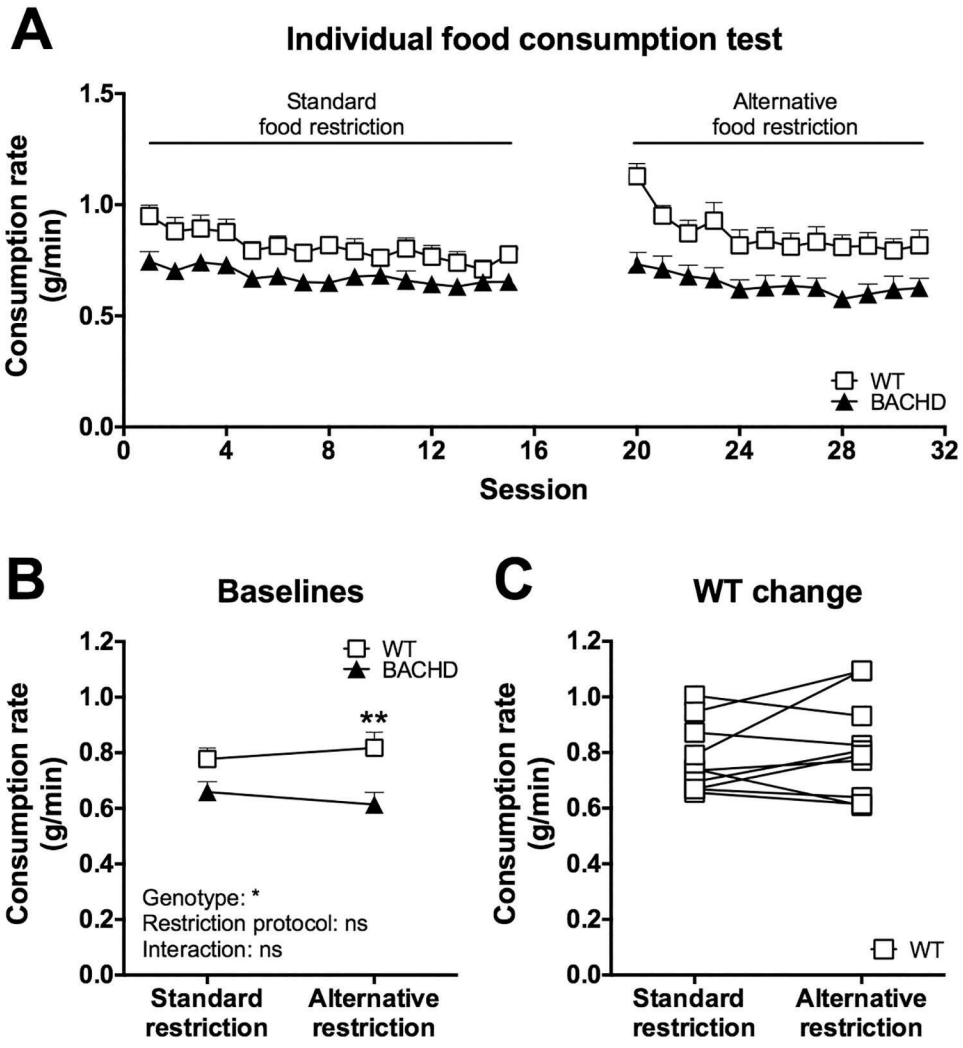

Fig 13. Food consumption rates in the individual food consumption test. Group II's performance in the individual food consumption test at 12 months of age on standard and alternative food restriction is displayed. (A) shows the performance on individual sessions, while (B) and (C) show comparisons of baseline performance during the different food restriction protocols. In (A) and (B) the symbols indicate group mean plus standard error, in (C) the symbols indicate individual WT rats. For (B), repeated two-way ANOVA results are indicated inside the graph, and results from *post-hoc* analysis are displayed in case WT and BACHD differed significantly. For (C), significant results from paired *t*-test is indicated. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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between the scored behaviors whenever a switch between biting and chewing episodes occurred. Video analysis of the first session indicated that BACHD rats needed more time than WT rats to consume the food pellet (Fig 14A). In addition, BACHD rats required more bites compared to WT rats (Fig 14B) and consequently had a smaller estimated bite size (Fig 14C). Although there was no difference in the mean duration of individual biting episodes (Fig 14D), curves showing the biting episode duration distribution still clearly indicated a behavioral difference between the rats (Fig 14E and 14H). While WT rats had a small range of relatively fast bites, BACHD rats showed a slightly right-shifted and broadened peak, indicating that they had slightly longer biting episodes compared to WT rats (Fig 14E and 14H). There was no difference between the genotypes in the mean chewing episode duration (Fig 14F). Detailed analysis of the chewing episode duration distribution indicated that BACHD rats had a higher number of short chewing episodes compared to WT rats (Fig 14G), although the relative distribution of chewing episodes did not indicate any behavioral differences between the genotypes (Fig 14I).

As noted above, the food consumption rate phenotype was noticeably weaker during baseline performance. This was also true for the phenotypes found in the video scoring. BACHD rats still needed more time than WT rats to consume the food pellet (S7A Fig), but there was no longer any statistical difference in the number of bites (S7B Fig) or the estimated bite size (S7C Fig). BACHD rats still showed a shift towards making longer biting episodes compared to WT rats (S7E Fig), although it was less pronounced than during the first test session (Fig 14E). BACHD rats did again not show any indications of having a changed chewing behavior during baseline performance (S7F, S7G and S7I Fig). When splitting the total time needed to consume the food pellet (S8A Fig) into the total time spent biting (S8B Fig) and the total time spent chewing (S8C Fig), BACHD rats spent specifically more time chewing compared to WT rats. Additional analysis of chewing episode distribution, using a different set of bins, indicated that BACHD rats had more chewing episodes of intermediate duration (1.6–5.0 s) compared to WT rats (S8D Fig). BACHD rats also showed an increased total amount of time chewing specifically within this range of chewing episodes (S8E Fig), without showing a difference in mean chewing episode duration (S8E Fig).

Finally, the BACHD rats of Group II were found to have shorter heads compared to their WT littermates (S9A Fig). However, this did not appear to have any major influence on the rats' food consumption rates (S9C and S9D Fig).

Discussion

Progressive ratio performance and motivational phenotype of BACHD rats

One of the aims of the current study was to evaluate if our initial findings concerning the BACHD rats' performance in the progressive ratio test [18] were reproducible at older ages. This was clearly the case. At all investigated ages, BACHD rats were less motivated than WT rats to perform the test when the standard food restriction protocol was used. When the alternative food restriction protocol was used, WT and BACHD rats reliably showed comparable motivation to perform the test. Ultimately, the results are likely to also be reproducible with other groups of BACHD rats, as they do not appear to be caused by unspecific variations in performance.

Our initial interpretation regarding the motivational deficit in the BACHD rat was that it was likely to be caused by metabolic, rather than psychiatric disturbances [18]. We hypothesized that when the rats were maintained on standard food restriction, WT rats were hungrier than BACHD rats, resulting in them being more motivated to perform lever pushes for a food

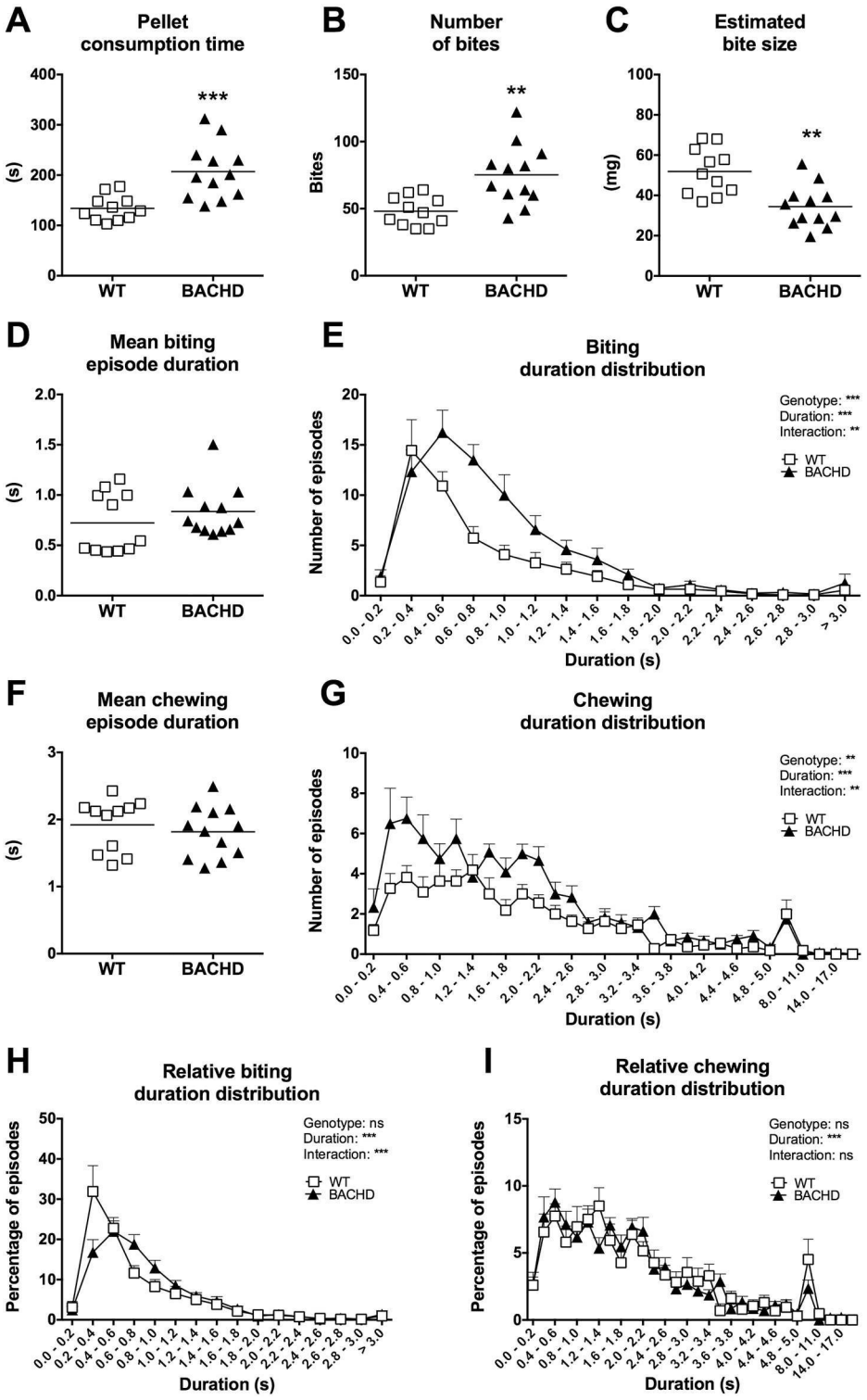

Fig 14. Video scoring of the individual food consumption test during alternative food restriction. Group II's performance on the first session of the individual food consumption test during the alternative food restriction protocol was subjected to detailed video analysis. (A)–(D) and (F) indicate the performance of individual rats. Significant results from *t*-test or Mann-Whitney test are shown. (E), (G), (H) and (I) show frequency distribution curves for biting and chewing episodes of different durations, indicating group mean plus standard error. The bins used are described in detail in the Material and Methods section. Note that the x-

axis in (G) and (I) only labels every other bin. Results from repeated two-way ANOVA are displayed inside the graphs. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

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reward. The alternative food restriction protocol sought to adjust the food restriction levels of the rats, so that they became equally hungry. As this reliably resolved the motivational deficit in the progressive ratio test, we considered it unlikely that the initial phenotype had been caused by psychiatric deficits. In the current study, we aimed at further evaluating this idea by performing the progressive ratio test while the rats had free access to food. This constituted a second feeding condition (on top of the alternative food restriction), where WT and BACHD rats should be equally hungry (i.e. in this case satiated). However, in contrast to their behavior during the alternative food restriction, BACHD rats were found to be less motivated than the WT rats in this setting (i.e. similar to the rats' behavior during the standard food restriction). Importantly, this did not appear to be due to BACHD rats becoming satiated or fatigued at an earlier point than WT rats, as performance on the FR5 control test (where rats of both genotypes performed more pushes and consumed more pellets compared to the progressive ratio sessions) did not differ between the genotypes (for the same reason, the BACHD rats' reduced motivation during the standard food restriction is likely not caused by fatigue or satiety). Ultimately, a difference in hunger levels is unlikely to fully explain the motivational deficit found in BACHD rats performing the progressive ratio test. Still, the phenotype might be otherwise connected to the rats' metabolic disturbances.

As previously noted, male BACHD rats are obese [18]. Leptin, an endocrine hormone secreted from white adipose tissue [23], has been shown to affect rats' motivation to perform the progressive ratio test. Specifically, increased leptin signaling has been found to reduce motivation [24,25], while knock-down of leptin receptors has been found to increase motivation [26]. Interestingly, leptin has been shown to decrease motivation in progressive ratio tests both at satiety [24,25] and during food restriction [26]. Because of this, we hypothesized that the motivational deficit seen in BACHD rats during satiety and the standard food restriction was caused by an obesity-related increase in serum leptin levels. We further hypothesized that this phenotype would be resolved through the use of the alternative food restriction protocol. To evaluate this, we measured serum leptin levels in Group I during their different performance baselines. The results clearly indicated that BACHD rats had higher leptin levels than WT rats both at satiety and when the standard food restriction protocol was used. However, although the difference was less apparent during the alternative food restriction, it was not fully resolved. In line with this, the dissection results clearly showed that the BACHD rats still carried more adipose tissue than WT rats when they were maintained on the alternative food restriction. Thus, the results appear to argue against the hypothesis that the BACHD rats' motivational deficit is caused primarily by their obesity. Still, it is not known how large the difference in leptin levels would have to be in order to result in such a phenotype. In relation to this, it is also unknown, if the neuronal circuits necessary for leptin signaling are intact in BACHD rats. To better understand the current results, it would therefore be of interest to investigate dose-response curves for leptin's effect on BACHD and WT rats' progressive ratio performance. In addition, it would be important to study the expression of leptin receptors in the BACHD rats' mid- and hindbrain, as these regions appear to be involved in progressive ratio performance [24,26,34] (interestingly, leptin receptors in the hypothalamus appear to be of less importance [26]).

However, further studies of the integrity of the BACHD rats' leptin system are unlikely to offer any final conclusions regarding whether or not their motivational deficit is caused by their obesity. For this, efforts should be made to elucidate the cause of the rats' obesity, so that

lean BACHD rats might be obtained and subsequently assessed in the progressive ratio test. Interestingly, inactivating mutant huntingtin expression in the hypothalamus of BACHD mice completely resolved their obesity phenotype [35]. Although the cause for the obesity phenotypes might differ between BACHD mice and BACHD rats (obesity in the mouse model has been suggested to be due to overeating [35], while this does not appear to be the case in the rat model [17,18]), both could be due to hypothalamic pathologies, involving different subregions [36]. Regarding BACHD rats, the arcuate nucleus is particularly interesting, as lesioning this region has been shown to result in obesity without associated hyperphagia [36–40]. Interestingly, the lesions appear to target neuron populations that are involved in regulating the release of growth hormone [40–43]. In line with this, down-regulation of growth hormone signaling has been found to result in growth impairments coupled with obesity [44–46], i.e. specifically the phenotypes that we have previously noted in male BACHD rats [18]. Moreover, one of the peripheral functions of growth hormone is to stimulate the release of IGF-1 from the liver [47], and we have repeatedly found that male BACHD rats have lower serum levels of IGF-1 (unpublished data). Thus, the growth hormone signaling axis and the integrity of the arcuate nucleus are of great interest for future work with the BACHD rats. In connection to this, detailed investigation of the similarities and differences in male and female BACHD rats' physiologies would be of importance.

The phenotype of reduced motivation among BACHD rats remained arguably stable when the rats were retested at older ages. Still, there were some indications that a subtle progressive worsening of the phenotype might be present (i.e. the *post-hoc* analysis shown in Fig 2B and the results from the three-way ANOVA Genotype x Age interaction effect). However, additional longitudinal studies of the BACHD rats' progressive ratio performance would be necessary to conclude if this is truly the case. Based on HD's clinical presentation, one would expect disease-related phenotypes in animal models to progressively worsen with age. In line with this, other phenotypes found in the BACHD rats have shown strong progressive change even at ages below four months [17,48,49], which is well within the ages investigated in the current study. Still, it is worth noting that the obesity phenotype did not appear to change with age during our previous study [18], so if that indeed causes the motivational phenotypes one would expect the latter to remain reasonably stable as well. Still, not all psychiatric symptoms in HD patients clearly progress with age either [50]. For example, while apathy appears to progressively worsen, depression does not [22,50–52]. Performance in the progressive ratio test at satiety has been suggested to be primarily affected by the rats' hedonic value of the food reward, while motivation to perform the test during food restriction is thought to be more governed by the induced energy imbalance (i.e. hunger) [53, 54]. As the BACHD rats were less motivated to perform the test at satiety, it is possible that their motivational phenotype is at least to some extent due to anhedonia, which is an aspect of depression that has been implicated in HD [55]. In the end, the apparent lack of progression seen in the BACHD rats' motivational deficit does not offer any clear insight into the specific nature of the phenotype.

Although the alternative food restriction protocol only changed the WT rats' restriction conditions, BACHD rats also showed drops in motivation between the performance baselines established on the standard and alternative restriction protocols. During the 7–9 months test, this was primarily caused by the set of prefeeding tests described in the Material and methods section. As noted, both WT and BACHD rats failed to return to their initial performance baseline between the prefeeding tests, and their motivation instead dropped with each session. As the prefeeding tests were run between the establishment of performance baselines on the standard and alternative restriction, the BACHD rats show a clear drop in motivation when the two are compared directly. As the same issue concerned the WT rats, the change in restriction protocol appeared to have a particularly pronounced effect on performance during the 7–9

months test (as indicated by the significant Restriction protocol x Age interaction effect revealed by the three-way ANOVA). The drop in motivation that was seen among BACHD rats during the later test ages was instead likely related to a specific aspect of the food restriction. As noted, both the standard and alternative food restriction protocols took natural growth into account, which meant that the amount of food given to the rats was continuously adjusted. We have in other studies found that the current calculations result in a slight overcorrection of the food restriction (due to the expected growth being overestimated). The error increases with experiment duration, although we have not found any strong behavioral effects of this and have reliably been able to establish stable performance baselines. Still, this is likely the reason for the small drop in motivation seen among BACHD rats when directly comparing their baselines from the current study.

One final and important aspect to consider in the current progressive ratio results concerns the readouts that were not directly related to the rats' motivation, as these indicated that BACHD rats might suffer from striatal impairments. First, there is the slowed food pellet retrieval seen among BACHD rats. From the several Skinner box-based tests that we have run so far at our institute, this phenotype is found in almost all test protocols and animal groups (largely unpublished, but see [18]). Thus, it offers an interesting and reproducible phenotype to work with, although it is at this point unclear if the impairment is caused by motoric or psychiatric deficits. Similar phenotypes have been found in the TgHD rat model of HD [56] and rats with lesions to the dorsolateral striatum [57]. Second, BACHD rats were found to perform an increased number of excessive (i.e. perseverative) lever pushes. This has also been seen in rats with lesions to the dorsal striatum [57]. Interestingly, such lesions do not appear to affect the rats' overall motivation to perform the progressive ratio test [57]. Thus, the slowed pellet retrieval latency and the increase in perseverative lever pushes suggest that the BACHD rats suffer from some kind of striatal dysfunction, which is likely separate from what causes their motivational impairment. In line with this, the slowed pellet retrieval and perseverative responding were present on both standard and alternative food restriction.

Food consumption rate phenotypes of BACHD rats

In our initial study [18], we used a food consumption test (the standard food consumption test) in order to estimate the rats' apparent hunger and food interest, as similar methods had been used by others [28–33]. In the current study, we sought to extend our initial work by adding a video-based scoring of the rats' behavior in the standard food consumption test, and also assess how they consume individual food pieces (individual food consumption test). When the standard food restriction protocol was used, BACHD rats were found to have a lower food consumption rate compared to WT rats in both tests. In contrast, when the alternative food restriction protocol was used, there was no difference in the rats' food consumption rate in the standard food consumption test, while BACHD rats were still slower than WT rats in the individual food consumption test.

BACHD rats were found to require more biting episodes than WT rats in order to consume the food pellets in the individual food consumption test. As all rats were given food pellets of comparable size, the results suggest that BACHD rats also took smaller bites compared to WT rats. This phenotype could be related to BACHD rats having problems with biting larger pieces off from the food pellet, with keeping a large amount of food inside their mouths or with efficiently chewing and swallowing a large food piece. Importantly, the deficit did not appear to be due to the BACHD rats' smaller heads, and did not seem to be strongly influenced by hunger. In addition to requiring a higher number of biting episodes to consume the pellets, the BACHD rats' biting episodes were slightly longer than the WT

rats'. It is possible that BACHD and WT rats used similar techniques for biting pieces off from the food pellet. If so, the BACHD rats' longer biting episodes might indeed indicate that they had problems biting pieces off. However, the results might also be due to BACHD rats preferring more time-consuming techniques (such as gnawing rather than performing distinct bites) compared to WT rats. More detailed scoring would be required to determine if that was the case. Further characterization work would also be necessary in order to determine if this could explain the BACHD rats' smaller bite size, and whether or not it would be related to a motoric impairment. The duration of single biting episodes among both WT and BACHD rats were still short compared to the chewing episodes. Therefore, the latter likely had a stronger impact and probably contributed more to the BACHD rats' food consumption rate phenotype. In line with the hypothesis that BACHD rats made smaller bites compared to WT rats, analysis revealed that they showed a higher absolute (but not relative) number of short chewing episodes. If BACHD rats were as skillful as WT rats at chewing and swallowing, while managing smaller volumes of food during each chewing episode, one might have expected them to show a more pronounced shift towards shorter chewing episodes. The apparently unchanged frequency distribution of chewing episodes could thus indicate that BACHD rats indeed have problems with chewing and swallowing. In this regard, it is worth considering that the smaller bite size discussed above might constitute a compensatory mechanism, allowing BACHD rats to maintain optimal (i.e. seemingly unchanged) chewing. Impaired chewing and swallowing could be due to motoric impairments, although other possibilities should also be considered. Specifically, we have found that BACHD rats have disproportionally small salivary glands (unpublished results), which might impair their ability to form a convenient food bolus. HD patients often suffer from problems regarding eating, with particularly frequent problems when swallowing [58–61]. We have repeatedly performed tests where WT and BACHD rats are allowed to consume a large amount of the reward pellets used in the Skinner boxes (see [18] for a published example). Typically, BACHD rats are slightly slower than WT rats during initial sessions of this, but quickly reach a comparable consumption rate. Importantly, consumption of these small reward pellets appears to involve very limited chewing behavior, suggesting that other aspects (such as tongue protrusion and swallowing) are more important determinants of the food consumption rate in this test. Given the BACHD rats' generally unimpaired performance in these tests, their ability to swallow is most likely not strongly impaired. In addition, we have performed several tests where BACHD rats were allowed to consume spaghetti pieces (unpublished data). Feeding behavior under these circumstances appears to involve biting primarily with the incisors, and once again limited chewing. Again, BACHD rats have generally been found to show comparable consumption rates to WT rats in this test. Thus, the key factor causing the BACHD rats' slowed food consumption in the individual food consumption test might concern the test's strong dependency on chewing and/or the formation of a convenient bolus for swallowing. Further efforts should be made to characterize the noted consumption rate deficit, as it constitutes an interesting and robust phenotype seen among the BACHD rats.

Our initial interpretation of the slowed consumption rate among BACHD rats in the standard food consumption test was that they were less hungry compared to WT rats, and that the alternative restriction protocol resolved this difference. The results from the current study do not strongly support this idea, but do not necessarily refute them either. When the standard restriction protocol was used, BACHD and WT rats generally behaved in a comparable way. They showed similar amounts of food-oriented behaviors and spent the same amount of time on both paying attention to the food in the food crib and actively trying to bite pieces off from it. As it is clear that BACHD rats still consumed less food than WT rats, it is reasonable to

assume that their biting behavior was less efficient. The analysis also indicated that BACHD rats performed more but shorter biting episodes compared to WT rats. Based on the results from the individual food consumption test, it seems fair to assume that this might be due to them taking a high number of smaller bites, while WT rats made a low number of larger bites. This is further supported by the fact that episodes where rats consumed a separate food piece did not differ in length between the genotypes. If BACHD and WT rats had bitten off pieces of comparable size, one would expect these consumption episodes to be longer among BACHD rats (according to the results of the individual food consumption test). Thus, the nature of the reduced food consumption rate among BACHD rats seems at first glance to be comparable between the standard and individual food consumption tests. Ultimately, this suggests that the phenotype seen in the standard food consumption tests during standard food restriction is primarily due to them taking smaller bites, which (based on the results from the individual food consumption test) might not be strongly affected by hunger.

The alternative food restriction protocol sought to match WT and BACHD rats' food consumption rates (with the assumption that this represented the rats' hunger level). This primarily focused on giving more food to WT rats, and as a consequence, there was a clear change in their behavior. Most notably, the amount of time spent on food-oriented behaviors and paying attention to the food crib dropped below the level of BACHD rats. This appeared to be largely a result of WT rats making shorter visits to the food crib, rather than fewer. This, in turn, seemed to be due to the WT rats showing a reduced latency to initiate biting episodes. In contrast, the time spent biting at the food remained unchanged and comparable to both their behavior during the standard food restriction protocol and to that of the BACHD rats. As the WT rats still consumed less food under these circumstances, the results suggest that their biting behavior had now become less efficient. Due to the limitations of the video quality, it remains unclear if the unidentified hunger-sensitive behaviors that modulated the WT rats' biting efficiency were the same as the ones causing the BACHD rats' reduced food consumption rate during the standard food restriction protocol. As noted, a reduced bite size might be the cause of the BACHD rats' reduced consumption rate in the standard food consumption test. A change in bite size could theoretically also explain the change in the WT rats' consumption rate during the alternative food restriction. The latter is, in contrast to the former, not supported by the results from the individual food consumption test, as bite size appeared to be unaffected by the change in food restriction protocol. Still, it should be noted that food consumption behavior in these two tests might not be directly comparable. In the standard food consumption test, the rats remain in their home cage and are allowed to consume food if they are interested. In contrast, in the individual food consumption test the rats are more or less forced to consume the food piece before being allowed to return to their home cage. Thus, the latter test might have conditioned the rats to eat as fast as possible, rather than based on how hungry they were. This might have resulted in the evaluated parameters' (e.g. bite size) apparent resistance to a change in hunger levels. Importantly, it is clear that hunger was not the only factor that affected the rats' performance in the individual consumption test, as both WT and BACHD rats showed very high consumption rates during early sessions and needed several sessions to approach a stable baseline performance. This was despite the fact that the rats were maintained on a constant feeding regimen. Thus, while bite size appears to be unaffected by hunger in the individual food consumption test, it might still be sensitive to hunger in the standard food consumption test. Ultimately, it is therefore still possible that the less efficient biting behavior of BACHD rats in the standard food consumption test during standard food restriction is caused by them being less hungry compared to WT rats. Additional work is needed before a final conclusion regarding this matter can be reached.

The more extensive investigation of WT and BACHD rats' food consumption behavior in the current study was in part performed to better understand the progressive ratio phenotype of the BACHD rats. As noted, the BACHD rats' motivational deficit cannot be fully explained by a difference in hunger or leptin levels. Based on the results above, it can be further argued that the standard food restriction constitutes a suitable protocol, as it seems to induce similar food interest among WT and BACHD rats. In addition, one can argue that the lower food consumption rates among BACHD rats could primarily be caused by non-hunger related differences in feeding behavior. If so, the alternative food restriction protocol would only serve to mask the underlying phenotype rather than to resolve it. This would also suggest that the apparent lack of a motivational deficit in the progressive ratio test during the alternative food restriction is coincidental. Ultimately, the true phenotype of the BACHD rats would be a reduced motivation to perform the progressive ratio test, likely based on a psychiatric deficit. However, as noted above, the influence of the BACHD rats' obesity and increased leptin levels on their progressive ratio performance is not clear, and conclusive results still have to be obtained. Likewise, the exact nature of the food consumption rate phenotype in the standard food consumption test remains unclear, and could still involve more discreet hunger-related behaviors than the ones scored here.

Connection to previously noted motor impairments of the BACHD rats

Other studies have sought to directly investigate the presence of motor impairments among the BACHD rats [17,48,49]. These have revealed early (onset at one to two months of age) progressive deficits in the BACHD rats' ability to maintain balance on a rotating rod [17,48,49], and late (onset at twelve to fourteen months) deficits in unhindered gait [17,48]. The results from the current study suggest that yet another kind of motor function (i.e. orofacial) might be disturbed in the BACHD rat, and is worth investigating further. From the results we have gathered so far, these impairments appear to show an early onset [18], without any clear progression with age. All together, the results suggest that BACHD rats might suffer from a range of different motor impairments, which become apparent and progress differently throughout their disease development. However, the influence that possible confounding factors (such as repeated exposure to the stressful rotarod test and the BACHD rats' changed physiology) have had on these motor impairments has not been investigated. Thus, additional work is needed before conclusions on the overall picture of the BACHD rats' motor impairments can be drawn.

Assessing BACHD rats' performance in food-based tests

One of the overarching aims of our research is to investigate the presence of cognitive impairments in the BACHD rat. A large concern when considering this has been the BACHD rats' metabolic phenotypes and the possibility that these could confound the readouts of a given behavioral protocol. Although it remains unclear if the obesity phenotype is the main cause of the BACHD rats' reduced motivation to perform the progressive ratio test, the consistent difference in motivation is of importance when considering other behavioral protocols. Notably, differences in motivation have been found to result in remarkable differences in behavior [20]. In our initial publication [18], we argued that the alternative food restriction protocol constitutes a good approach to achieve an experimental setting where WT and BACHD rats are comparably motivated to perform a given food-reinforced behavioral test. Although the current results also largely argue for that, the use of the alternative food restriction protocol can no longer be fully supported. This is primarily due to the fact that it is based on matching the rats' food consumption rates, with the assumption that this represents a good measurement of

hunger. As noted, the exact nature of the BACHD rats' reduced food consumption rates is not clear, and it might be influenced by non-hunger related feeding impairments. However, the standard food restriction protocol clearly results in WT and BACHD rats having different metabolic characteristics, and would likely result in them being differently interested in performing a given food-reinforced test. As neither protocol is optimal on its own, we suggest that any behavioral characterization performed with BACHD rats in food-reinforced tests should include appropriate control tests. These should aim at investigating how the readouts of the given test are affected by changes in motivation. If phenotypes are found in parameters that are sensitive to changes in motivation, interpretations should be made carefully.

Another option is to use cognitive tests that do not rely on food reinforcements. Specifically, tests that make use of larger maze setups frequently use the possibility of returning to the home cage as an incentive for rats to perform the given task. Such a protocol has previously been used for evaluating reversal learning in BACHD rats [62] (the authors specifically argued that avoiding food restriction would be preferable when considering the difference in *ad libitum* food consumption first described in [17]). Still, this should also be done with some caution, as BACHD rats have repeatedly been found to show reduced anxiety in a test of exploration behavior [17,49]. Such a phenotype might under some circumstances result in them having a reduced interest in returning to their home cage compared to WT rats. Thus, further investigation of the use of this kind of reinforcement should also be made before considering it a better alternative.

Conclusions and final remarks

The current study does not offer any final conclusions regarding the reduced motivation and food consumption rate found among male BACHD rats. It does, however, support the results of our initial study [18], indicating that BACHD rats are likely to be less motivated than WT rats to perform food-reinforced tasks when standard food restriction protocols are used.

In addition, detailed analysis of progressive ratio performance revealed that BACHD rats were reliably slower at retrieving the reward pellets, and had an increased tendency to perform excessive lever pushes. Both phenotypes appeared to be unrelated to their lower motivation, and might be indicators of striatal dysfunction.

We further found clear indications that male BACHD rats are slower than WT rats in consuming single pieces of standard rodent chow, suggesting a non hunger-related feeding impairment reminiscent of eating problems in HD patients. Because of this, we no longer consider it advisable to use the standard food consumption test as a test of hunger when working with BACHD rats.

As the presence of motivational differences between WT and BACHD rats is a possible confounding factor when working with food-based tests, and as the alternative food restriction protocol is not necessarily better than the standard restriction protocol, we suggest that any work with BACHD rats and food-reinforced tests should include appropriate control tests.

Supporting information

S1 Fig. Pushes on the non-reinforced lever during the progressive ratio test. Age progression of the number of pushes on the non-reinforced lever during the progressive ratio test performed with Group I is shown. (A) shows performance during the standard food restriction protocol, while (B) shows performance during the alternative food restriction protocol. The graphs indicate group mean plus standard error. Repeated two-way ANOVA results are displayed in each graph, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and

($P < 0.001$) ***.
(TIFF)

S2 Fig. FR5 phase of the progressive ratio test on standard and alternative food restriction. Group I's performance during the FR5 phase of the progressive ratio test, during both food restriction protocols, is shown. Data was created based on the overall performance on all test ages, as no consistent change with age was found for the parameters. Detailed information on how the different parameters were measured is given in the Material and Methods section. (A) indicates the performance of individual rats and group mean. Significant results from Mann-Whitney test are shown inside the graphs. (B)–(D) show group mean plus standard error. Repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S3 Fig. The effect of food restriction on break point 600. The graphs show comparisons of the number of ratios completed at break point 600 for Group I during their progressive ratio baselines at satiety and the standard food restriction protocol. (A) shows data from the tests performed at 12–14 months of age. (B) shows data from the tests performed at 17–19 months of age. The curves indicate group mean plus standard error, repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were found. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S4 Fig. Performance in the standard food consumption test using different food placements. When Group II was maintained on the standard food restriction protocol, one session of the standard food consumption test was run with the food placed inside of the cage (on the cage floor) instead of in the food crib. Data from this session is compared to the performance baseline of the standard food consumption test. The curve indicates group mean plus standard error, repeated two-way ANOVA results are displayed inside the graph. *Post-hoc* analysis did not reveal significant genotype differences. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S5 Fig. Effect of the change in food restriction protocol on the behavior in the standard food consumption test (part I). The graphs show the change in Group II's behavior in the standard food consumption test, when the food restriction protocol was changed from the standard to the alternative approach. Graphs indicate group mean plus standard error. (A) displays results from repeated two-way ANOVA inside the graph and *post-hoc* analysis at data points where performance between the genotypes differed significantly. (B)–(G) concern the total amount of time spent on the different scored behaviors, and show significant results from *t*-test or Mann-Whitney test for single comparisons between the genotypes on either restriction protocol (see also Figs 10B and 11B). ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S6 Fig. Effect of the change in food restriction protocol on the behavior in the standard food consumption test (part II). The graphs show the change in Group II's behavior in the standard food consumption test, when the food restriction protocol was changed from the standard to the alternative approach. Graphs indicate group mean plus standard error. The graphs concern details regarding the number of behavioral episodes, their mean duration, frequency and initiation latency of the different scored behaviors. Significant results from *t*-test

or Mann-Whitney test for single comparisons between the genotypes on either restriction protocol are shown (see also Figs 10C–10E and 11C–11E). ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S7 Fig. Video scoring of individual food consumption test baseline during alternative food restriction protocol. Group II's mean performance on session 5–7 of the individual food consumption test during the alternative food restriction protocol was subjected to detailed video analysis in order to investigate baseline behavior. (A)–(D) and (F) indicate the performance of individual rats. Significant results from *t*-test or Mann-Whitney test are shown in case significant genotype differences were found. (E), (G), (H) and (I) show frequency distribution curves for biting and chewing episodes of different durations, indicating group mean plus standard error. The bins used are described in detail in the Material and Methods section. Note that the x-axis in (G) and (I) only labels every other bin. Results from repeated two-way ANOVA are displayed inside the graphs. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S8 Fig. Further analysis of the performance difference found in the individual food consumption test. Group II's performance on session 5–7 of the individual food consumption test during the alternative food restriction protocol was subjected to detailed video analysis in order to investigate baseline behavior. As the initial analysis of these sessions (see S7 Fig) did not clearly reveal the same phenotypes as found in the first session (see Fig 14), additional parameters were analyzed. These particularly concerned the total time spent biting (B) and chewing (C) the food, as well as the frequency distribution of chewing episodes of different durations, using different bins (E) (compare to Fig 14E, 14I and S7E, S7I Fig). Graphs indicate the performance of individual rats and group mean. Significant results from *t*-test or Mann-Whitney test are shown. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

S9 Fig. Head size phenotype and its influence on the individual food consumption test. The head size of the rats in Group II was measured at the endpoint of the study, and a brief analysis was made to evaluate if this parameter had any strong influence on the rats' performance. For this, the food consumption of a subgroup of rats with comparable head size was investigated. As noted in previous studies [18], BACHD rats were found to have smaller heads than WT rats (A). (B) displays the comparable head sizes in the subgroup used for further analyses. (C) displays the mean food consumption rates of both the full groups and the subgroups with comparable head sizes (see also Fig 12). (D) shows the mean food consumption rate during baseline performance for the subgroup. (A), (B) and (D) indicate data from individual rats. (C) indicates group mean plus standard error. For (A), (B) and (D), significant results from *t*-test or Mann-Whitney test are shown. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.
(TIFF)

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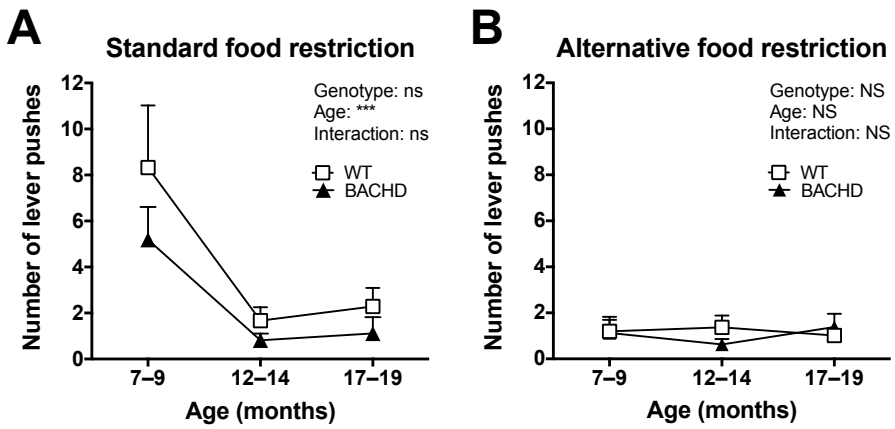
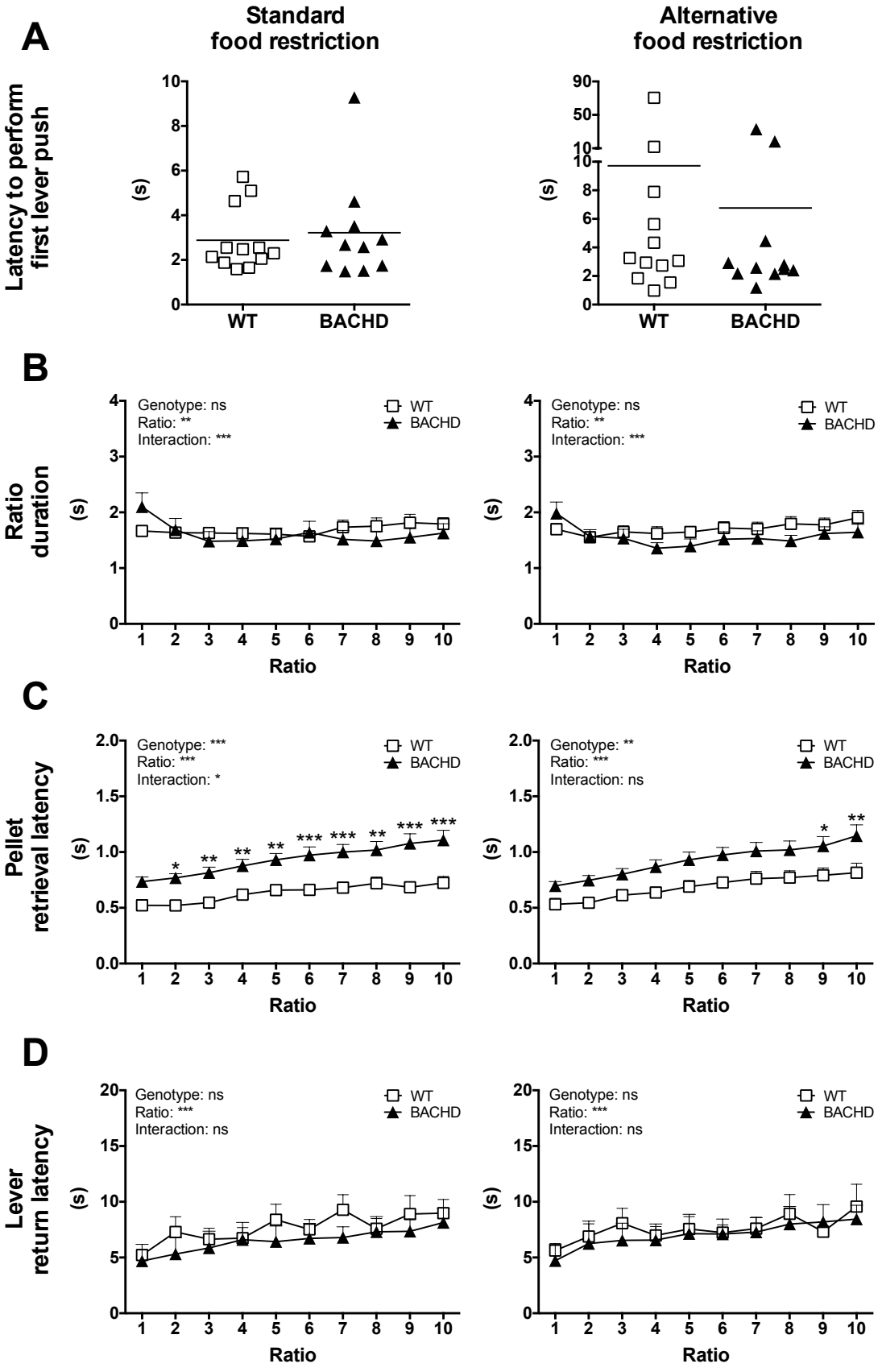
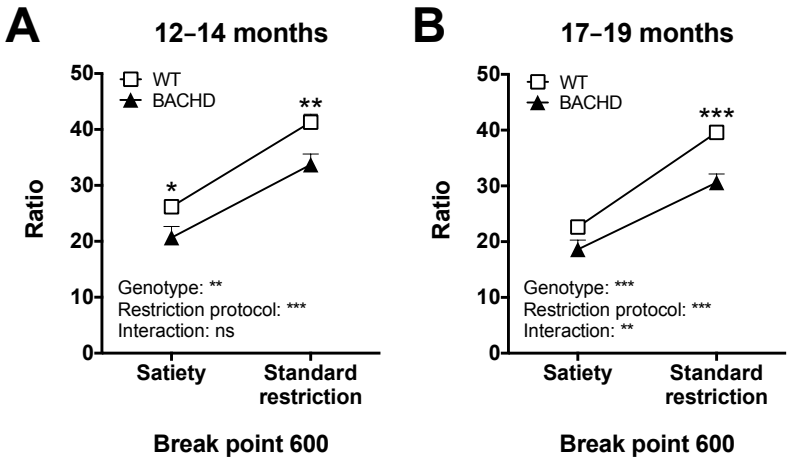

A
Standard food restriction
Number of lever pushes
12
10
8
6
4
2
0
Genotype: ns
Age: ***
Interaction: ns
WT
BACHD
7–9
12–14
17–19
Age (months)
B
Alternative food restriction
Number of lever pushes
12
10
8
6
4
2
0
Genotype: NS
Age: NS
Interaction: NS
WT
BACHD
7–9
12–14
17–19
Age (months)

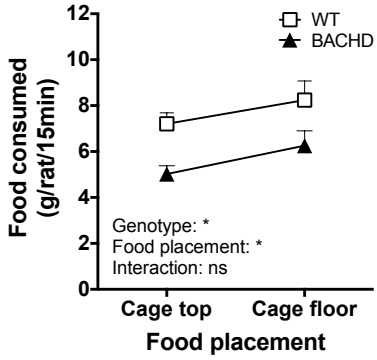
S1 Fig. Pushes on the non-reinforced lever during the progressive ratio test.
Age progression of the number of pushes on the non-reinforced lever during the progressive ratio test performed with Group I is shown. (A) shows performance during the standard food restriction protocol, while (B) shows performance during the alternative food restriction protocol. The graphs indicate group mean plus standard error. Repeated two-way ANOVA results are displayed in each graph, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
Standard food restriction
Alternative food restriction
Latency to perform first lever push
(s)
WT
BACHD
B
Ratio duration
Genotype: ns
Ratio: **
Interaction: ***
Ratio
C
Pellet retrieval latency
Genotype: ***
Ratio: ***
Interaction: *
Genotype: **
Ratio: ***
Interaction: ns
D
Lever return latency
Genotype: ns
Ratio: ***
Interaction: ns

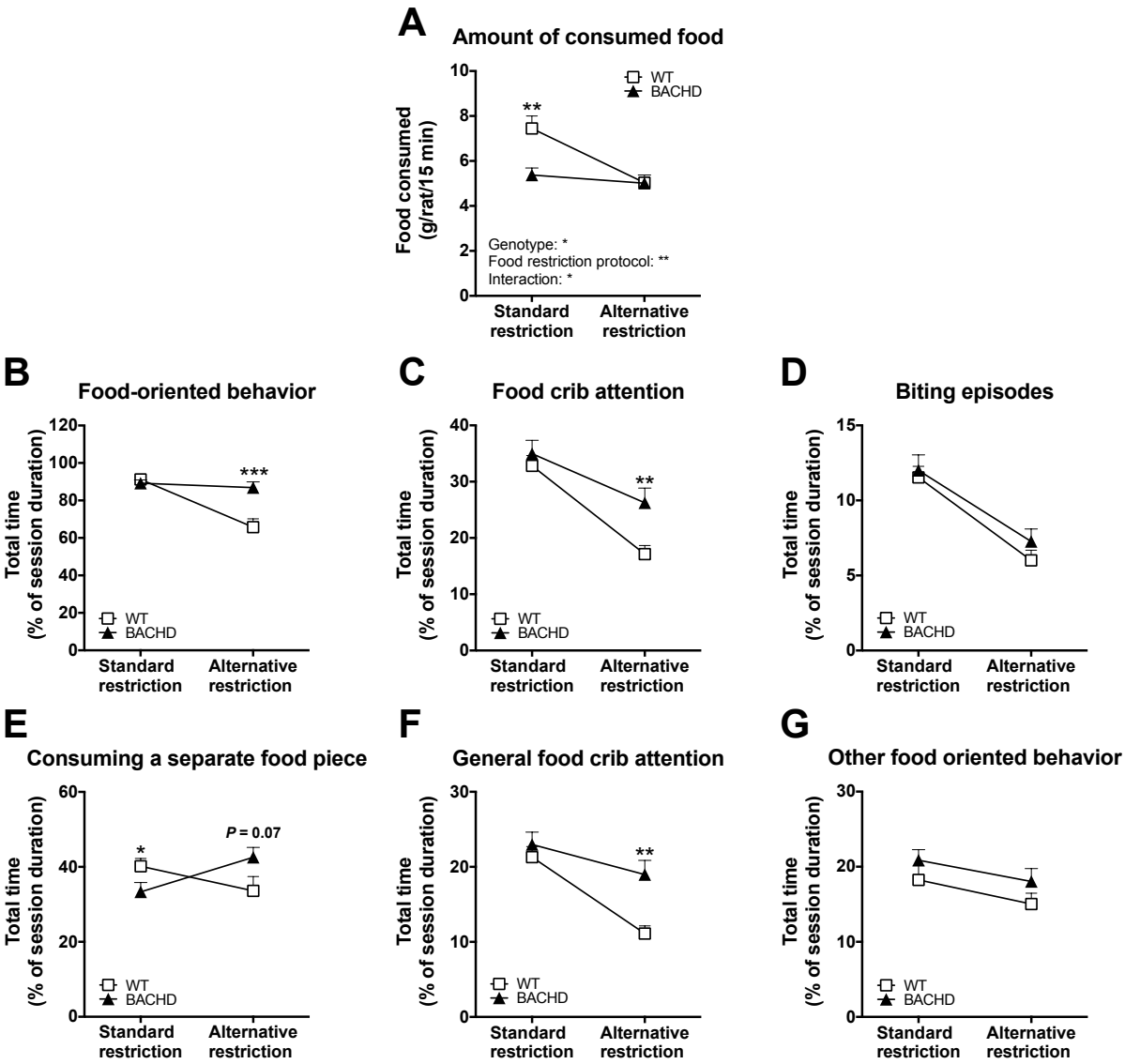
S2 Fig. FR5 phase of the progressive ratio test on standard and alternative food restriction
Group I's performance during the FR5 phase of the progressive ratio test, during both food restriction protocols, is shown. Data was created based on the overall performance on all test ages, as no consistent change with age was found for the parameters. Detailed information on how the different parameters were measured is given in the Material and Methods section. (A) indicates the performance of individual rats and group mean. Significant results from Mann-Whitney test are shown inside the graphs. (B) – (D) show group mean plus standard error. Repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were detected. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
12–14 months
WT
BACHD
Ratio
50
40
30
20
10
0
*
**
Genotype: **
Restriction protocol: ***
Interaction: ns
Satiety
Standard restriction
Break point 600
B
17–19 months
WT
BACHD
Ratio
50
40
30
20
10
0
***
Genotype: ***
Restriction protocol: ***
Interaction: **
Satiety
Standard restriction
Break point 600

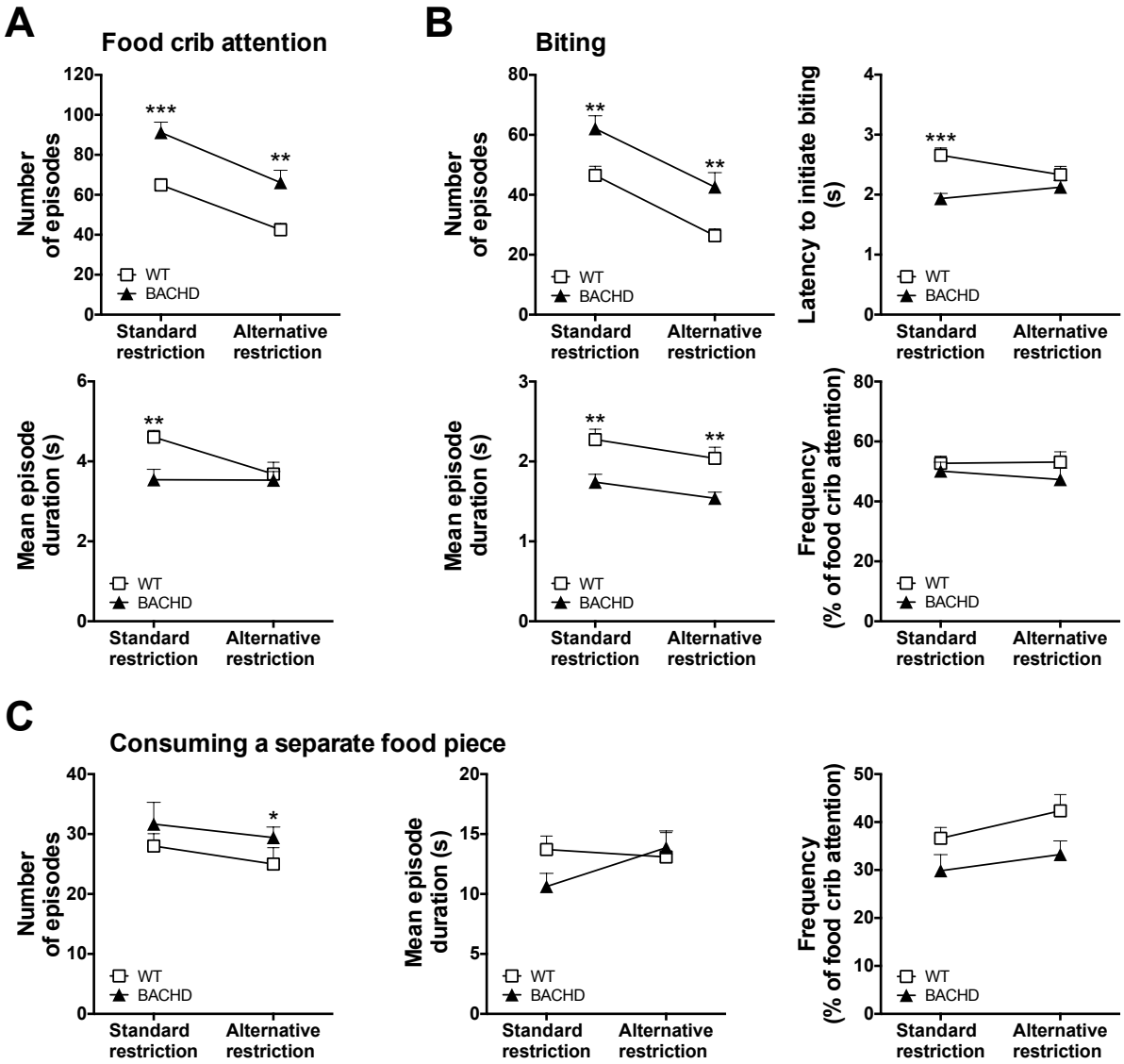
S3 Fig. The effect of food restriction on break point 600
The graphs show comparisons of the number of ratios completed at break point 600 for Group I during their progressive ratio baselines at satiety and the standard food restriction protocol. (A) shows data from the tests performed at 12–14 months of age. (B) shows data from the tests performed at 17–19 months of age. The curves indicate group mean plus standard error, repeated two-way ANOVA results are displayed inside the graphs, and results from *post-hoc* analysis are shown for individual data points in case significant genotype differences were found. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


WT
BACHD
Food consumed (g/rat/15min)
12
10
8
6
4
2
0
Genotype: *
Food placement: *
Interaction: ns
Cage top
Cage floor
Food placement

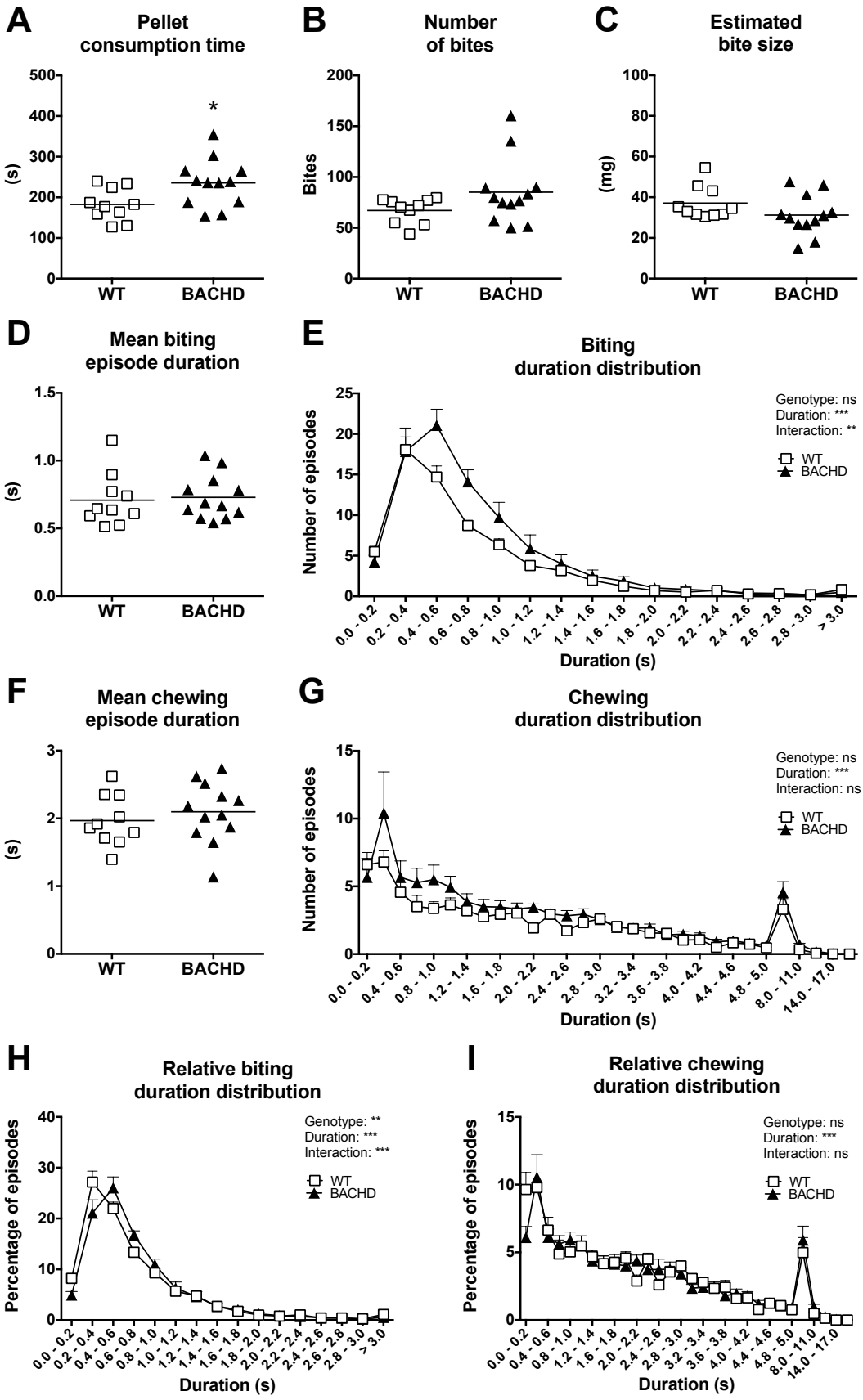
S4 Fig. Performance in the standard food consumption test using different food placements
When Group II was maintained on the standard food restriction protocol, one session of the standard food consumption test was run with the food placed inside of the cage (on the cage floor) instead of in the food crib. Data from this session is compared to the performance baseline of the standard food consumption test. The curve indicates group mean plus standard error, repeated two-way ANOVA results are displayed inside the graph. *Post-hoc* analysis did not reveal significant genotype differences. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
Amount of consumed food
Food consumed (g/rat/15 min)
WT
BACHD
**
Genotype: *
Food restriction protocol: **
Interaction: *
Standard restriction
Alternative restriction
B
Food-oriented behavior
Total time (% of session duration)
***
C
Food crib attention
**
D
Biting episodes
E
Consuming a separate food piece
*
P = 0.07
F
General food crib attention
**
G
Other food oriented behavior

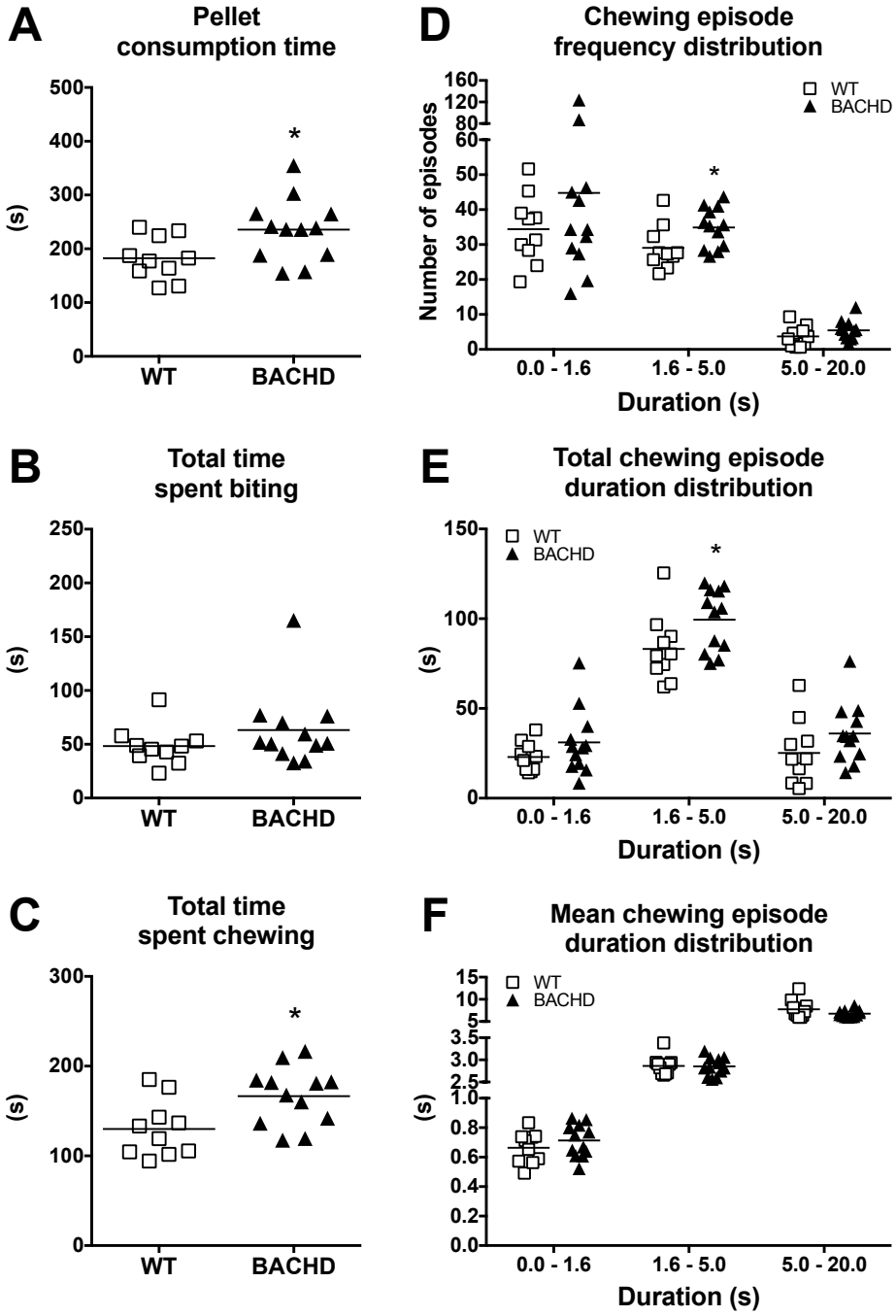
S5 Fig. Effect of the change in food restriction protocol on the behavior in the standard food consumption test (part I)
The graphs show the change in Group II's behavior in the standard food consumption test, when the food restriction protocol was changed from the standard to the alternative approach. Graphs indicate group mean plus standard error. (A) displays results from repeated two-way ANOVA inside the graph and *post-hoc* analysis at data points where performance between the genotypes differed significantly. (B) – (G) concern the total amount of time spent on the different scored behaviors, and show significant results from *t*-test or Mann-Whitney test for single comparisons between the genotypes on either restriction protocol (see also Figs 10B and 11B). ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
Food crib attention
Number of episodes
Mean episode duration (s)
B
Biting
Number of episodes
Mean episode duration (s)
Latency to initiate biting (s)
Frequency (% of food crib attention)
C
Consuming a separate food piece
Number of episodes
Mean episode duration (s)
Frequency (% of food crib attention)
WT
BACHD
Standard restriction
Alternative restriction

S6 Fig. Effect of the change in food restriction protocol on the behavior in the standard food consumption test (part II)
The graphs show the change in Group II's behavior in the standard food consumption test, when the food restriction protocol was changed from the standard to the alternative approach. Graphs indicate group mean plus standard error. The graphs concern details regarding the number of behavioral episodes, their mean duration, frequency and initiation latency of the different scored behaviors. Significant results from *t*-test or Mann-Whitney test for single comparisons between the genotypes on either restriction protocol are shown (see also Figs 10C-E and 11C-E). ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

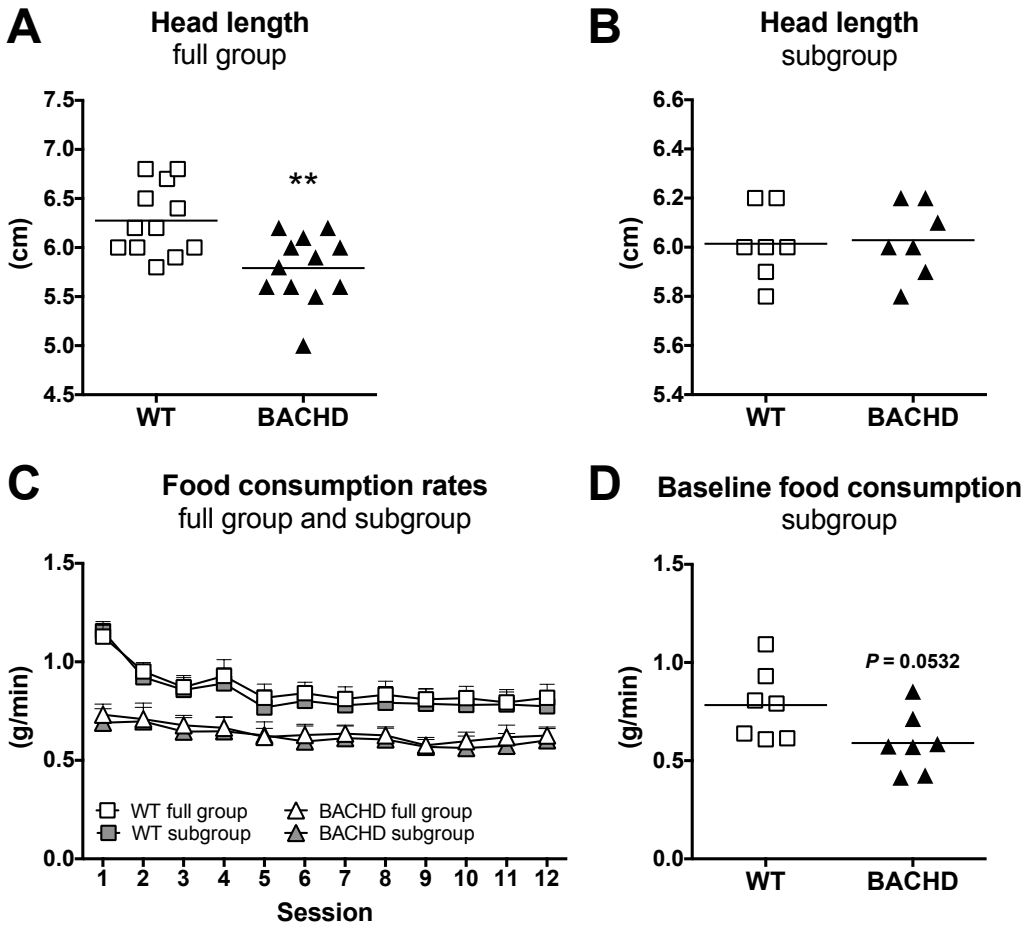

A
Pellet consumption time
(s)
WT
BACHD
B
Number of bites
Bites
C
Estimated bite size
(mg)
D
Mean biting episode duration
E
Biting duration distribution
Number of episodes
Duration (s)
Genotype: ns
Duration: ***
Interaction: **
F
Mean chewing episode duration
G
Chewing duration distribution
Genotype: ns
Duration: ***
Interaction: ns
H
Relative biting duration distribution
Percentage of episodes
Genotype: **
Duration: ***
Interaction: ***
I
Relative chewing duration distribution
Genotype: ns
Duration: ***
Interaction: ns

S7 Fig. Video scoring of individual food consumption test baseline during alternative food restriction protocol.
Group II's mean performance on session 5–7 of the individual food consumption test during the alternative food restriction protocol was subjected to detailed video analysis in order to investigate baseline behavior. (A) – (D) and (F) indicate the performance of individual rats. Significant results from *t*-test or Mann-Whitney test are shown in case significant genotype differences were found. (E), (G), (H) and (I) show frequency distribution curves for biting and chewing episodes of different durations, indicating group mean plus standard error. The bins used are described in detail in the Material and Methods section. Note that the x-axis in (G) and (I) only labels every other bin. Results from repeated two-way ANOVA are displayed inside the graphs. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
Pellet consumption time
(s)
WT
BACHD
B
Total time spent biting
(s)
WT
BACHD
C
Total time spent chewing
(s)
WT
BACHD
D
Chewing episode frequency distribution
Number of episodes
WT
BACHD
0.0 - 1.6
1.6 - 5.0
5.0 - 20.0
Duration (s)
E
Total chewing episode duration distribution
(s)
WT
BACHD
0.0 - 1.6
1.6 - 5.0
5.0 - 20.0
Duration (s)
F
Mean chewing episode duration distribution
(s)
WT
BACHD
0.0 - 1.6
1.6 - 5.0
5.0 - 20.0
Duration (s)

S8 Fig. Further analysis of the performance difference found in the individual food consumption test

Group II's performance on session 5–7 of the individual food consumption test during the alternative food restriction protocol was subjected to detailed video analysis in order to investigate baseline behavior. As the initial analysis of these sessions (see S7 Fig) did not clearly reveal the same phenotypes as found in the first session (see Fig 14), additional parameters were analyzed. These particularly concerned the total time spent biting (B) and chewing (C) the food, as well as the frequency distribution of chewing episodes of different durations, using different bins (E) (compare to Fig 14E,I and S7E,I Fig). Graphs indicate the performance of individual rats and group mean. Significant results from *t*-test or Mann-Whitney test are shown. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.


A
Head length
full group
(cm)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
**
WT
BACHD
B
Head length
subgroup
(cm)
6.6
6.4
6.2
6.0
5.8
5.6
5.4
WT
BACHD
C
Food consumption rates
full group and subgroup
(g/min)
1.5
1.0
0.5
0.0
WT full group
WT subgroup
BACHD full group
BACHD subgroup
1 2 3 4 5 6 7 8 9 10 11 12
Session
D
Baseline food consumption
subgroup
(g/min)
1.5
1.0
0.5
0.0
P = 0.0532
WT
BACHD

S9 Fig. Head size phenotype and its influence on the individual food consumption test
The head size of the rats in Group II was measured at the endpoint of the study, and a brief analysis was made to evaluate if this parameter had any strong influence on the rats' performance. For this, the food consumption of a subgroup of rats with comparable head size was investigated. As noted in previous studies [18], BACHD rats were found to have smaller heads than WT rats (A). (B) displays the comparable head sizes in the subgroup used for further analyses. (C) displays the mean food consumption rates of both the full groups and the subgroups with comparable head sizes (see also Fig 12). (D) shows the mean food consumption rate during baseline performance for the subgroup. (A), (B) and (D) indicate data from individual rats. (C) indicates group mean plus standard error. For (A), (B) and (D), significant results from *t*-test or Mann-Whitney test are shown. ($P < 0.05$) *, ($P < 0.01$) ** and ($P < 0.001$) ***.

Publication III

PLOS ONE

RESEARCH ARTICLE

The BACHD Rat Model of Huntington Disease Shows Signs of Fronto-Striatal Dysfunction in Two Operant Conditioning Tests of Short-Term Memory

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Abstract

The BACHD rat is a recently developed transgenic animal model of Huntington disease, a progressive neurodegenerative disorder characterized by extensive loss of striatal neurons. Cognitive impairments are common among patients, and characterization of similar deficits in animal models of the disease is therefore of interest. The present study assessed the BACHD rats' performance in the delayed alternation and the delayed non-matching to position test, two Skinner box-based tests of short-term memory function. The transgenic rats showed impaired performance in both tests, indicating general problems with handling basic aspects of the tests, while short-term memory appeared to be intact. Similar phenotypes have been found in rats with fronto-striatal lesions, suggesting that Huntington disease-related neuropathology might be present in the BACHD rats. Further analyses indicated that the performance deficit in the delayed alternation test might be due to impaired inhibitory control, which has also been implicated in Huntington disease patients. The study ultimately suggests that the BACHD rats might suffer from neuropathology and cognitive impairments reminiscent of those of Huntington disease patients.

Introduction

Huntington disease (HD) is an autosomal hereditary neurodegenerative disease, which is caused by a specific mutation in the gene for the huntingtin protein [1,2]. The gene contains a CAG repeat sequence in its first exon, which codes for a stretch of glutamines that is present in the translated protein. Patients who carry an allele with a CAG repeat sequence that is 40 repeats or longer invariably develop HD. As the disease manifests and progresses there is extensive neuronal loss throughout the brain. This is first evident in the caudate nucleus of the striatum, although it eventually affects most brain regions. This result in a wide range of clinical signs that are commonly grouped into motor, psychiatric, cognitive and metabolic

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Competing Interests: The authors have declared that no competing interests exist. Laura Clemensson's affiliation with QPS Austria is not considered a competing interest, as her contribution to the study was separate from her work at QPS Austria. QPS Austria had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Laura Clemensson's involvement in the current study should thus be considered the work of an independent researcher, rather than a representative of a commercial company. The affiliation with QPS Austria also does not alter our adherence to PLoS One policies on sharing data and materials.

symptoms. There are currently no disease-modifying treatments available for HD, and the disease is invariably fatal.

HD patients have been found to suffer from a range of different cognitive impairments [3–14]. Among these there are frequent findings indicating impaired executive function [10–14], which is commonly considered to be dependent on specific regions of the prefrontal cortex and their connections to various subcortical nuclei [15–18]. In line with this, some of the executive function impairments seen in HD appear to be related to fronto-striatal pathology [19–22]. Due to the single disease-causing gene of HD, there are several relevant transgenic animal models of the disease. Our group works primarily with the BACHD rat, a recently developed model that is currently being characterized in order to understand its advantages and disadvantages concerning modeling of HD. In the current study, we investigated the rats' performance in two operant conditioning protocols called the delayed alternation and the delayed non-matching to position tests. Both are frequently used for assessing short-term memory in rodents, and commonly utilize operant conditioning chambers equipped with two retractable levers [23–29]. In the alternation test, the rats have to learn to alternate their responses between the two levers, when these are presented on discrete trials. In the non-matching test, trials are divided into two parts. During the first part, the rats are presented with one randomly chosen sample lever. During the second part, the rats are presented with both levers and should respond to the lever that was not presented as a sample. Successful performance in either protocol is rewarded with small food pellets. In order to evaluate the rats' short-term memory, delays are introduced in the protocols to evaluate how long the rats remember which lever to respond to. As successful performance in both the delayed alternation and the delayed non-matching to position test is sensitive to various lesions of prefrontal and striatal brain regions [23–29], they offer a good set of tests to evaluate the presence of HD-related pathology in the BACHD rats.

Materials and Methods

Animals

A total of 48 male rats were used for the study. These were acquired from two separate in-house breeding events with hemizygous BACHD males from the TG5 line [30] paired with WT females (Charles River, Germany). All animals were on Sprague-Dawley background. Animals were genotyped according to previously published protocols [30] and housed in genotype-matched groups of three in type IV cages (38×55 cm), with high lids (24.5 cm from cage floor). During tests, rats were food restricted according to the two protocols described below. During both protocols, each cage was given a specific daily amount of food (SNIFF V1534-000 standard chow) to maintain appropriate restriction levels. Rats had free access to food between the tests. Rats had free access to water through the entire study. During tests, body weight was measured daily to track the rats' relative food restriction level and assess basic health. Between tests, body weight was measured weekly.

The animal facility kept 21–23˚C, 55–10% humidity, and was set to a partially inverted light/dark cycle with lights on/off at 02:00/14:00 during summer, and 01:00/13:00 during winter.

Two groups of 24 rats were formed from the total of 48. The birth dates of the rats in these two groups were spaced roughly two months apart. Each group was composed of 12 WT and 12 BACHD rats. One group was used for a longitudinal study of performance on the delayed alternation protocol, while the other one was used for a longitudinal study of performance on the delayed non-matching to position protocol. The groups were run in an alternating fashion so that the testing ages were the same for both groups. Behavioral evaluation was thus

performed at 4, 9, 14 and 19 months of age. It should, however, be noted that training was initiated approximately two months before the set ages, as the rats had to progress through several steps before reaching the final test protocols. Thus, the actual test ages were 2–4, 7–9, 12–14 and 17–19 months of age.

During the late test ages, several rats had to be sacrificed due to illnesses (the exact number of rats is specified in the Results section). Decision to sacrifice was always made together with the local veterinarians, after careful examination of the rat. End points considered unidentified illnesses causing weight loss past 80% of free-feeding body weight, or critically reduced welfare according to commonly used indicators (i.e. tumorous swellings that clearly impaired the rats' ability to eat, move and clean themselves, labored breathing, poor grooming, lethargy, disturbed gait, sensitivity to handling or reduced appetite). In such cases, these rats were euthanized in a CO_2 inhalation chamber. No other methods were used to alleviate suffering.

All tests were approved by the local ethics committee (Regierungspraesidium Tuebingen) and carried out in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU).

Food restriction protocols

Two different food restriction protocols were used during the study. The first one focused on restricting the animals to a specific relative body weight. During this, both BACHD and WT rats were restricted until they reached 85% of their respective free-feeding body weight. This relative body weight, or food restriction level, was calculated using previously gathered data from growth curves of free-feeding BACHD and WT rats. Thus, the calculations of restriction levels were made with gender, age and genotype-matched values and took normal growth into account. This protocol was used as the start point at all test ages and will be referred to as the standard food restriction protocol.

We have previously found that male BACHD rats are obese, but have comparable body weights to WT rats [31]. Interestingly, the transgenic rats still reliably consume less food than their WT littermates [30,31]. It is currently unclear to what extent these phenotypes affect the BACHD rats' motivation to perform food-oriented tasks in general, although it has been shown that they are less motivated than WT rats to perform a progressive ratio task (a classical test of motivation) when standard food restriction protocols are used [31]. Because of this, we sought to evaluate the impact of motivation on the readouts from the protocols used in the current study. Thus, once data from performance during the standard food restriction protocol had been gathered, the restriction protocol was changed to an alternative protocol. During this, the amount of food given to WT rats was increased so that they reached 95% of their free-feeding weight rather than the previous 85%. When they had reached the new restriction level, data for a second baseline was gathered. BACHD rats were during this given continuous training (but were kept at 85% of their free-feeding body weights) to validate that any effects seen in the WT rats were indeed due to the change in food restriction level.

It should be noted that it was rarely possible to give the exact same amount of food to either of the genotypes during extended periods of time, as both the standard and alternative restriction protocol had to take natural growth into account. We have, however, found that these smaller adjustments have little impact on the rats' performance.

Operant conditioning setup

A bank of six operant conditioning boxes (Coulbourn Instruments, H10-11R-TC) was used to run the test. Each chamber was equipped with two retractable levers, one on either side of a

central pellet delivery trough that was equipped with a yellow light. This light was used to signal the delivery of a reward pellet during the protocols. Above each lever was a single white cue light. The boxes further contained a red house light on the wall opposite from the levers and pellet delivery trough, which shone during the full duration of the training sessions. A water bottle was also available on this wall to ensure *ad libitum* access to water during testing. The protocols were designed and run with Graphic State 4.1.04. Rats were given single daily sessions, meaning that a total of four daily runs with all six operant chambers were needed to assess a full group. Each run assessed three WT and three BACHD rats in a determined order so that a given rat was trained on the same time of day through all tests. Each rat was assigned to a specific operant chamber, although this was arranged so that each operant chamber was used to assess equal numbers of WT and BACHD rats.

Behavioral assessment started approximately six hours after dark phase onset, in a room separate from the animals' housing room, using soft red light. Rats received their daily amount of regular food one hour after the completion of the last run of the day.

Operant conditioning protocols

At each test age, the rats were first put on food restriction for approximately 14 days before any training occurred. This aimed at restricting both WT and BACHD rats to 85% of their respective free-feeding body weights, as described above. During the first test age, this step was also used to familiarize the rats with the reward pellets that were used in the operant conditioning boxes. This was done by adding a spoonful of reward pellets (Bio-serv, Dustless Precision Pellets® F0021, purchased through Bilaney Consultants, Duesseldorf, Germany) to the daily amount of food given to each cage. It was not necessary to repeat this when the rats were reassessed at older ages.

Before reaching the final operant conditioning protocols of interest, the rats had to be trained in a series of separate protocols. The first protocols aimed at habituating the rats to the operant conditioning boxes, and at training them to reliably respond to the levers. These first steps were similar for the two rat groups and were only run during the first test age. The specific protocols are described below.

Habituation. All rats were given two habituation sessions in order for them to familiarize themselves to the operant conditioning boxes and the pellet trough where food rewards could be retrieved. During these sessions, both levers were retracted and a single reward pellet was delivered to the pellet trough at 10-, 15-, 20-, 25-, or 30-second intervals. The pellet delivery interval varied in a pseudo-randomized fashion so that each set of five deliveries used each interval once. Pellet retrieval, or failure to retrieve the pellet within five seconds after delivery, lead to the start of the next pellet delivery interval. Pellet deliveries were signaled by the light in the pellet trough being switched on. The light was switched off when the pellet was retrieved, or when five seconds had passed and the next interval started. Sessions lasted until 100 pellets had been delivered, which took roughly 30 minutes.

Continuous reinforcement (CRF) with help. The aim of these sessions was to train the rats to reliably perform lever pushes to obtain reward pellets. During the sessions one of the two levers was inserted into the box and remained inserted until the end of the session. Each lever push resulted in the delivery of a single reward pellet. At the start of the session, the lever was baited with a paste made by mashing some reward pellets in water. The experimenter then manually delivered rewards when the rats approached, sniffed and touched the inserted lever. Through this, the rats eventually performed a few accidental responses and soon developed a reliable lever-pushing behavior. Sessions ended either after 30 minutes had passed or after 100 pellets had been delivered. Training continued until the rats had managed to perform 100

pushes within one session without any help from the experimenter. Training was organized so that half of the rats from each genotype group were trained on the right lever, while the other half was trained on the left lever.

CRF on the second lever. Once the rats had passed the criterion for CRF performance on the first lever, the same training was done for the second lever. Thus, the lever the rats were initially trained on was retracted, while the other lever was inserted. The new lever was also baited at the start of the trial, but the experimenter only manually delivered reward pellets if rats had clear problems understanding what to do. Session durations and criteria were the same as during the initial CRF training.

Forced alternation and non-matching to position sequence training. At this point, the rats of the two groups were trained on slightly different protocols. Both protocols aimed at training the rats to reliably start the individual trials that made up the delayed alternation and delayed non-matching to position sessions. In addition, the protocols sought to familiarize the rats with the main concept of the tasks they were going to perform (i.e. alternation and non-matching to position).

The rats in the delayed alternation group were trained on a forced alternation protocol. For this protocol, each session was split into a series of trials, separated by brief (2 s) inter-trial intervals (ITIs). The sessions started with an ITI step, with both levers retracted, the house light switched on and all cue lights off. At the end of the ITI the light in the pellet trough would start to shine. When the rats entered the pellet trough with their head, the light was switched off and either the left or right lever was inserted. The lever remained inserted until the rats performed a response. The lever retracted and a reward pellet was delivered at the off signal of a lever response. Delivery of a reward pellet was signaled with the light in the pellet trough shining once again. The trial ended either when the rats collected the reward pellet or when five seconds had passed since the reward pellet had been delivered. Either event triggered the start of a new ITI. On the first trial of the session, the protocol was set to randomly insert either the left or the right lever. On all subsequent trials, the inserted lever would be on the opposite side of the lever used during the previous trial. Through this, the rats were forced to alternate their responses between the left and right lever. The sessions lasted either until the rats had completed 100 trials or until 45 minutes had passed. Rats were trained until they completed 100 trials within the session duration limit without any help from the experimenter.

The rats in the delayed non-matching to position group were trained on a non-matching to position sequence training protocol. The sessions of this protocol were also split into a series of trials separated by ITIs. The duration of these ITIs varied in a pseudo-randomized fashion between 5, 7, 9 and 11 seconds so that each block of four ITIs used each duration once. The sessions started with an ITI step, with both levers retracted, the house light switched on and all cue lights off. At the end of the ITI the light in the pellet trough started to shine. When the rats entered the pellet trough with their head, the light was switched off and either the left or right lever was inserted. The protocol followed a pseudo-randomized structure so that each block of six trials used three trials with the left lever and three trials with the right lever. This also meant that a given trial type (i.e. left or right lever) could maximally appear six times in a row. The lever remained inserted until the rats performed a lever response. The lever retracted and the food trough light started to shine again at the off signal of a lever response. Notably, no reward pellet was given for this response. When the rats entered the pellet trough again, the pellet trough light went out, both lever cue lights shone and the lever on the opposite side from the first one was inserted. The lever once again stayed inserted until the rats made a response. The lever retracted, the lever cue lights stopped shining and a reward pellet was delivered at the off signal of a lever response. As in previous steps, the delivery of a reward pellet was signaled by the light in the pellet trough starting to shine. The trial ended either when the rats collected the

reward pellet, or when five seconds had passed since the reward pellet was delivered. Either event triggered the next ITI. The sessions lasted either until rats had completed 100 trials or until 45 minutes had passed. Rats were trained until they completed 100 trials within the session duration limit without any help from the experimenter.

Once rats had reached the performance criterion on their respective protocols, omission limits were added in the protocols to make sure that the rats performed the desired responses at a proper pace. For the forced alternation protocols, these limits were set for starting a trial and responding to the inserted levers. For the non-matching to position sequence learning, the limits were set for the trial start, responding to the first lever, returning to the pellet trough and responding to the second lever. On those steps, if a rat failed to perform the required response within ten seconds, the protocol went into an omission state, in which all lights were switched off and all levers retracted. After ten seconds the protocols went into ITIs that ensured that the rats would be given an identical trial to the one they had just failed to complete. These protocols were run until the rats performed less than 5 omissions in total, while completing 100 trials within the session duration. Importantly, omitted trials were not counted towards the 100-trial limits of the sessions, as they were not considered to be completed trials.

Free alternation and non-matching to position. The next set of protocols were the first ones where rats were able to make mistakes, and also served as the starting point when rats were retrained at older ages. The basic structure of the protocols were similar to the forced alternation and the non-matching to position sequence learning protocols. Thus, they used the same basic structure concerning the start and stop of the individual trials as well as the ITI setup described above. In addition, both protocols still ended either after 100 completed trials or 45 minutes. As above, omitted trials did not count towards this 100-trial limit, while both successful and failed trials did. The outlines of the two tests are shown in Figs 1 and 2.

The main difference between the forced and free alternation protocols was that both levers were inserted during each trial of the latter protocol. On the first trial of each session, the rats were rewarded for pushing either the left or right lever. On all subsequent trials, however, the rats were only rewarded for pushing the lever they did not respond to on the previous trial. Importantly, this was independent of whether the previous trial was successful or not. Thus, in order to continuously receive rewards, the rats had to alternate their responses between the left and right levers and avoid making repeated responses on one of the levers. A mistake resulted in a brief timeout (3 s) during which the house light was switched off. At the end of the timeout, the protocol returned to an ITI state, which was followed by a new trial. On occasions where the rats performed an omission, the protocol reset to a starting position. Thus, on the next trial the rats were rewarded for pushing either lever, and this trial set the start point for the next series of alternations. Importantly, the first trial of the session, and trials that directly followed omissions, were not counted towards the 100-trial limit of the session and were also not included in the success rate calculations. Training continued until the rats showed an 85% or higher success rate during three consecutive sessions.

The free non-matching to position test used the same two-part structure as the non-matching to position sequence learning protocol. Thus, after the trial start, the rats were prompted to push a single non-reinforced lever, which will be referred to as the sample lever. After responding to the sample lever and returning to the pellet trough, both levers were now inserted into the box. This second part of the non-matching trials will be referred to as the choice step. Similar to the previous training step, the rats were rewarded for pushing the lever that was not presented during the sample step. Pushing the same lever as the sample lever resulted in a ten-second timeout similar to the one described for the free alternation protocol. During the first testing age, the rats were trained until they showed an 85% success rate or higher during three consecutive sessions. This was, however, reduced to two consecutive sessions when the rats

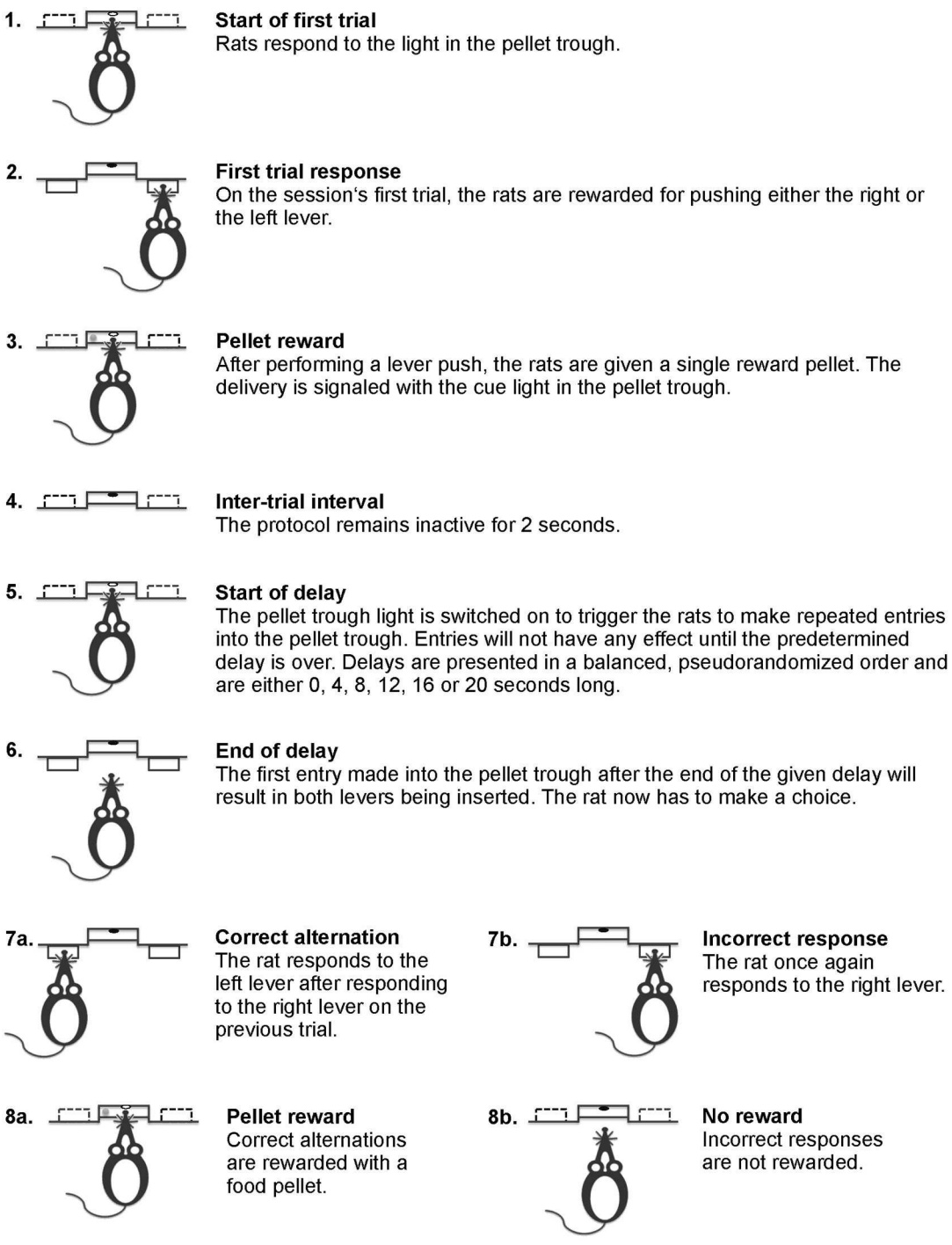

Fig 1. Delayed alternation protocol. The figure describes the steps that make up individual trials in the delayed alternation test.

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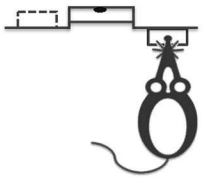
1. 

Start of trial
Rats respond to the light in the pellet trough.

2. 

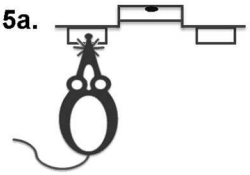
Sample lever presentation
Either the left or the right lever is inserted, and the rat pushes it. The protocol presents left and right sample levers in a balanced, pseudorandomized order.

3. 

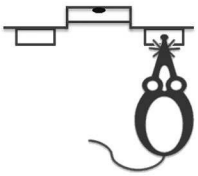
Start of delay
No reward is given for pushing the sample lever. Instead, the lever retracts and the light in the pellet trough starts to shine in order to trigger the rats to make repeated entries into the pellet trough. In addition, the delay timer is started. Entries have no effect until the predetermined delay is over. Delays are presented in a balanced, pseudorandomized order and are either 0, 5, 10, 15 or 25 seconds long.

4. 

Choice step
The first entry made into the pellet trough after the end of the given delay will result in both levers being inserted. The rat now has to make a choice.

5a. 

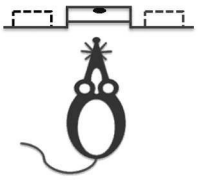
Correct non-matching
The rat responds to the left lever on a trial where the right lever was presented as sample lever.

5b. 

Incorrect response
The rat responds to the same lever that was presented as sample lever.

6a. 

Pellet reward
Correct non-matchings are rewarded with a food pellet.

6b. 

No reward
Incorrect responses are not rewarded.

7. 

Inter-trial interval
The protocol remains inactive for 5, 7, 9 or 11 seconds.

8. **Continuation of protocol**
The protocol loops back to step 1.

Fig 2. Delayed non-matching to position protocol. The figure describes the steps that make up individual trials in the delayed non-matching to position protocol.

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were retested, as the rats showed little problem with handling the test. Trials using the left and right sample lever were pseudo-randomized as described above. Omissions resulted in the rats being presented with a trial using the same sample lever again.

Delayed alternation and non-matching to position. When the rats had learned to handle their respective basic task, delays were introduced into the protocols. The aim of this was to assess the rats' short-term memory function. In the alternation protocol, the delays were introduced at the start of the trials, at the point when the pellet trough light started to shine. As explained above, a head entry at that point usually triggered the continuation of the trial (i.e. lever insertion). During the delayed alternation, however, head entries had no effect until the end of the set delay. Once the delay was over, the first head entry resulted in the levers being inserted. Delays were introduced in a similar manner in the non-matching to position protocol. Specifically, they were used during the step where the rats returned to the pellet trough after responding to the sample lever. As described above, making the second entry into the pellet trough would usually result in triggering the choice step of the protocol. But during the delayed non-matching to position protocol, head entries had no effect until the delay was over. Similarly to the delayed alternation protocol, the first head entry performed after the end of the delay would trigger the choice step. Through these delays, rats were thus forced to perform responses in the two protocols with certain specific spacing in relation to either the previous trial or their sample lever response. The omission limits that were set for trial start and initiation of the choice step were applied at the end of the delays. Thus, if a rat had not performed a head entry response within ten seconds after the end of the delay on either protocol, the trial was aborted.

The sessions were initially made up of 100 trials and used a set of five different delays, leading to five different trial types. These were presented in a pseudo-randomized fashion so that each block of 20 trials used each delay four times. This also meant that a given delay could be presented a maximum of eight times in a row. If rats had performed an omission the protocols were designed so that the rat had to rerun a trial with the same delay. The pseudo-randomization of sample levers in the delayed non-matching to position protocol was also changed compared to before. Specifically, each block of four trials used each sample lever twice. The rats were trained on several protocols with gradually increasing delay durations. The aim of this was to find a delay set where a clear drop in the rats' success rate could be seen between trials with the shortest and longest delay. For the delayed alternation protocol, the delay sets were as follows: 0, 1, 2, 3, 4 seconds / 0, 1, 3, 6, 9 seconds / 0, 1, 4, 8, 12 seconds / 0, 2, 5, 10, 15 seconds / 0, 4, 8, 12, 16, 20 seconds. During the first test, the age rats progressed from one protocol to another when they had shown above 80% success on three consecutive sessions. During retesting at older ages, this criterion was reduced to rats performing above 80% success on two consecutive sessions. Exceptions to this performance-based criterion were the two last delay sets. Specifically, rats were given three training sessions on the second last delay set, regardless of their success rate. Training on the last delay set continued until rats showed a stable performance, as defined below. The delay sets used for the delayed non-matching to position test were: 0, 1, 2, 3, 4 seconds / 0, 1, 3, 6, 9 seconds / 0, 1, 4, 8, 12 seconds / 0, 2, 5, 10, 15 seconds / 0, 5, 10, 15, 20 seconds / 0, 5, 10, 15, 20, 25 seconds. The same criterions as described above were used for progressing through these delay sets. Notably, the last delay set for both the delayed alternation and the delayed non-matching to position protocol used six delays rather than five. To accommodate this, the number of trials per session and the pseudo-randomization were adjusted. The delayed alternation sessions were set to last 120 trials or 60 minutes. The trials were organized so that each block of 12 trials used each delay twice. For the delayed non-matching to position protocol, the number of trials was initially set to 96, but was reduced to 48 for a large part of the study (all baselines except the ones for the 4 months performance

at standard food restriction and both baselines from the 19 months test). The reason for this was that many rats were not motivated enough to perform 96 trials and we sought to minimize differences in possible within-session training effects. The protocol was still set so that each block of four trials used each sample lever twice, while the delays were organized in the same way as the delayed alternation trials. It should be noted that the pseudo-randomization limits described above were not completely reliable. However, they functioned well enough to ensure that rats experienced comparable numbers of each trial type on any given session (+/- 3 trials). In addition, the baselines were constructed from performance over several consecutive sessions, thus minimizing the effect that slight differences in the frequency of a given trial type would have had on the overall performance.

As noted, the rats were trained on the final delay sets until they showed a stable performance. When this was achieved, data from a number of consecutive sessions were used to create the baseline data that was used for detailed analysis. At each test age this baseline data was first gathered while the rats were maintained on the standard food restriction protocol. Afterwards, the food restriction protocol was changed. Rats of both genotypes were continuously given daily sessions through the restriction adjustment. When the alternative food restriction levels had been established and rats were once again performing stably, data for a second performance baseline was gathered. Once the data had been gathered, the rats were once again given free access to food and the test ended.

Both the delayed alternation and the delayed non-matching to position tests are well described in literature [23–29,32]. Our protocols were based on the general consensus and small optimizations of these references.

Operant conditioning protocol parameters

The operant conditioning system created individual log files for each training session and rat. These log files were run through a series of in-house designed analysis scripts written in R, to obtain a large set of parameters that were used for subsequent analysis.

The number of sessions required to reach the various performance criteria served as a major parameter for evaluating how animals learned the given tasks and progressed through the series of protocols. Success rate (i.e. the percent of trials with successful responses) was calculated differently depending on the protocols used. During the free alternation and the non-matching to position sequence learning protocols, the calculation included all completed trials to give a single success rate value for each session. For protocols where delays were present, separate success rates were calculated for each trial type (i.e. trials with different delays) so that curves plotting success rate against delay durations could be created for each session. These curves served as the main readout of the tests and were used to determine when the rats had reached stable performance on the final delay sets. During testing, the rats' mean performance on each block of three consecutive sessions was calculated. When statistical analysis showed no significant change between several consecutive session blocks, the rats were considered to have reached stable performance. As noted, the sessions within the blocks where stable performance was found were used for detailed analysis of baseline performance. Although the exact number of sessions included in these analyses varied between baselines, it stayed between 9 and 12 sessions. As noted above, only completed trials (i.e. trials where the rats performed either a correct or incorrect response) were included in success rate calculations. The number and frequency of omission trials (trials where rats failed to perform a head entry or lever push within the set time limit) constituted their own analysis.

The protocols offered several parameters regarding the rats' latency to perform specific responses. For the free alternation protocol, this primarily included the latency to start trials

(measured from the pellet trough light being switched on to the rat entering the pellet trough, triggering lever insertion) and the latency to respond to the inserted levers (measured from lever insertion to lever push). The free non-matching to position protocol included similar parameters, with trial start latency (measured from the pellet trough light being switched on, to the rat entering the pellet trough, triggering insertion of the sample lever), latency to respond to the sample lever (measured from lever insertion to lever push), latency to return to the pellet trough (measured from release of sample lever to the rat entering the pellet trough, triggering the choice step) and the latency to perform a lever response during the choice step (as above, measured from lever insertion to lever push). When delays were added to the protocols, the exact measurement made by some of these parameters were slightly modified and additional parameters were added to ensure a comprehensive analysis of the rats' behavior. For the delayed non-matching to position protocol the latencies to start trials and respond to levers were measured in the same way as during the free non-matching to position protocol. The latency to return to the pellet trough after responding to the sample lever was, however, replaced by the parameters for the latency to perform the first head entry of the delay (measured from release of sample lever to first entry) and the latency to trigger the choice step (measured from the end of the delay, to the point when rats performed the entry that triggered insertion of both levers). It is important to note that trials with 0 second delays were only included in the latter analysis. For the delayed alternation, the lever response latency was measured in the same way as during the free alternation protocol. However, the trial start latency was now measured from the end of the delay to the point when rats performed the entry that triggered lever insertion. Similarly to the delayed non-matching to position protocol, a measurement for the latency to perform the first head entry of the delay (measured from the pellet trough light being switched on to the point when rats performed the first entry) was added. The distinction of these various parameters is important to consider when comparing the performance between the various protocols. Thus, the trial start latency in the free alternation, free non-matching to position and delayed non-matching to position can be considered a measurement of how fast the rats respond to the light in the pellet trough being switched on. However, in the delayed alternation protocol, this behavior is best described by the latency to perform the first head entry of the delay rather than the trial start latency. Further, the trial start latency of the delayed alternation protocol is closely connected to the rats' interest in the pellet trough during the delays, and is comparable to the latency to trigger the choice step in the delayed non-matching to position protocol. The lever response latency in the alternation protocols is comparable to the choice lever response latency in the non-matching to position protocols. Finally, the latency to respond to the sample lever and perform the first entry of the delay during the delayed non-matching to position protocol lack direct counterparts in the delayed alternation protocols. Additional parameters were used to investigate the rats' behavior during delay steps. These measured the mean number of entries and the total time spent inside the pellet trough during delay steps, as well as the mean duration of individual entries. Trials with the longest delays were subjected to further analysis. Specifically, the mean number of head entries performed during discrete segments of these delays was evaluated in order to investigate if the rats' interest in the pellet trough changed with time. The latency to retrieve reward pellets was investigated for all tests. This was measured from the point of releasing the reinforced lever to entering the pellet trough.

As with the success rate analysis, the parameters described above were only evaluated for completed trials. For alternation protocols, the first trial of the session and the first trial following omissions (i.e. trials where any lever response would be reinforced) were also excluded. Analysis of free alternation and free non-matching to position performance was made over all completed trials. In contrast, separate analysis of trials with different delay durations was

performed for most of the parameters in the delayed alternation and delayed non-matching protocols. Analysis was primarily made over all trials regardless of outcome, although separate analyses for successful and failed trials were also performed.

Video scoring

As noted above, several parameters were used to evaluate the rats' behavior during delay steps. All these parameters used readouts from the entry sensor in the pellet trough. Interpretations of these parameters can occasionally be difficult, as the signaled number of entries does not always correspond to the actual number of entries. Thus, several videos were recorded during the last test age in order to manually score their behaviors during delays. Video scoring was performed with the Observer XT software (v.12.5.927, Noldus, The Netherlands, Wageningen). The following behaviors were scored during delays:

Time spent in pellet trough. This considered all occasions where a rat had anything from its nose to its entire head inside the pellet trough.

Time spent in a central position. This considered all occasions where a rat had its head inside the pellet trough. It also included all occasions where a rat was sitting in front of the pellet trough, keeping its head outside, while still appearing to focus on it. In addition, it included occasions where a rat investigated the wall portion that was positioned directly above the pellet trough.

Body shifts towards the left or right side. With quite high frequency, the rats would exit the pellet trough to briefly investigate the wall portions to the right or left of the pellet trough, and then return. These body shifts occurred in several different forms. Some were short, and the rat only quickly indicated an interest to either the right or left side. Others were longer and could include both direct investigation of the lever slots or more general investigation of the surrounding wall area. All body shifts, regardless of duration or specific nature, were included in the analysis. During analysis, separate scores were given for shifts to the left and right side.

All scoring focused on noting start and stop point of each occasion where a rat displayed the above mentioned behaviors. The logs from the Observer XT software were later combined with the log files from the operant conditioning system. These were run through in-house designed R scripts to obtain detailed analysis. Through this, the number of behavioral episodes, their mean duration and the total time spent on the different behaviors could be evaluated for individual delay steps. The estimated amount of time spent investigating other parts of the operant conditioning boxes during delays was also calculated. These calculations primarily considered time spent investigating the back wall as well as the back halves of the left and right wall of the operant conditioning box. The calculations were based on the total time for all behaviors noted above and the known delay durations. The body shifts were initially scored as being made either to the left or right side of the pellet trough, although they were later relabeled depending on if they were made towards the correct or incorrect lever, or if they were made towards the lever that the rats eventually responded to. The latter was initially used to assess whether the body shifts at all constituted a form of strategy. It was further used to evaluate how rats established, maintained and shifted focus during delays. For this, the rats were considered to have established a focus for one particular lever based on their first body shift during a given delay step. The rats were then considered to have maintained or changed it, if the last body shift during the delay step was made towards the same or the opposite side, respectively. Thus, the focus behavior during each delay step was classified as having no focus (no body shifts occurred), established focus (only one body shift occurred), maintained focus (first and last body shifts of delay were made towards the same side) or changed focus (first and last body shifts of delay were made towards different sides). Further scores were made to

evaluate if the initial focus had been made towards the correct or incorrect lever. Finally, specific analysis was made for trials with the longest delay steps. For this, the relative amount of time spent around the correct lever during segments of the delay was analyzed separately for successful and non-successful trials. In addition to the behaviors that were scored during delays, the rats' behavior during lever responses was also investigated. Specifically, it was noted if rats had responded to the chosen lever without showing any interest in the other lever (direct responses), if the rats first headed for one lever but changed their mind and ultimately responded to the other lever (corrections) or if the rats went back and forth between the two levers a few times before finally deciding on one (uncertain responses). In addition to investigating the frequency of the different behaviors, theoretical success rate curves were created to assess the importance of the corrections. For this, the hypothetical results of rats responding according to the lever they first showed interest in during correction trials was considered.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism v.6.01 (GraphPad Software, San Diego California USA, http://www.graphpad.com).

The results from most parameters were investigated with different types of two-way repeated measures ANOVAs. Most of these were aimed at investigating genotype differences, and thus focused on data where genotype was used as the non-repeated factor, while either age, delay duration, type of baseline or specific protocol step served as the repeated factor. Certain analyses, however, were performed within genotype groups, and aimed at investigating performance differences between baselines at different ages, baselines at different food restriction protocols or performance on successful or failed trials. All these analyses used two-way repeated measures ANOVAs where both delay and the other given factor were considered to be repeated factors. This kind of ANOVA was also used when evaluating if rats had reached a stable performance baseline. The results from video scoring the frequency of different behaviors during delay and lever steps of the delayed alternation and delayed non-matching tests were analyzed with separate two-way ANOVAs for the different behaviors. As above, these used genotype as the non-repeated factor and delay as the repeated factor. Sidak's multiple comparison *post-hoc* test was used to follow up any significant effects found in the two-way ANOVAs. The number of sessions required to progress through the set of delayed alternation and delayed non-matching to position protocols with gradually increasing delay durations was analyzed in several single comparisons between WT and BACHD rats. For these, t-test, t-test with Welch's correction or Mann-Whitney test were used, depending on the data's apparent distribution.

During testing there were occasionally rats that fell ill and had to be sacrificed. Thus, the n of the analyses changed as follows. For the delayed alternation group, 2–4 months (WT:12, BACHD: 12), 4–9 months (WT: 12, BACHD: 12), 12–14 months (WT: 11, BACHD: 11) and 17–19 months (WT: 9, BACHD: 8 during standard food restriction protocol, 7 during alternative food restriction protocol). For the delayed non-matching to position group, 2–4 months (WT:12, BACHD: 12), 4–9 months (WT: 12, BACHD: 12), 12–14 months (WT: 12, BACHD: 12) and 17–19 months (WT: 12 during standard food restriction protocol, 11 during alternative food restriction protocol, BACHD: 11). Video scored behavior concerned (WT: 9, BACHD: 7) and (WT: 11, BACHD: 11) for the alternation and non-matching tests, respectively. Age development analyses excluded data from animals that were not assessed at all ages. No other exclusion criteria were used. As described in the Results section, there was very rarely any clear effect of age found on the various parameters. Thus, for most baseline parameters the analysis was performed on the mean performance of all evaluated ages to maintain an n of 12.

Alpha for all analyses was set to 0.05.

Results

Survival

Most rats remained healthy through the entire duration of the tests, although some rats had to be sacrificed due to illness. All in all, three WT and five BACHD rats were sacrificed from the delayed alternation group, and one rat of each genotype was sacrificed from the delayed non-matching to position group. In most cases, the illnesses concerned tumors. The change in n for the different groups is described in detail in the Material and Methods section.

Basic operant conditioning protocols

There were no consistent or overt performance differences between BACHD and WT rats during habituation, CRF training, forced alternation training or non-matching to position sequence training (data not shown). All rats quickly progressed through their specific set of protocols, and rarely required more than a single session per step. BACHD rats were during CRF training occasionally found to be slower than WT rats when returning to the reinforced lever after retrieving a pellet reward (data not shown).

Free and delayed alternation performance

During the first three test ages, BACHD and WT rats completed comparable numbers of trials on all of the investigated protocols described below. During the 19-month test age, BACHD rats tended to complete fewer trials than WT rats on the protocol with the final delay set, although the difference did not reach statistical significance (data not shown). There were at no point any differences concerning the ratio of completed Left-Right and Right-Left alternations between WT and BACHD rats (data not shown).

The number of sessions needed to reach criterion on the free alternation protocol decreased with repeated testing for rats of both genotypes (Fig 3A) (age effect: $F_{(3,45)} = 253.8$, $P < 0.001$). BACHD rats required more sessions compared to WT rats during the first two test ages, as indicated by a significant genotype effect ($F_{(1,15)} = 19.15$, $P < 0.001$), genotype x age interaction effect ($F_{(3,45)} = 10.96$, $P < 0.001$) and post-test results (4 months: $P < 0.001$; 9 months: $P < 0.05$) (Fig 3A). During criterion-level performance, there were no consistent differences between WT and BACHD rats in terms of success rate (Fig 3B), trial start latency (Fig 3C), lever response latency (Fig 3D) or number of omissions (Fig 3E). BACHD rats did, however, become progressively slower at retrieving the reward pellets, resulting in them showing significantly longer latencies compared to WT rats at the last test age (Fig 3F) (post-test result at 19 months: $P < 0.05$).

Most rats reliably reached the performance criterion on each delayed alternation protocol, and thus progressed properly through the series of delay sets. A total of four BACHD rats did not consistently reach each performance criterion and would occasionally get stuck on a particular delay set (two to three out of the four rats at each test age). The rats would in these cases show no clear indication of improving their performance, despite being given extensive training (up to ten sessions with arguably stable performance). Their performance typically remained close to criterion, being just above or below it on more or less alternating sessions. These rats were still allowed to continue through the series of delayed alternation protocols, as they were deemed to simply have reached their maximum performance. The rats were, however, excluded from the analysis of the number of sessions required to progress through all the protocols. This analysis showed that rats of both genotypes required a high number of sessions

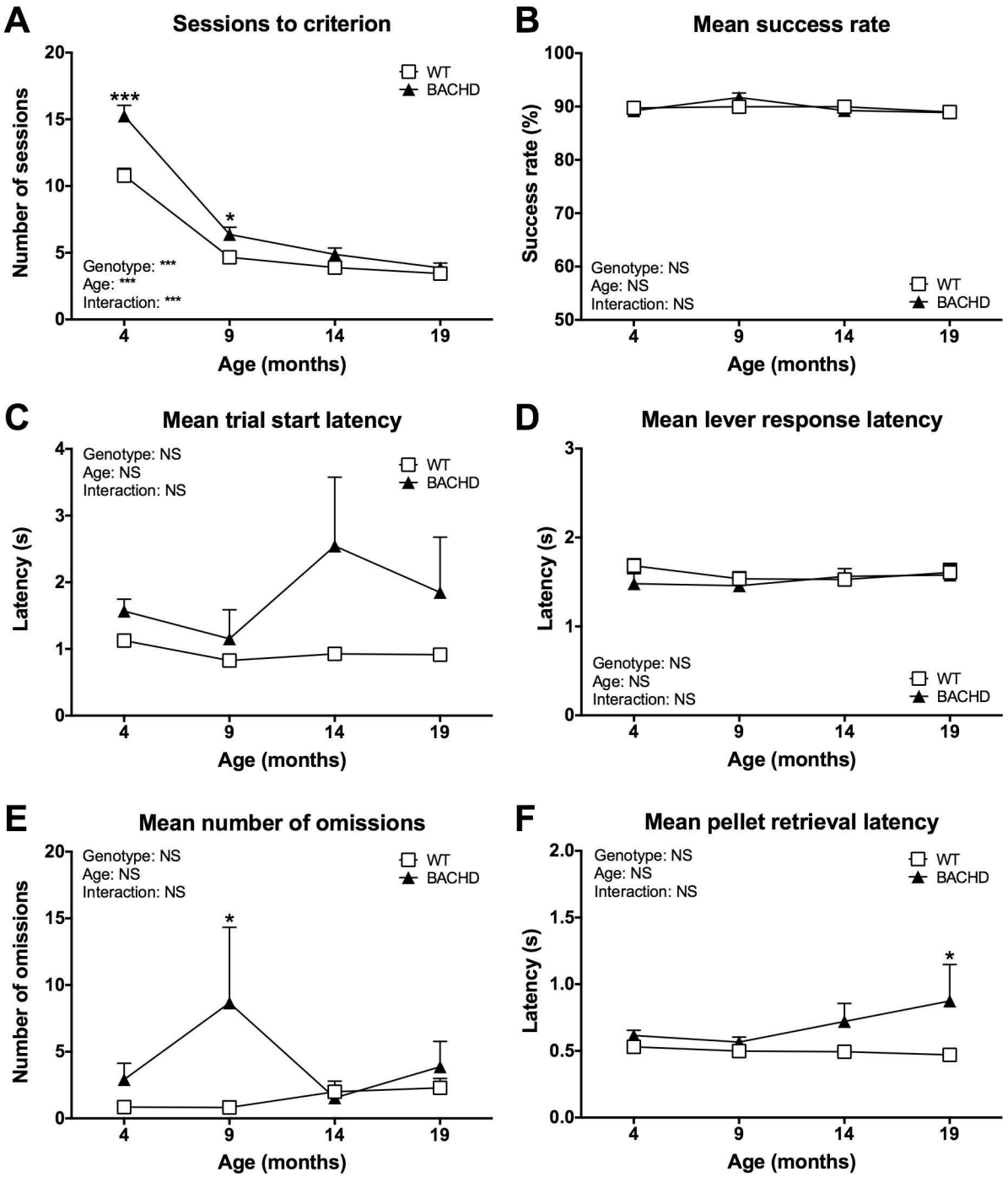

Fig 3. Age development of free alternation performance. The graphs show the main readouts of the free alternation protocol over the four test ages. (**A**) shows the number of training sessions required for reaching criterion. (**B**)–(**F**) show the mean performance of rats during sessions where their success rate was at criterion level. Curves show group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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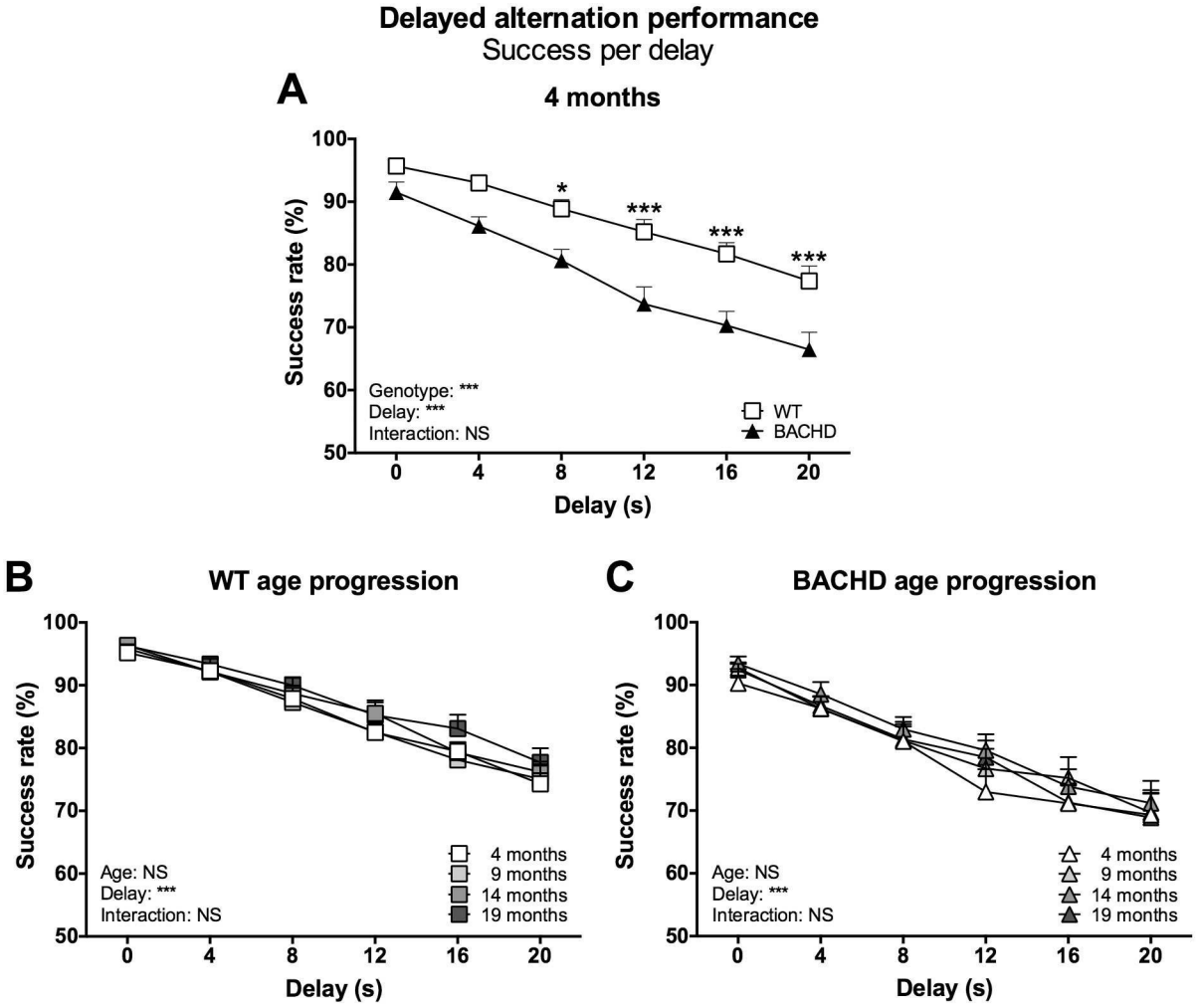

Fig 4. Success rate per delay in the delayed alternation test. The graphs show the success rate on trials preceded by delays of different durations in the delayed alternation test. (A) shows the stable baseline performance of rats maintained on the standard food restriction protocol at four months of age. (B) and (C) show the age progression of performance for WT and BACHD rats. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. For (A), results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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during the first test age, but then dropped to a relatively stable level during retesting (S1 Fig). BACHD rats needed significantly more sessions than WT rats to progress through the series of delayed alternation protocols during the first three test ages, although the phenotype was strongest during the first test age (S1 Fig) (single comparisons: 4 months: $P < 0.001$; 9 months: $P < 0.05$; 14 months: $P < 0.05$).

The main parameter of interest for the delayed alternation test concerned the success rate on the different trial types. Analysis of this parameter showed that rats of both genotypes maintained a high success rate when trials were preceded by a delay of zero seconds, but dropped as the delay duration increased (Fig 4A for 4-month data, S2 Fig for 9-, 14- and 19-month data) (delay effect at 4 months: $F_{(5,110)} = 66.14$, $P < 0.001$; 9 months: $F_{(5,110)} = 81.59$, $P < 0.001$; 14 months: $F_{(5,100)} = 103.4$, $P < 0.001$; 19 months: $F_{(5,75)} = 70.50$, $P < 0.001$). BACHD rats performed generally worse than WT rats at all investigated ages, as indicated by significant genotype effects for all baseline comparisons (genotype effect at 4 months: $F_{(1,22)} = 19.99$,

$P < 0.001$; 9 months: $F_{(5,110)} = 13.66$, $P < 0.01$; 14 months: $F_{(5,20)} = 11.20$, $P < 0.01$; 19 months: $F_{(5,15)} = 8.79$, $P < 0.01$) without statistically significant genotype x delay interaction. Still, trends and *post-hoc* analysis indicated that the reduced success rate among BACHD rats was more pronounced on trials preceded by longer delays. Performance and phenotypes did not notably change with age when rats were retested (Fig 4B and 4C, S2 Fig).

Several additional parameters concerning delayed alternation performance were investigated. One set of parameters concerned the entries made into the pellet trough during delays (Fig 5). These parameters did not appear to change with age and therefore, although initial analyses were made for individual test ages, only mean performance over all test ages is displayed and discussed here. The latency to perform the first head entry during the delay increased with delay duration (Fig 5A) (delay effect: $F_{(4,88)} = 13.41$, $P < 0.001$). The ANOVA did not reveal an overall genotype difference. However, BACHD rats were slower than WT rats during the longest delay step, as indicated by a significant genotype x delay interaction ($F_{(4,88)} = 2.763$, $P < 0.05$) as well as a significant genotype difference in *post-hoc* analysis of that data point (head entry latency at 20 months: $P < 0.05$). The number of entries made during delays increased with delay duration (Fig 5B) (delay effect: $F_{(4,88)} = 152.4$, $P < 0.001$). BACHD rats made generally fewer entries compared to WT rats (genotype effect: $F_{(1,22)} = 6.715$, $P < 0.05$), although the phenotype was more pronounced during longer delays, as indicated by a significant genotype x delay interaction effect ($F_{(4,88)} = 7.554$, $P < 0.001$) and significant genotype differences in *post-hoc* analyses (16-second delay: $P < 0.01$, 20-second delay: $P < 0.001$). To gain further insight into the behavior, the number of entries made during segments of the 20-second delay was analyzed (Fig 5C). This indicated that BACHD rats made fewer entries than WT rats on all segments of the delay (genotype effect: $F_{(1,22)} = 7.852$, $P < 0.05$, post-test result: $P < 0.05$ for the 5–8, 9–12, and 13–16 seconds delay segments). However, WT and BACHD rats still spent comparable amounts of time inside the pellet trough (Fig 5D), as BACHD rats made generally longer entries compared to WT rats (Fig 5E) (genotype effect: $F_{(1,22)} = 33.34$, $P < 0.001$, post-test result: $P < 0.001$ for all delays).

During the 4-month test age, there was no difference between WT and BACHD rats regarding their trial start latencies (S3A Fig) or number of trial start omissions (S3B Fig). However, a peculiar performance difference developed during retesting. Specifically, BACHD rats showed longer trial start latencies compared to WT rats on trials that were preceded by intermediate delays, but not on trials preceded by 0- or 20-second delays (S3C Fig) (genotype difference in *post-hoc* analysis 4-second delay: $P < 0.05$, 8-second delay: $P < 0.001$, 12-second delay: $P < 0.01$). The behavioral basis for this phenotype was discovered during video scoring and is discussed further below. Specifically, it was found that BACHD rats frequently turned away from interactive wall in order to drink. The same behavior also caused BACHD rats to perform a higher number of trial start omissions than WT rats on trials preceded by short delays (S3D Fig) (genotype difference in *post-hoc* analysis 0-second delay: $P < 0.01$, 4-second delay: $P < 0.001$, 8-second delay: $P < 0.05$).

There were no overt differences in lever response latencies between the genotypes, although BACHD rats appeared to be a bit slower than WT rats at responding during trials preceded by a 0-second delay (S4A Fig). Finally, BACHD rats were slower than WT rats at retrieving the reward pellets (S4B Fig). The phenotype became more apparent with age, and the age progression analysis shown in S4 Fig only found a significant phenotype during the last test age (genotype difference in *post-hoc* analysis 19 months: $P < 0.05$). It should, however, be noted that single comparisons at each test age reliably revealed a significant genotype difference (data not shown).

The results described above were from analyses that included all completed trials, i.e. both successful and failed trials (although excluding omitted ones). Analysis of each parameter was,

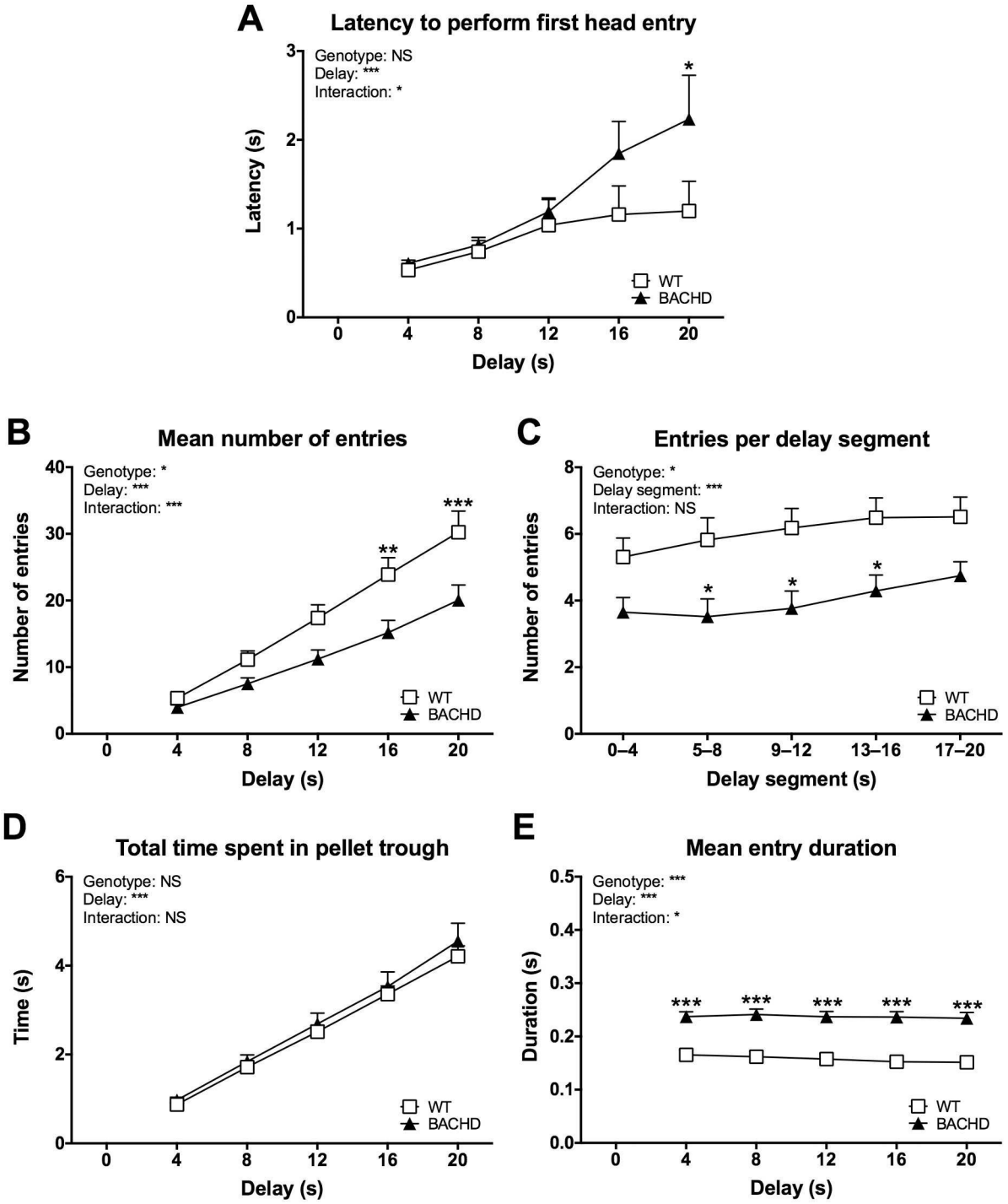

Fig 5. Head entry behavior during delays of the delayed alternation protocol. The graphs show several aspects of head entries made into the pellet trough during delay steps of the delayed alternation test. Curves were created based on the overall performance at all test ages, as no significant change with age was found for the parameters. (C) concerns the 20-second delay step. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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however, also performed based on trial outcome (S5 Fig). This was done to evaluate, if the BACHD rats' lower success rate is connected to the other noted behavioral differences. Among BACHD rats, failed trials were preceded by delays with slightly fewer entries (S5A Fig) (trial type difference in *post-hoc* analysis 8-second delay: $P < 0.05$, 12-second delay: $P < 0.05$, 16-second delay: $P < 0.01$, 20-second delay: $P < 0.01$) and slightly less time spent in the pellet trough (S5B Fig) (trial type difference in *post-hoc* analysis 8-second delay: $P < 0.05$, 16-second delay: $P < 0.05$, 20-second delay: $P < 0.05$). However, these differences were small and not consistently present at all individual test ages, and thus unlikely to be of major importance. Trial start latencies (S5C Fig) and lever response latencies (S5D Fig) instead appeared to have stronger impact on the trial outcome. On trials preceded by short delays, failing appeared to be related to long trial start latencies for BACHD rats (trial type difference in *post-hoc* analysis 0-second delay: $P < 0.001$, 4-second delay: $P < 0.001$, 8-second delay: $P < 0.05$), while being related to long lever response latencies for WT rats (trial type difference in *post-hoc* analysis 0-second delay: $P < 0.001$, 4-second delay: $P < 0.001$).

Changing the food restriction protocol so that the WT rats' food restriction level increased from 85% (standard food restriction) to 95% (alternative food restriction) did not markedly change the rats' behavior. They still completed all trials of the sessions and performed comparable number of Left-Right and Right-Left alternations (data not shown). The success rate per delay remained completely unchanged by the adjustment of food restriction at all ages (S6 Fig). The shift also did not have any overt effects on the other parameters of the delayed alternation protocol (S7 Fig). Still, trial start latencies (S7C Fig) and lever response latencies (S7D Fig) appeared to become longer after adjustment (specific effect among WT rats being changed to a lower restriction level, not seen for BACHD rats during the extended training) (food restriction effect on trial start latency in WT rats: $F_{(1,11)} = 11.91$, $P < 0.01$; food restriction effect on lever response latency in WT rats: $F_{(1,11)} = 9.55$, $P < 0.05$). For the trial start latencies, the change primarily concerned the intermediate delays (food restriction difference in *post-hoc* analysis 4-second delay: $P < 0.001$, 8-second delay: $P < 0.001$, 12-second delay: $P < 0.001$; 16-second delay: $P < 0.01$). Despite this, the aforementioned genotype difference in trial start latency largely remained the same (data not shown). The number of omissions was affected both by the motivational shift due to food restriction adjustment and by extended training (S8A Fig). Specifically, WT rats performed more omissions, while BACHD rats performed fewer ones, resulting in a significant genotype x baseline interaction effect ($F_{(1,22)} = 15.42$, $P < 0.001$). The change among BACHD rats appeared to be connected to a slightly lower omission rate on trials preceded by no delay (S8B Fig) (baseline difference in *post-hoc* analysis 0-second delay: $P < 0.01$), while the change among WT rats concerned an increase in their omission rates on all other trial types (baseline difference in *post-hoc* analysis: $P < 0.001$ for 4-, 8-, 12-, 16-, and 20-second delays). Despite these changes, the initial phenotype of BACHD rats performing more omissions than WT rats was not resolved (data not shown).

Free and delayed non-matching to position performance

BACHD rats tended to complete fewer trials than WT rats (data not shown), although this could not be investigated in detail, as the session duration was adjusted so that rats of both genotypes would complete comparable numbers of trials. Despite these efforts, BACHD rats were found to complete significantly fewer trials than WT rats during the 19-month test age (data not shown). At that point, BACHD rats completed on average 84 trials, while WT rats completed 96 trials. There was at no point any difference concerning the ratio of completed Left-Right and Right-Left trials between WT and BACHD rats (data not shown).

The free non-matching to position protocol was, in contrast to the free alternation, very easy for the rats to learn. Thus, after the initial response sequence training and upon retesting at older ages, most rats performed at criterion level from the first session onwards. This resulted in rats needing very few sessions to reach the performance criterion, and no difference was found between the genotypes concerning this parameter at any age (Fig 6A). During performance at criterion level, there were no differences regarding success rate (Fig 6B), trial start latency (Fig 6C), sample lever response latency (Fig 6D), pellet trough return latency (Fig 6E), choice lever response latency (Fig 6F) or number of omissions (Fig 6G). BACHD rats were, however, found to be slower than WT rats at retrieving the reward pellets (Fig 6H). This phenotype was most pronounced during the last two ages and not present during the first test age, as indicated by a significant genotype effect ($F_{(1,21)} = 5.265$, $P < 0.05$), significant genotype x age interaction effect ($F_{(3,63)} = 6.338$, $P < 0.001$) and results from *post-hoc* analysis (significant genotype differences at 14 and 19 months: $P < 0.05$).

Most rats progressed properly through the delayed non-matching to position protocols with increasing delay durations by reaching the performance criterion of each protocol. There were, however, a total of three BACHD rats that did not reliably manage to reach each criterion and thus would occasionally get stuck at a particular delay set despite extensive training. The rats did not consistently show the problems, meaning that at each given test age there were between zero and three out of those three BACHD rats that did not manage all performance criteria. The rats were handled like the ones in the delayed alternation. Thus, they were allowed to continue through the series of protocols, were part of the main performance analysis, but not the specific analysis concerning the number of sessions required to progress through the series of delay sets. This sessions to criterion analysis indicated that rats needed a relatively stable number of sessions to reach the final delay step, and the two genotypes required similar numbers of sessions at all test ages (S9 Fig).

The main parameter of interest was once again the success rate on trials with different delay durations. Analysis of this parameter showed that rats of both genotypes maintained a high, close to 100%, success rate on trials with a 0-second delay, but dropped as the delay duration increased (Fig 7A for 4-month data, S10 Fig for 9-, 14- and 19-month data) (delay effect at 4 months: $F_{(5,110)} = 40.10$, $P < 0.001$; 9 months: $F_{(5,110)} = 32.51$, $P < 0.001$; 14 months: $F_{(5,110)} = 35.76$, $P < 0.001$; 19 months: $F_{(5,105)} = 48.53$, $P < 0.001$).

While there was no difference between the genotypes' performance on trials with 0-second delays, the BACHD rats' success rate dropped more than WT rats' on trials with 5- and 10-second long delays. Interestingly, the two genotypes appeared to show a comparable decline in success rate for trials with longer delays. Ultimately, while WT rats showed reasonably linear drops in success rate with increasing delays, BACHD rats appeared to show a biphasic curve. Still, statistical analysis failed to reliably detect significant differences in the rats' performance. No differences were found during the first two test ages, while the last two presented both significant genotype effects (14 months: $F_{(1,22)} = 11.01$, $P < 0.01$; 19 months: $F_{(1,21)} = 4.95$, $P < 0.05$) and genotype x delay interaction effects (14 months: $F_{(5,110)} = 4.49$, $P < 0.001$; 19 months: $F_{(5,105)} = 4.23$, $P < 0.01$). As this did not appear to be due to either of the genotypes changing their behavior with repeated testing (Fig 7B and 7C), and as the performance during the first test age still showed a quite strong genotype effect trend ($F_{(1,22)} = 3.44$, $P = 0.08$), this ultimately indicated a stable but discrete phenotype sensitive to small variations in the recorded data. The notion of a biphasic success rate curve for BACHD rats was supported by the fact that the genotype x delay interaction effect was also found when the analysis was limited to trials with 0- to 10-second delays (data not shown), while analysis of trials with 10- to 25-second delays did not reveal a significant genotype x delay but only an overall genotype

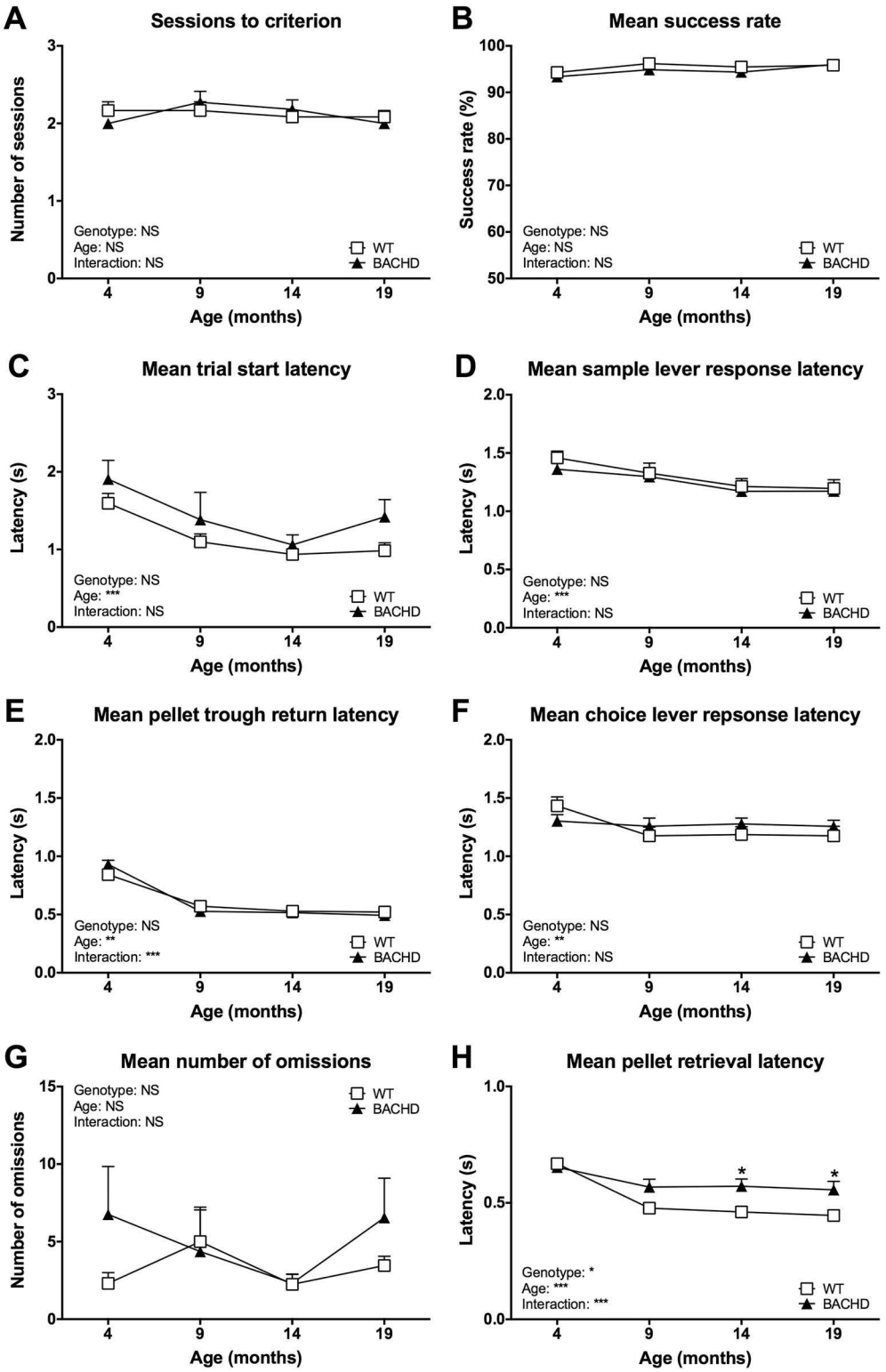

Fig 6. Age development of free non-matching to position performance. The graphs show the main readouts of the free non-matching to position protocol over the four test ages. (A) shows the number of training sessions required for reaching criterion. (B)–(H) show the mean performance of rats during sessions where their success rate was at criterion level. Session to criterion data was corrected for the change in criterion between the first test age and retesting. Curves show group mean plus standard error. Results from

two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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effect (data not shown). Thus, the phenotype was dependent on the presence of delays, although the extent of impairment was not directly related to their duration.

As with the delayed alternation protocol, several additional parameters concerning delayed non-matching to position performance were investigated. Once again, one set of parameters concerned the entries made into the pellet trough during delay periods (Fig 8). Performance on these parameters did not appear to change with age and therefore, although initial analyses were made for individual test ages, only the mean performance over all test ages is displayed and discussed here. The latency to perform the first head entry of the delay remained stable with delay duration and did not differ between WT and BACHD rats (Fig 8A). The number of

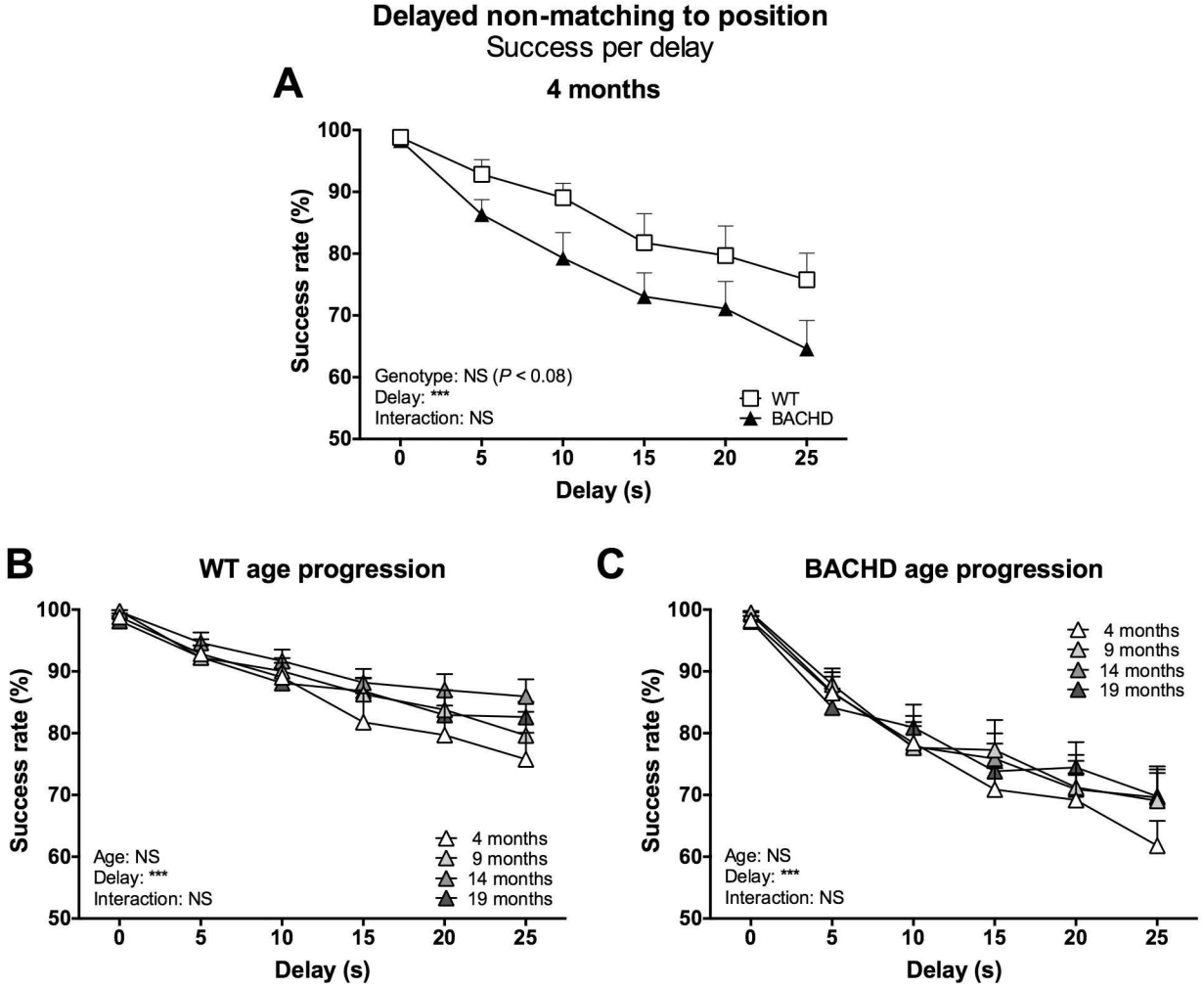

Fig 7. Success rate per delay in the delayed non-matching to position test. The graphs show the success rate on trials with delays of different durations in the delayed non-matching test. (A) shows the stable baseline performance of rats maintained on the standard food restriction protocol at four months of age. (B) and (C) show the age progression of performance for WT and BACHD rats, respectively. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. For (A), results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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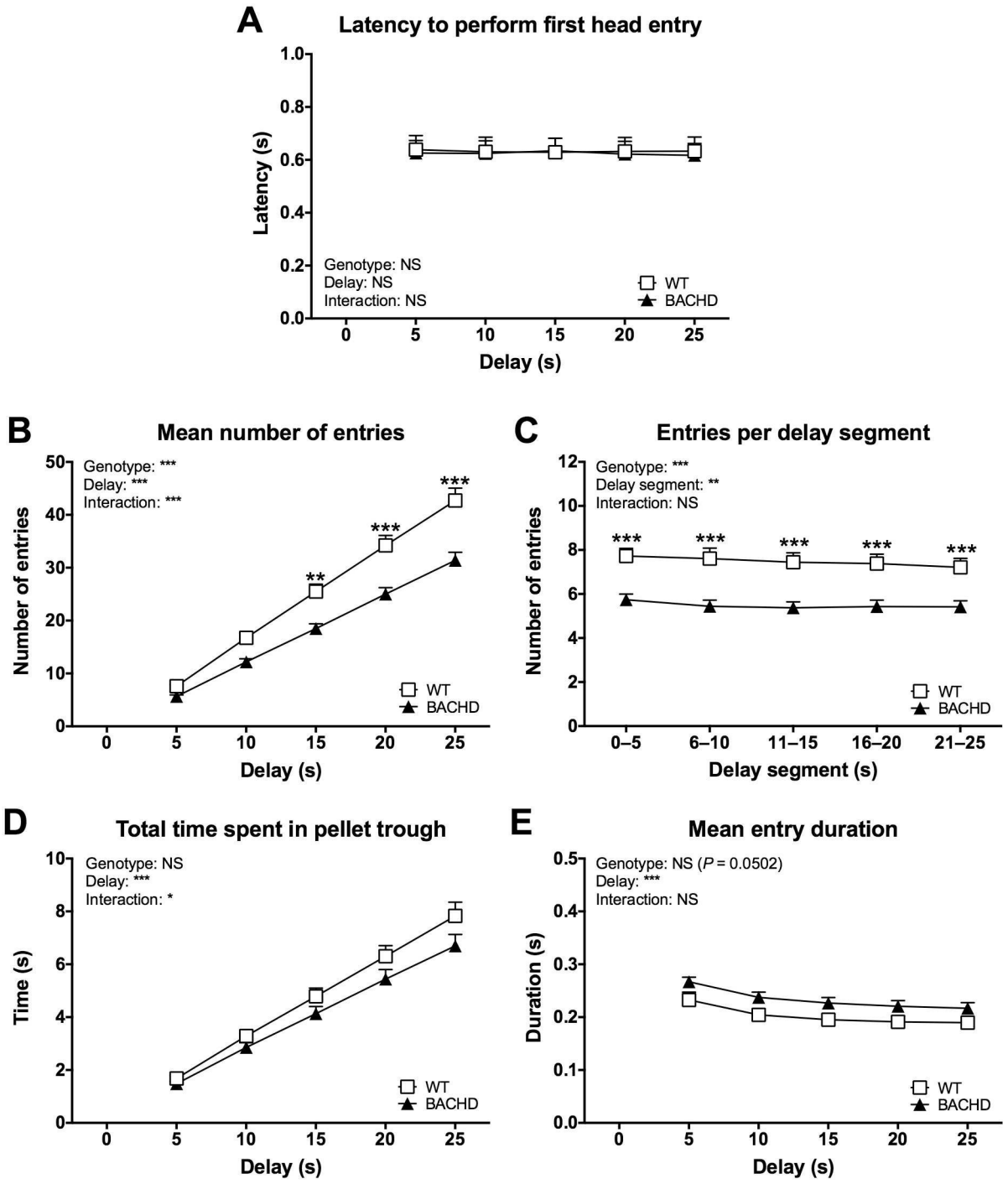

Fig 8. Head entry behavior during delays on the delayed non-matching to position protocol. The graphs show several aspects of head entries made into the pellet trough during delay steps of the delayed non-matching to position test. Curves were created based on the overall performance on all test ages, as no consistent change with age was found for the parameters. (C) concerns the 25-second delay step. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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entries made during delays increased with delay duration (Fig 8B) (delay effect: $F_{(4,88)} = 613.5$, $P < 0.001$). BACHD rats made generally fewer entries compared to WT rats (genotype effect: $F_{(1,22)} = 17.57$, $P < 0.001$), although the phenotype was more pronounced during longer delays, resulting in a significant genotype x delay interaction effect ($F_{(4,88)} = 14.62$, $P < 0.001$) and significant genotype differences in *post-hoc* analyses (15-second delay: $P < 0.01$, 20-second delay: $P < 0.001$, 25-second delay: $P < 0.001$). To gain further insight, the number of entries made during segments of the 25-second delay was analyzed (Fig 8C). This once again indicated that BACHD rats made fewer entries than WT rats throughout the delay, rather than during specific parts of it (genotype effect: $F_{(1,22)} = 17.00$, $P < 0.001$; genotype difference in *post-hoc* analyses: $P < 0.001$ for all delay segments). Through this, BACHD rats ended up spending slightly less time inside the pellet trough during the delays compared to WT rats (Fig 8D). Still, this phenotype was weak and only resulted in a significant genotype x delay interaction effect ($F_{(4,88)} = 2.58$, $P < 0.05$), but not in an overall genotype effect or significant differences in *post-hoc* analyses. This was likely due to the strong trend indicating that BACHD rats made generally longer head entries compared to WT rats (Fig 8E) (genotype effect: $F_{(1,22)} = 4.29$, $P = 0.0504$).

In contrast to the trial start latency during the delayed alternation protocol, the latency to trigger the choice step in the delayed non-matching to position test did not noticeably change with delay duration (S11A Fig). Both genotypes showed similar, and very short, latencies to trigger the choice step. In connection, neither genotype performed frequent omissions at this point of the protocol (S11B Fig). Rather, the main omission type in the delayed non-matching to position test concerned the trial starts, where BACHD rats performed slightly more omissions compared to WT rats (S11C Fig) (post-test result: $P < 0.05$). The latency to start individual trials was not different between WT and BACHD rats at any of the investigated ages (S12 Fig). Response latency to sample levers did not notably change with delay duration or differ between the genotypes (S13A Fig). Response latencies during choice steps were affected by delay duration, with rats of both genotypes being slightly slower at responding during trials with 0-second delays compared to all other delay durations (S13B Fig) (delay effect: $F_{(5,110)} = 11.88$, $P < 0.001$). Regardless of this effect, BACHD and WT rats showed identical choice response latencies. For both genotypes, response latencies during the choice step were generally shorter than response latencies to sample levers (S13C and S13D Fig) (trial step effect WT: $F_{(1,11)} = 8.501$, $P < 0.05$; trial step effect BACHD: $F_{(1,11)} = 9.547$, $P < 0.05$). Finally, BACHD rats were generally slower at retrieving the reward pellets during the delayed non-matching to position test (S14A Fig) (genotype effect: $F_{(1,21)} = 6.638$, $P < 0.05$). Although the exact retrieval latency differed with age (age effect: $F_{(3,63)} = 5.845$, $P < 0.01$), there was no significant genotype x age effect, suggesting that the phenotype was similarly apparent at all ages. Pellet retrieval and pellet trough return are two behaviors that depend on comparable motoric aspects. As noted, WT and BACHD rats performed similarly on the former parameter, but differed on the latter. A direct comparison of these two parameters suggested that BACHD rats showed similar latencies for both behaviors (S14B Fig). In contrast, WT rats were faster when they retrieved reward pellets, compared to when they were returning to the pellet trough after responding to the sample lever. This discrepancy resulted in a significant genotype x protocol step interaction effect ($F_{(1,22)} = 5.205$, $P < 0.05$).

The results described above were from analyses that included all completed trials, i.e. both successful and failed trials (although excluding omitted ones). Analysis of each parameter was, however, also performed based on trial outcome. This was used to evaluate if the noted behavioral differences were connected to the BACHD rats' lower success rate (S15 and S16 Figs). For WT rats (S15 Fig), failure on trials without delays was related to longer trial start latencies (S15A Fig) (trial type difference in *post-hoc* analysis of 0-second trials: $P < 0.001$), pellet trough

return latencies (S15C Fig) (trial type difference in *post-hoc* analysis of 0-second trials: $P < 0.001$), triggering of choice steps (S15F Fig) (trial type difference in *post-hoc* analysis of 0-second trials: $P < 0.01$) and choice lever responses (S15G Fig) (trial type difference in *post-hoc* analysis of 0-second trials: $P < 0.001$). Similar results were seen when analyzing BACHD rats (S16 Fig) (trial type difference in *post-hoc* analysis of 0-second trials: Trial start latency: $P < 0.05$; Pellet trough return latency: $P < 0.001$; Latency to trigger choice step: $P < 0.01$; Choice lever response latency: $P < 0.001$), although the effects appeared to be less pronounced for all parameters except for the choice lever response latency. Importantly, the number of entries and total time spent in the pellet trough during delays were not clearly connected to trial outcome for either genotype (S15D and S15E, S16D and S16E Figs).

Changing the food restriction protocol so that WT rats increased from 85% (standard restriction) to 95% (alternative restriction) of their free-feeding body weight did not markedly change the behavior of the rats. The aforementioned slight difference in the number of completed trials was resolved due to WT rats performing fewer trials after changing the food restriction protocol (data not shown). Rats still completed comparable numbers of Left-Right and Right-Left trials (data not shown). Success rate per delay remained completely unaffected by the change of food restriction protocol at all ages (S17 Fig). The shift did also not have any overt effects on the other parameters of the delayed non-matching to position protocol (S18 Fig). Small increases in the sample lever response latency (S18B Fig), the time spent in the pellet trough (S18E Fig) and the choice lever response latency (S18G Fig) were found. However, similar changes were seen among BACHD rats that were given extended training on the protocol (S19 Fig), suggesting that the changes were not necessarily related to a shift in motivation due to the change in food restriction protocol. The number of trial start omissions made during the test sessions was, however, specifically affected. While BACHD rats typically performed more omissions than WT rats during the initial baselines, this phenotype was resolved when WT rat were maintained on the alternative restriction protocol (S20 Fig). This was due to WT rats performing more trial start omissions than during the initial baselines, while BACHD rats remained unchanged (baseline effect: $F_{(1,22)} = 7.29$, $P < 0.05$; interaction effect: $F_{(1,22)} = 5.90$, $P < 0.05$). Other omission types were not notably affected, neither by the motivational change nor the extended training (data not shown).

Video scoring

As noted, video recordings were made during baseline performance of both the delayed alternation and delayed non-matching test, at the 17–19-month test age. During this, several videos of full training sessions were gathered for each animal. For the delayed non-matching to position test, a single video from each rat was selected for video scoring. Video selection was made so that the performance on the selected sessions (with regard to the parameters presented above) was comparable to the overall baseline. Video analysis of delayed alternation performance was more elaborate. Initial investigation of the rats' behavior during the test revealed that the BACHD rats frequently turned away from the interactive wall of the conditioning chamber to drink water. In order to focus the analysis on other types of behavior, trials where the rats had been drinking were excluded. To still get a comprehensive analysis that covered a full test session (i.e. 120 trials), data from several separate sessions were combined. Importantly, the gathered data set still recapitulated most of the phenotypes mentioned in the previous sections, including the lower success rate and lower number of head entries during delays seen among BACHD rats (data not shown). The increased number of omissions and longer trial start latencies seen for BACHD rats on trials with intermediate delays were, however, no longer present (data not shown), concluding that the drinking behavior was the underlying cause.

Video scoring of both tests indicated that the mean amount of time spent inside the pellet trough, in a central position, around the lever slots and in other parts of the operant conditioning chambers increased with delay duration (Fig 9). In the delayed alternation test, BACHD rats appeared to spend slightly less time than WT rats inside the pellet trough (Fig 9A) and in a central position (Fig 9C), while spending more time than WT rats around the levers (Fig 9E). These trends were, however, not strong and with the exception of a significant genotype x delay interaction regarding the time spent around the lever slots ($F_{(4,56)} = 2.54$, $P < 0.05$), there were no significant effects. The results from the delayed non-matching test showed similar behavioral differences between the genotypes, although much more pronounced. There, BACHD rats spent significantly less time than WT rats both inside the pellet trough (Fig 9B) (genotype effect: $F_{(1,20)} = 13.63$, $P < 0.01$) and in a central position (Fig 9D) (genotype effect: $F_{(1,20)} = 7.58$, $P < 0.05$) during delays. Both post-tests and genotype x delay interaction effects indicated that the phenotype was more apparent in trials with longer delays (interaction effect pellet trough: $F_{(1,20)} = 7.80$, $P < 0.001$; interaction effect central position: $F_{(4,80)} = 4.01$, $P < 0.01$). BACHD rats also spent significantly more time than WT rats investigating the wall portions around the lever slots during the delayed non-matching protocol (genotype effect: $F_{(1,20)} = 6.83$, $P < 0.05$). The phenotype was once again more pronounced for trials with longer delays, as indicated by post-tests and a genotype x delay interaction effect (interaction effect: $F_{(4,80)} = 2.57$, $P < 0.05$). The phenotype was primarily due to the BACHD rats performing a higher number of body shifts towards the different lever slots, while the duration of these body shifts were comparable between the genotypes (data not shown). There were no differences between the genotypes concerning the amount of time they spent in other compartments of the conditioning boxes in either test (Fig 9G and 9H). In the alternation test, there was no difference between genotypes regarding the mean duration of head entries, while BACHD rats were found to make shorter entries compared to WT rats during the delayed non-matching test (data not shown).

To further evaluate if WT and BACHD rats appeared to use different strategies when performing the tests, their body shifts and apparent focus towards a given side of the interactive wall were investigated in terms of their eventual lever choice. Rats in the delayed alternation test (Fig 10) showed a slight preference for making body shifts towards the lever they eventually responded to (Fig 10A). The preference remained stable with increasing delay, and although it appeared to be slightly stronger among BACHD rats, there were no significant genotype or genotype x delay interaction effects. In contrast, both WT and BACHD rats showed a strong preference for making body shifts towards the lever they eventually responded to during the non-matching to position test (Fig 11A). This preference dropped slightly with increasing delay duration (delay effect: $F_{(4,80)} = 3.89$, $P < 0.01$). Once again, there was no significant genotype or genotype x delay interaction effect. Additional analysis of the longest delay in each protocol was performed to evaluate the influence of the rats' apparent lever focus on trial outcome. For both tests and both genotypes, correct lever choices were associated with maintaining a preference for the correct lever throughout the entire delay (Figs 10B and 11B). As above, this preference was notably stronger during the non-matching protocol compared to the alternation protocol. During trials with incorrect lever choices, the rats initially showed proper interest in the correct lever, but switched towards focusing on the incorrect lever during later phases of the delay. Once again, this behavior was apparent for rats of both genotypes and during both tests. It should be pointed out that proper statistics could not be performed for this analysis due to the limited amount of data available. As the noted behaviors appeared to constitute clear strategies for achieving high success rates on the two tests, further parameters were investigated to evaluate if the reduced success rate seen among BACHD rats might be explained by impaired strategy development and/or use. As noted, the rats' initial focus

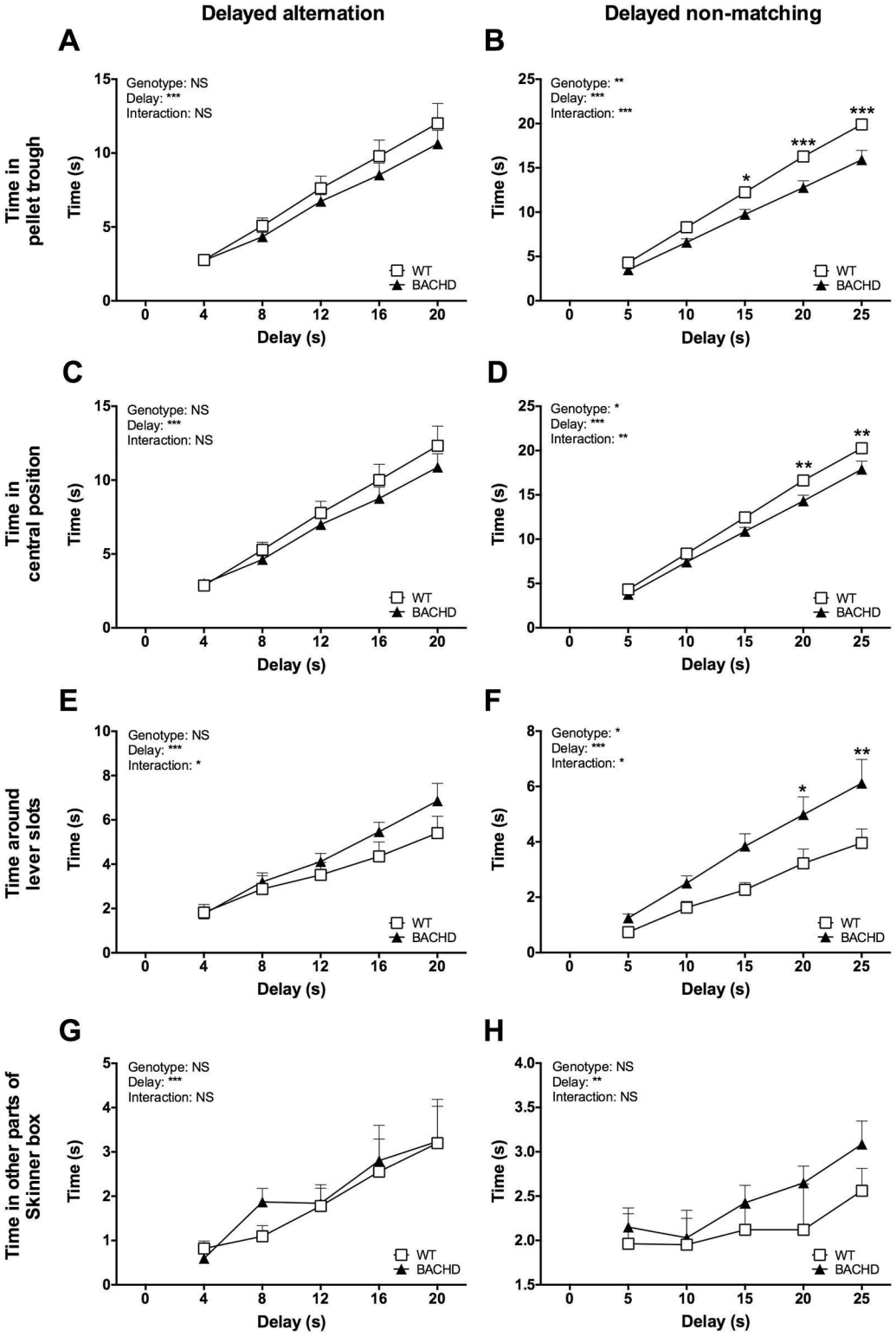

Fig 9. Time spent in different parts of the Skinner boxes during delay steps. The graphs show the time spent in different parts of the Skinner boxes during delays in the delayed alternation and delayed non-matching to position tests, as measured by video scoring. Specific details regarding the data and scoring method is available in the Material and Methods section. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

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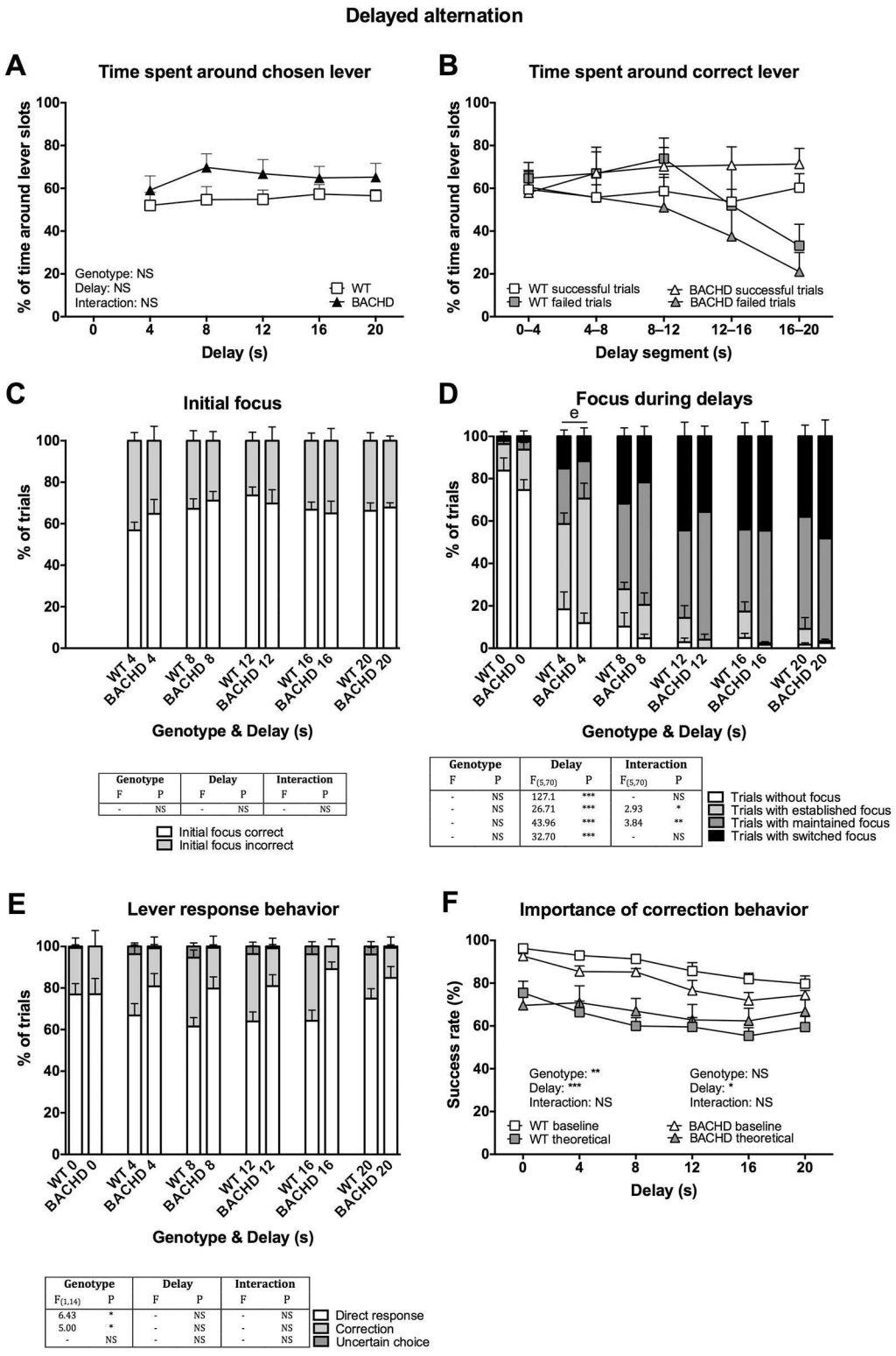

Fig 10. Video-scored behavior in relation to performance on delayed alternation. The graphs show various aspects of the behaviors scored from video recordings in relation to the rats' performance in the delayed alternation test. Graphs indicate group mean plus standard error. (B) concerns the 20-second delay step. In (F), the data that is labeled "theoretical" displays the theoretical success rates, as if the rats had responded according to their initial lever interest and not performed a correction behavior. Further details regarding the

scored behaviors are described in the Material and Methods section. Results from two-way repeated measures ANOVA are shown inside the graphs. For (C)–(E), separate two-way ANOVAs were performed for each kind of behavior, and the respective results are indicated in small tables. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$). In (D), 'e' notes a genotype difference ($P < 0.05$) for trials with established focus.

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during delays was frequently directed towards the correct lever. The frequency of trials with correct initial focus did not differ with delay duration or genotype for either test (Figs 10C and 11C) (data from trials with 0-second delays were excluded from the analysis due to the low number of trials with established focus). The frequencies of other focus-related behaviors were quantified as described in the Material and Methods section. During trials with 0-second delays, most rats did not establish a clear focus (Figs 10D and 11D). However, the frequency of this behavior dropped dramatically with delay duration (delay effect delayed alternation: $F_{(5,70)} = 127.10$, $P < 0.001$; delayed non-matching to position: $F_{(5,100)} = 264.30$, $P < 0.001$). The frequency of trials where a lever focus was only established (i.e. only one body shift motion was performed), was highest during trials with 4- and 5-second delays for the alternation and non-matching test, respectively. Like trials with no focus, their frequency clearly dropped with increasing delay duration (delay effect delayed alternation: $F_{(5,70)} = 26.71$, $P < 0.001$; delayed non-matching to position: $F_{(5,100)} = 66.66$, $P < 0.001$). In contrast, the frequency of trials with maintained or switched focus clearly increased with delay durations (delay effect of maintained focus delayed alternation: $F_{(5,70)} = 43.96$, $P < 0.001$; delay effect of maintained focus delayed non-matching to position: $F_{(5,100)} = 66.15$, $P < 0.001$; delay effect of switched focus delayed alternation: $F_{(5,70)} = 32.70$, $P < 0.001$; delay effect of switched focus delayed non-matching to position: $F_{(5,100)} = 30.40$, $P < 0.001$). There were trends indicating that WT rats showed a higher frequency of trials without focus compared to BACHD rats. This notion was supported by a significant genotype x interaction effect ($F_{(5,100)} = 3.21$, $P < 0.01$) for this parameter during the delayed non-matching test. Further, WT rats showed a lower frequency of trials with only established focus compared to BACHD rats, as indicated by significant genotype effects (delayed non-matching to position: $F_{(1,20)} = 9.79$, $P < 0.01$), genotype x delay interaction effects (delayed alternation: $F_{(5,70)} = 2.93$, $P < 0.05$), and post-test results ($P < 0.05/0.01$ on trials with 4- and 10-second delays for alternation and non-matching test, respectively). WT rats also showed a lower frequency of trials with maintained focus compared to BACHD rats, as indicated by significant genotype x delay interaction effects (delayed alternation: $F_{(5,70)} = 3.84$, $P < 0.01$; delayed non-matching to position: $F_{(5,100)} = 2.78$, $P < 0.05$) and significant *post-hoc* analysis results for trials with 5-second delays in the non-matching test ($P < 0.05$). There were no significant differences between genotypes in the frequency of trials with switched focus. It should be noted that although WT rats tended to show less trials with maintained focus, the ratio of maintained focus to switched focus trials did not differ between the genotypes (data not shown). Thus, the lower frequency of trials with maintained focus seen among WT rats in the analysis described above was likely a result of their lower tendency to perform body shifts (as indicated by the increased frequency of trials without focus).

The final behavioral aspect that was investigated concerned the rats' behavior while performing the final lever response. In both tests, the most common behavior for both genotypes and all delays were direct responses (Figs 10E and 11E). Thus, rats rarely showed any interest in the non-chosen lever. However, during the delayed alternation test, there were still a considerable amount of corrections (Fig 10E). Notably, BACHD rats showed a higher frequency of direct responses compared to WT rats (genotype effect: $F_{(1,14)} = 6.43$, $P < 0.05$), while WT rats showed a higher frequency of corrections (genotype effect: $F_{(1,14)} = 5.00$, $P < 0.05$). The frequency of uncertain choices was marginally higher among WT rats, although it failed to reach

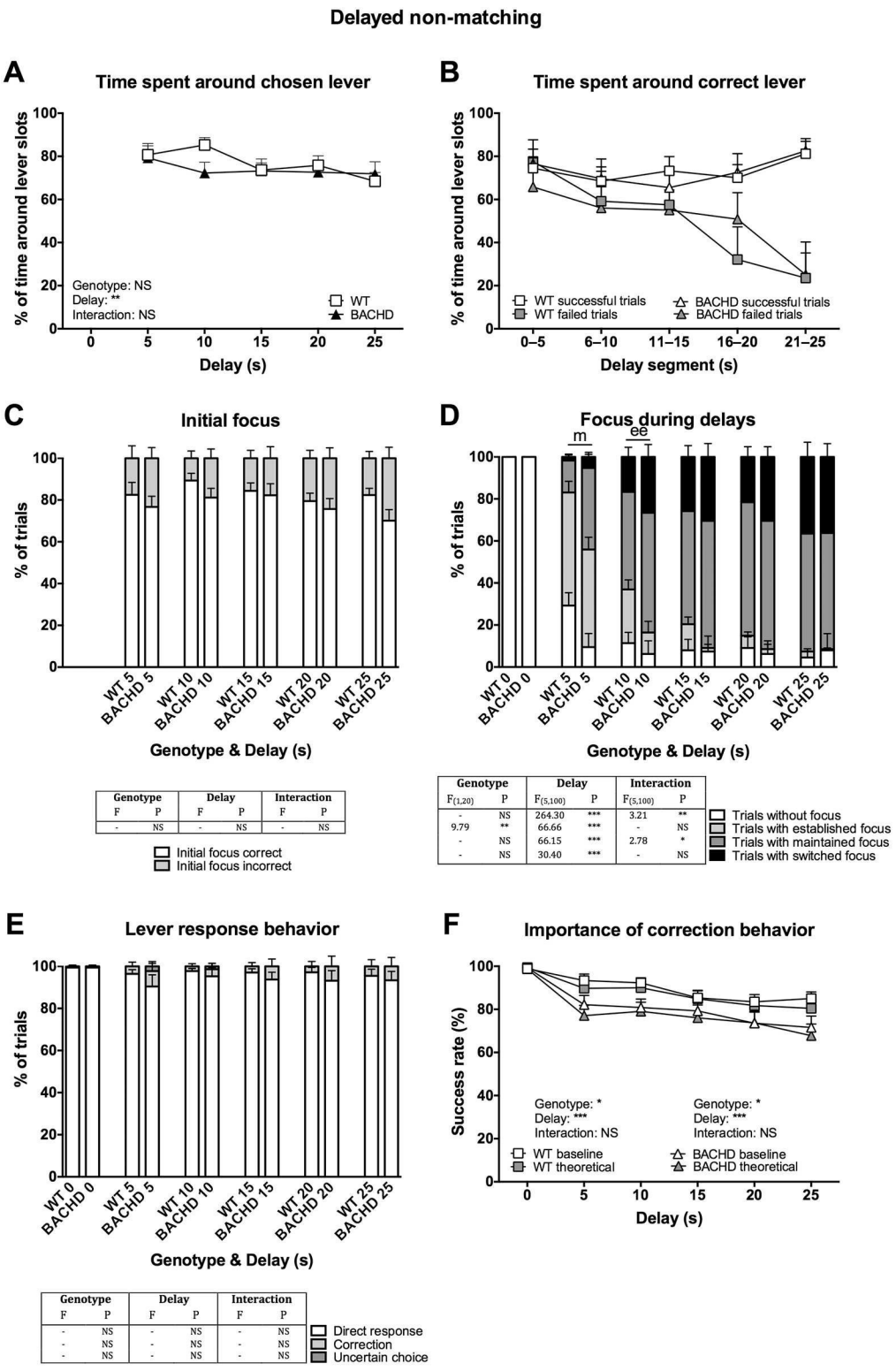

Fig 11. Video-scored behavior in relation to performance on delayed non-matching to position. The graphs show various aspects of the behaviors scored from video recordings in relation to the rats' performance in the delayed non-matching to position test. Graphs indicate group mean plus standard error. (B) concerns the 25-second delay step. In (F), the data that is labeled "theoretical" displays the theoretical success rates, as if the rats had responded according to their initial lever interest and not performed a correction behavior. Further

details regarding the scored behaviors are described in the Material and Methods section. Results from two-way repeated measures ANOVA are shown inside the graphs. For (C)–(E), separate two-way ANOVAs were performed for each kind of behavior, and the respective results are indicated in small tables. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$). In (D), 'ee' indicates a genotype difference ($P < 0.01$) for trials with established focus, and 'm' indicates a genotype difference ($P < 0.05$) for trials with maintained focus.

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statistical significance. As noted, the behaviors did not notably change with delay duration, although 0-second delay trials were the only ones where the aforementioned difference was not clearly present. The correction behavior still appeared to be important for the overall success rate of both WT and BACHD rats. Notably, the theoretical success rate of both genotypes (where the outcome of all correction trials had been adjusted to the hypothetical outcome of the response they initially intended to do) was markedly lower compared to their actual baseline (Fig 10F). While the actual baseline showed a similar performance deficit among BACHD rats as the one described above (genotype effect: $F_{(1,14)} = 8.88$, $P < 0.01$), there was no significant genotype difference in the theoretical data. Overall, these results were in clear contrast to the rats' behavior during the delayed non-matching to position test (Fig 11E). During that test, rats of both genotypes rarely displayed corrections and uncertain responses, and no genotype differences were found for these behaviors. In line with this, the rats' theoretical success rate did not clearly differ from their original baseline (Fig 11F). Thus, the BACHD rats' performance deficit was apparent in both data sets (genotype effect, recorded data: $F_{(1,20)} = 6.04$, $P < 0.05$; genotype effect, theoretical data: $F_{(1,20)} = 5.13$, $P < 0.05$).

An overview of the main results found in the delayed alternation and delayed non-matching to position tests are show in Tables 1 and 2.

Discussion

BACHD rats show no impairment when learning to perform simple instrumental response tasks

Our study did not reveal any overt differences between BACHD and WT rats during the initial habituation and lever training steps, with the exception of occasional indications that BACHD rats were slower at returning to the lever during CRF training. These findings are largely similar to what we have presented in previous publications [31], and what we have found in several studies that remain unpublished at this time. It should, however, be noted that in most of these studies the initial training steps were performed when the rats were 2–5 months old, and learning deficits might still be present in older animals. In line with this, it has been found that 18 months old BACHD rats (but not 2 and 8 months old rats) required more sessions than WT rats to reach criterion when learning to perform single nose pokes for food rewards [33]. However, no detailed analysis was performed to investigate if this phenotype was based on the rats having actual difficulties to associate the instrumental response with the delivery of a food pellet, or rather them being less interested in exploring the test chamber. Thus, while the BACHD rats' ability to learn simple instrumental response tasks appears to be reliably intact at young ages, it is still unclear if it deteriorates with age.

BACHD rats show slowed learning during alternation training, but not during non-matching to position training

Later training steps revealed clear differences between the alternation and the non-matching test. Specifically, while rats of both genotypes needed several training sessions before reaching criterion on the free alternation protocol, they required very little training to reach criterion

Table 1. Overview of results from the delayed alternation test.

Parameter	Results	Figure reference
Training needed to handle basic alternation task	BACHD rats required more training than WT rats during the first test age. The phenotype was less apparent but still present during the second test age and was fully resolved after further retesting.	Fig 3A
Training needed to progress through delay sets	BACHD rats required more training than WT rats during the first test age. The phenotype was less apparent but still present during the second and third test age and was fully resolved at the last test age.	S1 Fig
Overall success rate	BACHD rats showed a generally impaired performance with lower success rates compared to WT rats on all trial types.	Fig 4A, S2 Fig
Entries into pellet trough during delays	BACHD rats made fewer entries compared to WT rats. The phenotype was more pronounced on trials with long delays, although it did not appear to be due to BACHD rats gradually losing interest in the pellet trough with time.	Fig 5B and 5C
Time in pellet trough during delays	No overt difference was found between the genotypes in data recorded by the operant conditioning system, although a trend indicating that BACHD rats spent less time in the pellet trough compared to WT rats was found when manually scoring video recorded behaviors of the rats.	Figs 5D and 9A
Trial start latency	No difference was found between the genotypes during the first test age. During retesting, BACHD rats showed significantly longer trial start latencies compared to WT rats on trials with intermediate delay durations, which was found to be due to BACHD rats making frequent breaks for drinking.	S3A and S3C Fig
Lever response latency	No overt difference was found between the genotypes, although BACHD rats appeared to be slightly slower than WT rats during trials with 0-second delays.	S4A Fig
Pellet retrieval latency	No difference was found between the genotypes during the first two test ages. BACHD rats then appeared to become gradually slower with age, resulting in them being significantly slower than WT rats at the last test age.	S4B Fig
Omissions	No difference was found between the genotypes during the first test age. During retesting, BACHD rats showed significantly increased omission rates on trials with 0-, 4- and 8-second delays, which was found to be due to BACHD rats making frequent breaks for drinking.	S3B and S3D Fig
Video-scored behavior	During delays, rats of both genotypes were found to frequently exit the pellet trough and investigate the area around the retracted levers. There were discreet indications that this behavior functioned as a strategy for remembering which lever to respond to. There was no significant difference between the genotypes regarding this behavior, although BACHD rats tended to do it more frequently than WT rats. During lever responses, BACHD rats showed a reduced frequency of a type of correction behavior, and a corresponding increase in performing lever responses without hesitation, compared to WT rats. The correction behavior appeared to be important for maintaining a high success rate in the test.	Figs 9 and 10

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on the free non-matching test. It is possible that this was due to the alternation protocol putting more strain on the rats' inhibitory control. Essentially, it is reasonable to assume that rats have a strong tendency to return to a previously reinforced lever. During alternation protocols, this would cause them to have a high tendency to perform erroneous responses, and appropriate response inhibition would be required to achieve a high success rate. In contrast, the tendency to respond to the sample lever position during the non-matching protocols is likely low, as that response is never reinforced. Interestingly, BACHD rats showed a slowed learning compared to WT rats during the free alternation but not the free non-matching. This could have been due to them having specific problems with certain aspects of the alternation protocol (such as the suggested inhibitory control aspect), although it is also possible that the slowed learning represented a general learning deficit, which was not apparent during the non-matching test due to its relative simplicity. However, the latter hypothesis is unlikely to be true, as we have performed other complex cognitive tests without finding slowed learning among BACHD rats (unpublished results). In addition, the former hypothesis is to some extent supported by the BACHD rats' generally impaired performance in the delayed alternation test (further discussed below). It should also be noted that the slowed learning among BACHD rats was likely not related to any underlying motivational deficits, as there were no differences in the number completed trials.

Table 2. Overview of results from the delayed non-matching test.

Parameter	Brief description of phenotype	Figure reference
Training needed to handle basic non-matching task	No difference was found between the genotypes.	Fig 6A
Training needed to progress through delay sets	No difference was found between the genotypes.	S9 Fig
Overall success rate	BACHD rats showed unchanged success rate on trials without delays, while performance on trials with delays appeared to be generally impaired.	Fig 7A, S10 Fig
Trial start latency	No difference was found between the genotypes.	S12 Fig
Sample lever response latency	No difference was found between the genotypes.	S13A Fig
Food crib return latency	No difference was found between the genotypes.	Fig 8A
Entries into pellet trough during delays	BACHD rats made fewer entries compared to WT rats. The phenotype was more pronounced on trials with long delays, although it did not appear to be due to BACHD rats gradually losing interest in the pellet trough with time.	Fig 8B and 8C
Time in pellet trough during delays	No overt difference was found between the genotypes in data recorded by the operant conditioning system, although a trend indicated that BACHD rats spent less time in the pellet trough compared to WT rats. Manual video scoring of the behavior revealed a more pronounced phenotype of this nature.	Figs 8D and 9B
Latency to trigger choice step	No difference was found between the genotypes, although a trend indicated that BACHD rats were slightly slower than WT rats.	S11A Fig
Choice lever response latency	No difference was found between the genotypes.	S13B Fig
Pellet retrieval latency	BACHD rats were consistently slower than WT rats when retrieving the reward pellets.	S14A Fig
Omissions	BACHD rats showed a significantly higher number of trial start omissions, compared to WT rats	S11C Fig
Video-scored behavior	During delays, rats of both genotypes were found to frequently exit the pellet trough and investigate the area around the retracted levers. There were strong indications that this behavior functioned as a strategy for remembering which lever to respond to. BACHD rats performed this more frequently than WT rats. However, the video scoring did not reveal any behavioral differences that might explain the BACHD rats' reduced success rate in the test.	Figs 9 and 11

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Despite the slowed learning among BACHD rats, the performance during criterion sessions was comparable between the genotypes during both the free alternation and free non-matching. The only exception was the longer pellet retrieval latencies seen among BACHD rats, which was present in both protocols. This phenotype has been found in almost all operant conditioning tests that have been run with the BACHD rats at our institute (seven longitudinal studies) and is described in previous publications [31]. Interestingly, similar phenotypes have been found in transgenic rats that carry a fragment of the HD-causing gene (TgHD rats) [34]. In the current study, the non-matching to position protocol offered additional information regarding this phenotype. Specifically, it allowed direct comparison between the pellet retrieval latency and the pellet trough return latency. These two parameters measured the latency to perform comparable motor behaviors, but aimed towards a pellet trough that either contained a reward pellet or was empty. While WT rats were clearly faster at moving to the pellet trough when there was a reward pellet present, BACHD rats showed comparable latencies in both situations. This could indicate a form of emotional blunting among BACHD rats. However, it is also possible that the BACHD rats were already moving at their maximum speed. More focused investigation of this phenotype is needed to better understand if its nature is motoric or psychological. Interestingly, TgHD rats have shown indications of emotional blunting in a sucrose solution consumption test [35].

BACHD rats show reduced success rates in both the delayed alternation and delayed non-matching to position tests

The study's main finding of interest was the consistently lower success rates found among BACHD rats in both the delayed alternation and non-matching to position test. The

impairment seen in the delayed alternation test was not clearly affected by delay duration, suggesting that the rats had a general problem performing the basic task (i.e. alternation) rather than a short-term memory deficit. Similar phenotypes have been found in a knock-in HD mouse model [36] and rats with either striatal or prefrontal lesions [23–25]. The BACHD rats' slowed learning during the free alternation training also supports the idea that they have problems handling the basic alternation task. It should, however, be noted that there were occasional tendencies indicating that the BACHD rats' impairment became stronger with longer delay durations. Interestingly, such phenotypes have been found when lesioning specific fronto-striatal circuits [26], but also when lesioning the hippocampus [37]. Both pathologies could theoretically be present in the BACHD rats, as extensive protein aggregate formation has previously been found in the rats in these brain regions [30,38]. It should, however, also be noted that the apparent delay-dependent worsening of the phenotype might have been influenced by other aspects of the rats' behavior (further discussed below). The impairment seen in the delayed non-matching to position test was similar to that of the delayed alternation test, although not identical. Longer delays did once again not appear to result in a stronger deficit, while the general presence of delays seemed to be crucial. Thus, the phenotype once again appeared to be due to the BACHD rats having general problems with the basic task, although it specifically concerned the delayed task. To our knowledge, similar phenotypes have not been reported elsewhere. Striatal lesions have been found to result in slight learning impairments in the delayed non-matching test [27], which were not seen in the current study. Further, fornix lesions have been found to result in delay-dependent impairments [39,40], while lesions to the prefrontal cortex and hippocampus have been found to produce a general drop in success rate [28,29]. The latter kind of phenotype was also found in a recent study of the performance of an HD knock-in mouse in a slightly different version of the test [41]. As noted though, these phenotypes do not appear to be directly comparable to the apparent biphasic curves found in the current study. As a final note, it is interesting that HD patients have shown general problems to perform accurately in a delayed pattern matching to sample test, although the impairment did not become more pronounced with increased delays [42].

BACHD rats show other behavioral changes, although their influence on success rate is likely limited

Additional parameters were evaluated, as non-cognition based behavioral differences between BACHD and WT rats could have influenced the success rate in the two tests, and needed to be considered. The results revealed that there were indeed several behavioral differences between BACHD and WT rats, although few appeared to be directly related to the rats' success rates. During the delayed alternation test, BACHD rats were found to be slower at performing the first head entry of the delay, performed fewer head entries during the delays and showed longer trial start latencies for trials with intermediate delay durations. Out of these parameters, the difference in trial start latencies was the only factor that appeared to be related to failed trials. Still, BACHD and WT rats showed comparable trial start latencies during the first test age, while BACHD rats already presented an overall lower success rate. Similarly, when only trials without water consumption were considered, there was no difference between genotypes in terms of trial start latencies, while the reduced success rate among BACHD rats was still present. Thus, although the slowed trial start latency among BACHD rats most likely affected their performance to some extent, it was unlikely the main cause of their reduced success rate. There were fewer differences between the BACHD and WT rats' behavior during the delayed non-matching test. Most notably, BACHD rats again performed fewer head entries than WT rats during delays. However, detailed analysis suggested once again that this difference was

unlikely to explain the difference in success rate. As the reduced number of head entries was still consistently found in the two tests, it is worth noting that similar phenotypes have been found in rats with striatal lesions [24], although not consistently [23]. Ultimately, despite the various noted behavioral differences found between BACHD and WT rats it is likely that underlying cognitive changes were the main cause of their reduced success rates. However, additional non cognition-related behavioral differences likely still influenced the overall appearance of the phenotype to some extent. When considering this, it is also noteworthy that some parameters appeared to only affect certain trial types. Specifically, the relationship between trial start latencies and trial outcome in the delayed alternation test appeared to primarily concern trials with short delays, and was restricted to BACHD rats. In contrast, the WT rats' success on trials with short delays appeared to be more related to their lever response latencies. Specific parameters also appeared to be related to failure on trials with short delays during the delayed non-matching test (i.e. trial start latency, pellet trough return latency, latency to trigger choice step and choice lever response latency). These aspects complicate the task of assessing the appearance of the rats' actual cognitive impairment, making it possible that the current interpretations (i.e. cognitive impairments resulting in BACHD rats showing an overall impaired performance in the delayed alternation test, and a biphasic impairment in the delayed non-matching to position test) are not entirely true. Specifically, the BACHD rats' lower success rate on trials with 0-second delays during the delayed alternation test might have been related to non-cognitive behaviors influencing their trial start latencies. Thus, their true cognitive impairment might have been more comparable to the one seen in the delayed non-matching to position test, which would also be more in line with their unchanged success rate during the free alternation protocols.

Although the difference in the number of head entries performed during delays did not appear to be connected to the reduced success rate among BACHD rats, a difference in delay behavior might still indicate altered motivation, attention or strategy. Thus, additional analysis of this phenotype was of interest. The phenotypes were first validated with video scoring that indicated that BACHD rats indeed spent less time than WT rats being in an arguably central position during delays (although the phenotype was quite discreet during the delayed alternation test). Certain aspects argued against the phenotype being caused by motivational differences. First, the reduced number of entries was present on each segment of the longer delay steps, suggesting that the phenotype was not due to the BACHD rats simply losing interest as time passed. In addition, the number of entries was not strongly affected by changing the food restriction protocol of WT rats. BACHD rats were, however, found to frequently turn around to drink water during delays in the delayed alternation test, suggesting a change in their relative interest in food and water. It is worth noting that this behavior appeared to be the main cause of the BACHD rats' peculiar pattern of trial start latencies and omissions during the test. Further, although the trial start latencies themselves appeared to have only limited influence on the success rate (see above), it is still possible that the drinking behavior had caused the trends indicating that the BACHD rats' performance deficit worsened with longer delays. Regardless, although the drinking behavior might have affected the BACHD rats' success rate, and definitely contributed to their lower number of head entries performed during delays, both phenotypes were still present when trials with drinking were excluded.

BACHD and WT rats develop comparable strategies to maintain high success rates on the two tests

As noted, it was subsequently found that BACHD rats had a higher frequency of leaving the pellet trough to investigate the area around it. This could indicate a change in attention and

strategy, although there were no indications of either one in the current study. Both BACHD and WT rats preferentially made body shifts towards the lever they would eventually respond to, and appeared to focus on the lever that would give a correct response. Thus, although BACHD rats made these movements at a higher frequency than WT rats, their use and relevance to lever choices did not notably differ between the genotypes on either test. The increased use of body shifts seen among BACHD rats might, however, indicate that they were more dependent on the strategy than WT rats. This would be in line with the trend suggesting that BACHD rats had a slightly stronger preference for making body shifts towards the chosen lever during the delayed alternation test. This idea should be further evaluated by comparing performance in setups or protocols that make the use of this strategy more difficult, such as placing the levers and pellet trough on opposite walls, placing walls between the wall sections containing the lever slots and the pellet trough, or adding limits so that trials are cancelled if the rats exit the pellet trough. Still, the importance of the body shifts for accurate performance during the delayed alternation test is somewhat uncertain, as the rats did not show a particularly strong focus for the selected lever. Thus, although the rats clearly used the body shifts as part of a strategy during the delayed non-matching test, thee reason for at all performing them (and the consistent finding of BACHD rats performing them more frequently than WT rats) might be due to more general and not strategy-related behaviors. Interestingly, transgenic rats carrying a fragment of the HD-causing gene have been found to show a high frequency of early withdrawals from nose poke modules during a choice reaction time test, which was suggested to be due to impaired response inhibition [43]. This phenotype is arguably similar to the one found in the current study.

Strategies similar to the body shifts described here have been found in other studies of rats performing the delayed non-matching to position test [40]. That particular study also indicated that fronto-striatal lesions, which resulted in reduced success rate, also affected these mediating behaviors. Among other things, lesioned rats showed an increased frequency of changing focus from one lever to another during delays. Due to this, we investigated similar parameters in the current study. In both tests and genotypes, there were indications that maintaining focus on the correct lever throughout the delay was related to a successful outcome, while switching focus to the wrong lever was related to failed trials. However, there were no clear indications that BACHD rats switched focus more frequently than WT rats. In addition, there were no differences regarding how often the rats' initial focus was on the correct lever. It should, however, be noted that the scoring method used here (i.e. a rats' apparent focus being based on the first and last body shift) was limited. However, more elaborate scoring (such as judging the rat's apparent focus based on the percentage of time spent around a given lever) would have suffered from similar limitations due to the low number of body shifts that were performed (roughly four for WT and six for BACHD rats during the longest delay). Further insight into the rats' focus-shifting behavior might still be gained through the analysis of more data, using a more elaborate scoring protocol, but it is beyond the scope of the current study.

BACHD rats show a reduced frequency of correction behaviors during delayed alternation performance

The rats' behavior while performing lever pushes was also investigated. This scoring indicated that most rats responded without hesitation during the choice step of the delayed non-matching test. This was most likely due to a strong association between their body shift and the planned lever response. Thus, the main decision regarding which lever to push was likely made already during the delay step. This might also explain why both WT and BACHD rats

were faster at responding to choice levers than to sample levers. All in all, these aspects question to what extent the test really evaluated short-term memory rather than the rats' ability to establish and maintain focus on the correct lever. Direct responses also constituted the majority of responses made in the delayed alternation test. However, there was a considerable frequency of correction behaviors, where the rats would first start moving towards one lever but change their mind and respond to the other one. The importance of this behavior was indicated by the dramatically lower success rate found when the rats' theoretical performance was considered (i.e. success rate as if they had responded according to their initial lever choice). Importantly, the frequency of correction behaviors was higher among WT rats than BACHD rats. Further, there was no difference between WT and BACHD rats in their theoretical success rates. Thus, it is likely that the reduced frequency of correction behaviors among BACHD rats was connected to their lower success rate in the delayed alternation test. Still, in connection to the discussions above, it is noteworthy that there was no clear difference in the frequency of corrections during trials with 0-second delays. Regardless, the reduced frequency of correction behavior might be an indication that BACHD rats have difficulties inhibiting already initiated responses. This would suggest an impairment regarding a quite specific aspect of response inhibition, which should be further investigated in tests that probe this [44–46]. Evaluating the BACHD rats' performance in such tests might also help to determine if the impairment in the delayed alternation test truly concerned a failure to inhibit erroneous responses, as opposed to a failure to realize that the initiated responses would be erroneous. Interestingly, changes in neuronal signaling have been found in HD patients during performance of tests where they had to inhibit ongoing motor responses [47]. In addition, HD patients [48], HD mouse models [49] and BACHD rats [50] have all been found to show impaired performance in other tests of response inhibition. It should, however, be noted that the study performed on BACHD rats did not conclusively show that the response inhibition impairment concerned a baseline deficit rather than a response to a change in protocol.

The noted phenotypes generally remained stable with increasing age

As noted, the phenotypes found in the two tests did not appear to change with age. Due to the progressive nature of HD, one would typically expect that disease-related phenotypes in animal models would worsen when they grow older. Indeed, other phenotypes found in the BACHD rats have been shown to progressively worsen, already while the rats were a few months old [30]. However, the neuropathology of the BACHD rats has not been fully elucidated yet. Although loss of dopamine 2 receptors has been implicated in old animals, and although there is a gradual accumulation of huntingtin aggregates with age [30], it is not clear if this results in progressive loss of function in fronto-striatal circuits. The current results would suggest that it does not. Thus, the impairments found here might be due to neuropathology caused by the general presence of mutant huntingtin, rather than its progressive accumulation. Alternatively, the impairments might be due to neuropathology caused by developmental deficits. Specifically, male BACHD rats have been found to be smaller than their WT littermates [31] and consistently have smaller brains (unpublished results). At this point, it is unclear if this developmental deficit only regards size or also functionality. Finally, it should be considered that the rats in the current study spent roughly half of their life actively being assessed in the respective tests. This frequent behavioral evaluation might have acted as environmental enrichment, and might have counteracted any progression that would have occurred if less frequent training were used. To evaluate this further, additional tests should be run where test ages are spaced further apart or performed with separate test groups.

Conclusions and final remarks

BACHD rats showed impaired performance in both the delayed alternation and delayed non-matching test in the current study. The phenotypes were already present at 2–4 months of age and did not appear to progressively worsen with age. In both tests, the rats appeared to primarily have problems handling the basic task, while short-term memory remained intact. The impairment found in the delayed alternation test seemed to in part be caused by a failure to correct ongoing erroneous responses, which in turn could be due to deficits in attention and/or inhibitory control. It is currently unclear what specific behavioral differences caused the impaired performance in the delayed non-matching to position test, although it is likely related to the distinct mediating behaviors that both WT and BACHD rats used. Importantly, arguably similar performance deficits have been found in other HD models and rats with fronto-striatal lesions, suggesting that the BACHD rats' phenotypes are caused by HD-related neuropathology. In addition, the phenotypes were not affected by a change in motivation and hunger, suggesting that the impairments likely reflect true cognitive deficits rather than artifacts due to motivational differences between WT and BACHD rats.

As a side note, using water bottles during operant conditioning tests might not be optimal when working with BACHD rats. During delayed alternation training, the BACHD rats took frequent breaks to consume water, which dramatically affected their trial start latencies and omission rates. It is currently not clear why the rats developed this behavior, as it has not been found in other operant condition tasks performed at our institute. In addition, extensive control tests were run with the delayed alternation rats to investigate their thirst response to being fed reward pellets in various conditions. However, there were no indications that BACHD rats became thirstier than WT rats when consuming reward pellets.

Supporting Information

S1 Fig. Sessions required to progress through delayed alternation training. The graphs show the total number of sessions required for progressing through the series of delayed alternation protocols at the different test ages, with gradually increasing delay durations that were implemented before the training on the final delay set had started. The values were adjusted for the change in criterion that was made after the first test age. Rats, which did not reach criterion on each protocol, were excluded from the analysis. Plots indicate single values for individual rats. Note that the scale on the y-axis differs between the graphs. Results from *t*-test or Mann-Whitney U test are indicated in case significant genotype differences were present. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S2 Fig. Success rate per delay in the delayed alternation test during retesting. The graphs show the success rate on trial types with delays of different durations in the delayed alternation test. Each graph shows the stable baseline performance of rats maintained on the standard food restriction protocol. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. For (A), results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S3 Fig. Trial start latency and omissions during delayed alternation. The graphs show trial start latency and omissions during the delayed alternation protocol. (A) and (B) show the behavior at the four-month test age, while (C) and (D) show the mean performance at the three older ages. Graphs indicate group mean plus standard error. Results from two-way repeated

measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S4 Fig. Additional parameters of delayed alternation performance. The graphs show the last two parameters investigated for delayed alternation performance. (A) is based on the overall performance on all test ages, as no significant change with age was found for the parameter. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S5 Fig. Parameters indicating success or failure on delayed alternation. The graphs show some of the parameters of the delayed alternation protocol with performance of WT and BACHD rats separated for successful and failed trials. All graphs were constructed based on the mean performance over all test ages, as the relation to trial outcome did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S6 Fig. Effect of food restriction adjustment on success rate in delayed alternation test. The graphs show the WT rats' performance in the delayed alternation test during two different food restriction settings at the four investigated ages. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S7 Fig. Effect of food restriction adjustment and extended training on delayed alternation parameters. The graphs show some of the parameters of the delayed alternation protocol, comparing performance of WT and BACHD rats during their initial baseline with performance after changing food restriction protocol or given extended training, respectively. All graphs were constructed based on the mean performance over all test ages, as the effect of changing food restriction protocol or giving extended training did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant differences between baselines were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S8 Fig. Effect of food restriction adjustment on omissions during delayed alternation. The graphs show the effect of food restriction adjustment and extended training on the number of trial start omissions performed during the delayed alternation test. All graphs were constructed based on the mean performance over all test ages, as the effect of changing food restriction protocol or giving extended training did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant differences between baselines were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S9 Fig. Sessions required to progress through delayed non-matching to position training. The graphs show the total number of sessions required for progressing through the series of

delayed non-matching to position protocols with gradually increasing delay durations, which were implemented before the training on the final delay set had started. The values were adjusted for the change in criterion that was made after the first test age. Rats that did not reach criterion on each protocol were excluded from the analysis. Plots indicate single values for individual rats. Note that the scale on the y-axis differs between (A) and the remaining graphs. Results from *t*-test or Mann-Whitney U test are indicated in case significant genotype differences were present. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S10 Fig. Success rate per delay in the delayed non-matching to position test during retesting. The graphs show the age development of success rate on trial types with delays of different durations in the delayed non-matching test. Each graph shows the stable performance found when rats were maintained on the standard food restriction protocol. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S11 Fig. Latency to trigger choice step and omissions for delayed non-matching to position. The graphs show the latency to initiate the choice step, related omissions and omissions overview during the delayed non-matching to position protocol. Graphs display the mean performance over all ages, as no significant differences in the rats' behavior at different ages was found. (A) and (B) indicate group mean plus standard error. (C) indicates the performance of individual rats. For (A) and (B), results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. For (C), results from *t*-test or Mann-Whitney U test are indicated in case the genotypes differed significantly. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S12 Fig. Trial start latency in the delayed non-matching to position test. The graph shows the latency to initiate trials on the different test ages of the delayed non-matching to position protocol. The curve indicates group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graph, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S13 Fig. Lever response latencies during delayed non-matching to position. The graphs show the latencies to respond to a lever during either the sample step or the choice step of the delayed non-matching to position protocol. (A) and (B) display the comparison between WT and BACHD for both response latencies, while (C) and (D) display comparisons between the type of response latencies for both genotypes. Graphs display mean performance over all ages, as no significant differences in the rats' behavior at different ages was found. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated for data points where significant genotype differences were found. * ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$).
(TIFF)

S14 Fig. Reward pellet retrieval latency during delayed non-matching to position. (A) shows the mean pellet retrieval latency of WT and BACHD rats during the delayed non-matching to position protocol at all investigated ages. (B) shows a comparison of the mean pellet retrieval latency with the mean latency to return to the pellet trough after pushing the

sample lever. For this, the mean of all investigated ages and trial types were used, as the phenotypes or differences between latencies did not clearly change with age. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S15 Fig. Parameters indicating success or failure for WT rats in the delayed non-matching to position test. The graphs show some of the parameters of the delayed non-matching to position protocol performance of WT rats separated for successful and failed trials. All graphs were constructed using the mean performance over all ages, as the parameters' relation to trial outcome did not noticeably change between test ages. In addition, this was necessary to obtain data for failed 0-second delay trials for all rats. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S16 Fig. Parameters indicating success or failure for BACHD rats in the delayed non-matching to position test. The graphs show some of the parameters of the delayed non-matching to position protocol performance of BACHD rats separated for successful and failed trials. All graphs were constructed using the mean performance over all ages, as the parameters' relation to trial outcome did not noticeably change between test ages. In addition, this was necessary to obtain data for failed 0-second delay trials for all rats. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S17 Fig. Effect of food restriction adjustment on success rate in delayed non-matching to position. The graphs show the WT rats' performance in the delayed non-matching to position test during two different food restriction settings at the four investigated age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S18 Fig. Effect of food restriction adjustment of WT rats in the delayed non-matching to position test. The graphs show some of the parameters of the delayed non-matching to position protocol performance of WT rats separated for standard and alternative food restriction protocols. All graphs were constructed using the mean performance over all ages, as the parameters' relation to motivational state did not noticeably change between test ages. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S19 Fig. Effect of extended training of BACHD rats in the delayed non-matching to position test. The graphs show some of the parameters of the delayed non-matching to position protocol performance of BACHD rats separated for the baselines after initial and extended training. All graphs were constructed using the mean performance over all ages, as the

parameters' relation to the amount of training did not noticeably change between test ages. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from post-hoc analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

S20 Fig. Trial start omissions during different baselines of delayed non-matching to position. The graph shows the number of omissions during the initial baselines and after either a change in food restriction protocol or extended training on the delayed non-matching to position protocol. The graph was constructed using the mean performance over all ages, as the parameters' relation to food restriction or extended training did not noticeably change between test ages. The curve indicates group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graph, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).
(TIFF)

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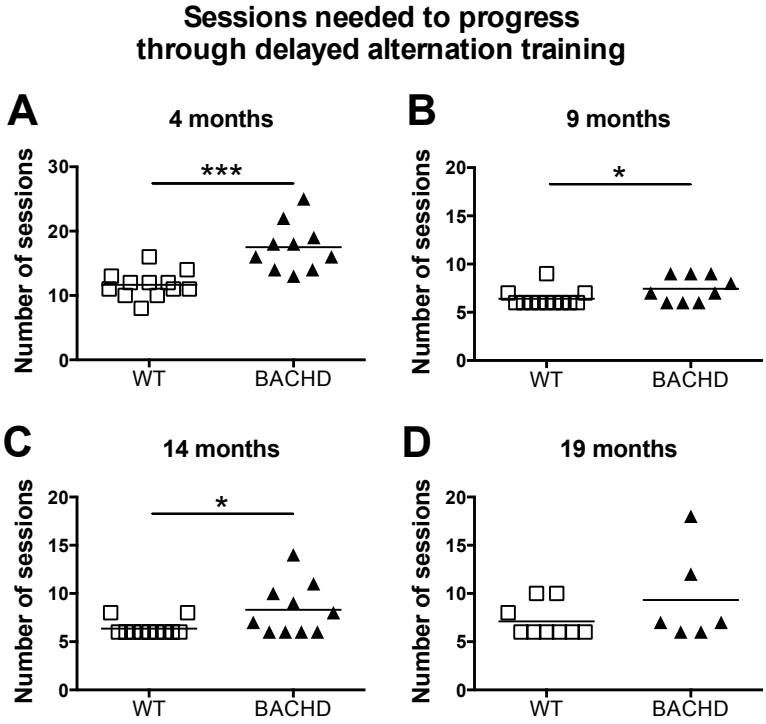
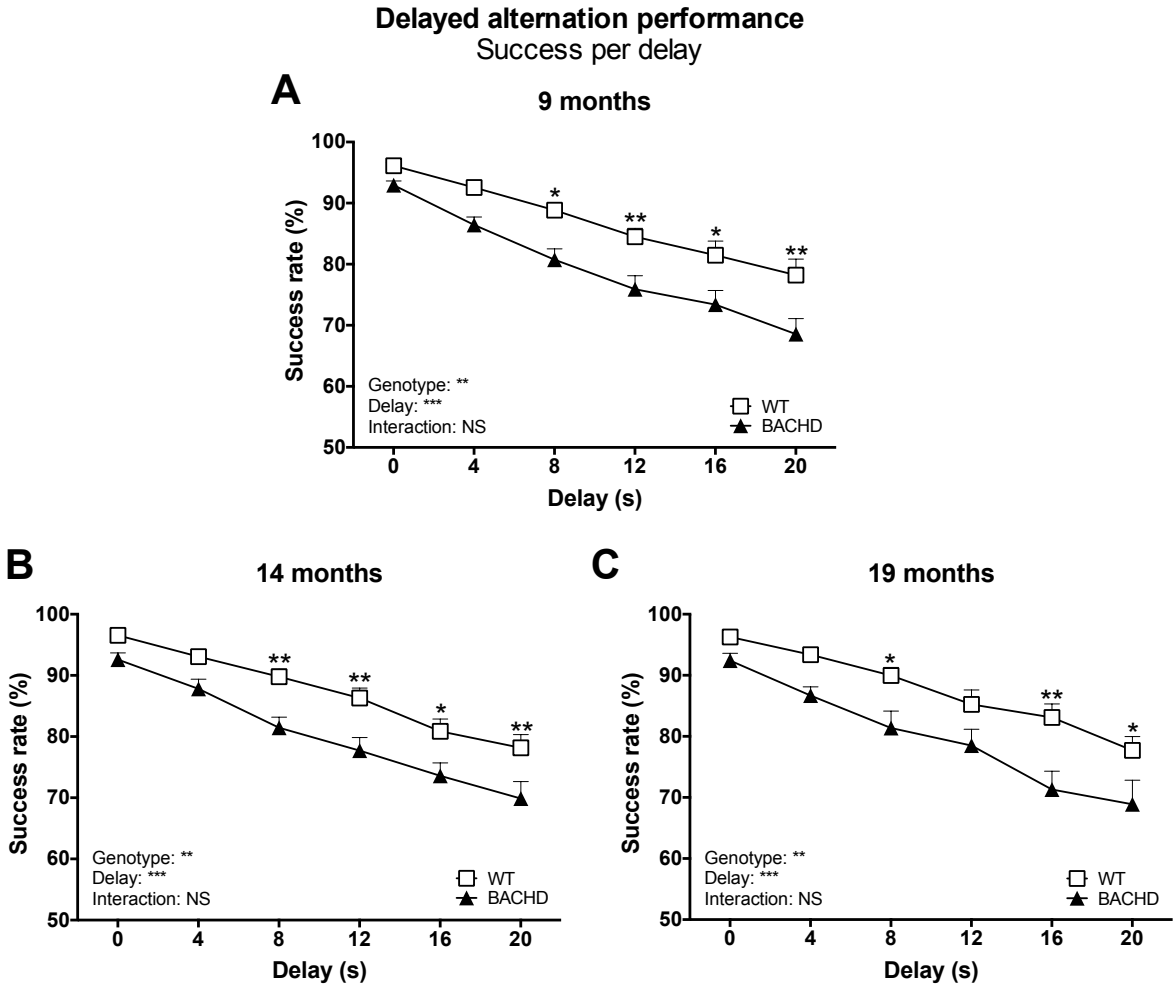
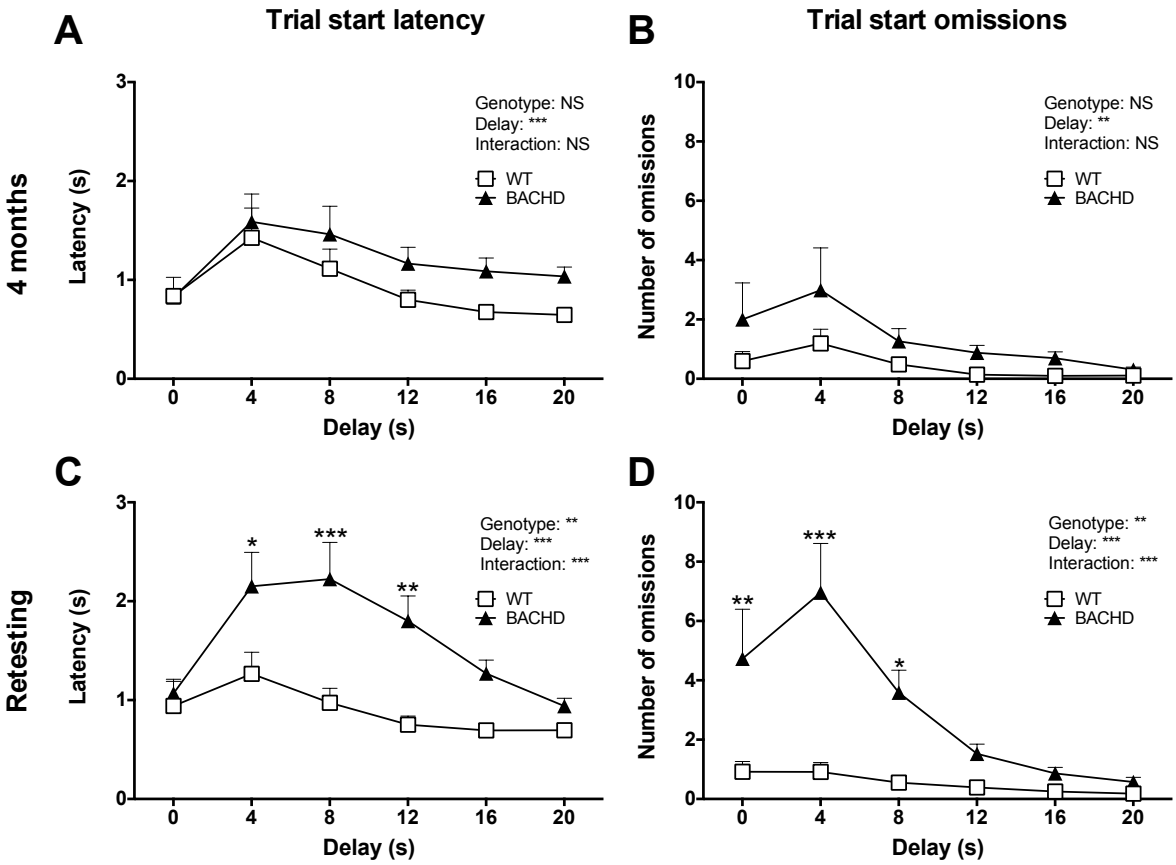

Sessions needed to progress
through delayed alternation training
A
4 months
***
Number of sessions
30
20
10
0
WT
BACHD
B
9 months
*
Number of sessions
20
15
10
5
0
WT
BACHD
C
14 months
*
Number of sessions
20
15
10
5
0
WT
BACHD
D
19 months
Number of sessions
20
15
10
5
0
WT
BACHD

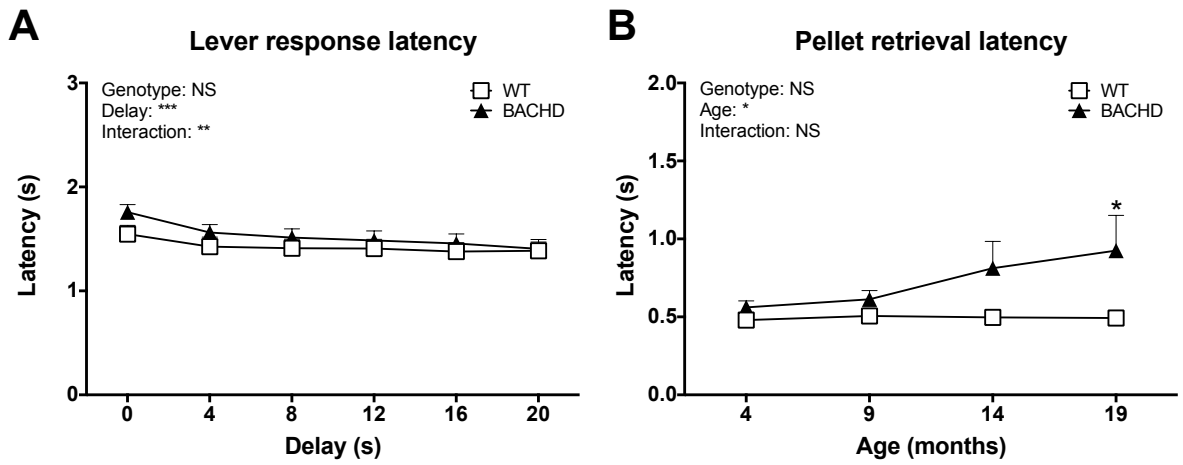
S1 Fig. Sessions required to progress through delayed alternation training
The graphs show the total number of sessions required for progressing through the series of delayed alternation protocols at the different test ages, with gradually increasing delay durations that were implemented before the training on the final delay set had started. The values were adjusted for the change in criterion that was made after the first test age. Rats, which did not reach criterion on each protocol, were excluded from the analysis. Plots indicate single values for individual rats. Note that the scale on the y-axis differs between the graphs. Results from *t*-test or Mann-Whitney U test are indicated in case significant genotype differences were present. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


Delayed alternation performance
Success per delay
A
9 months
Success rate (%)
Delay (s)
Genotype: **
Delay: ***
Interaction: NS
WT
BACHD
B
14 months
C
19 months

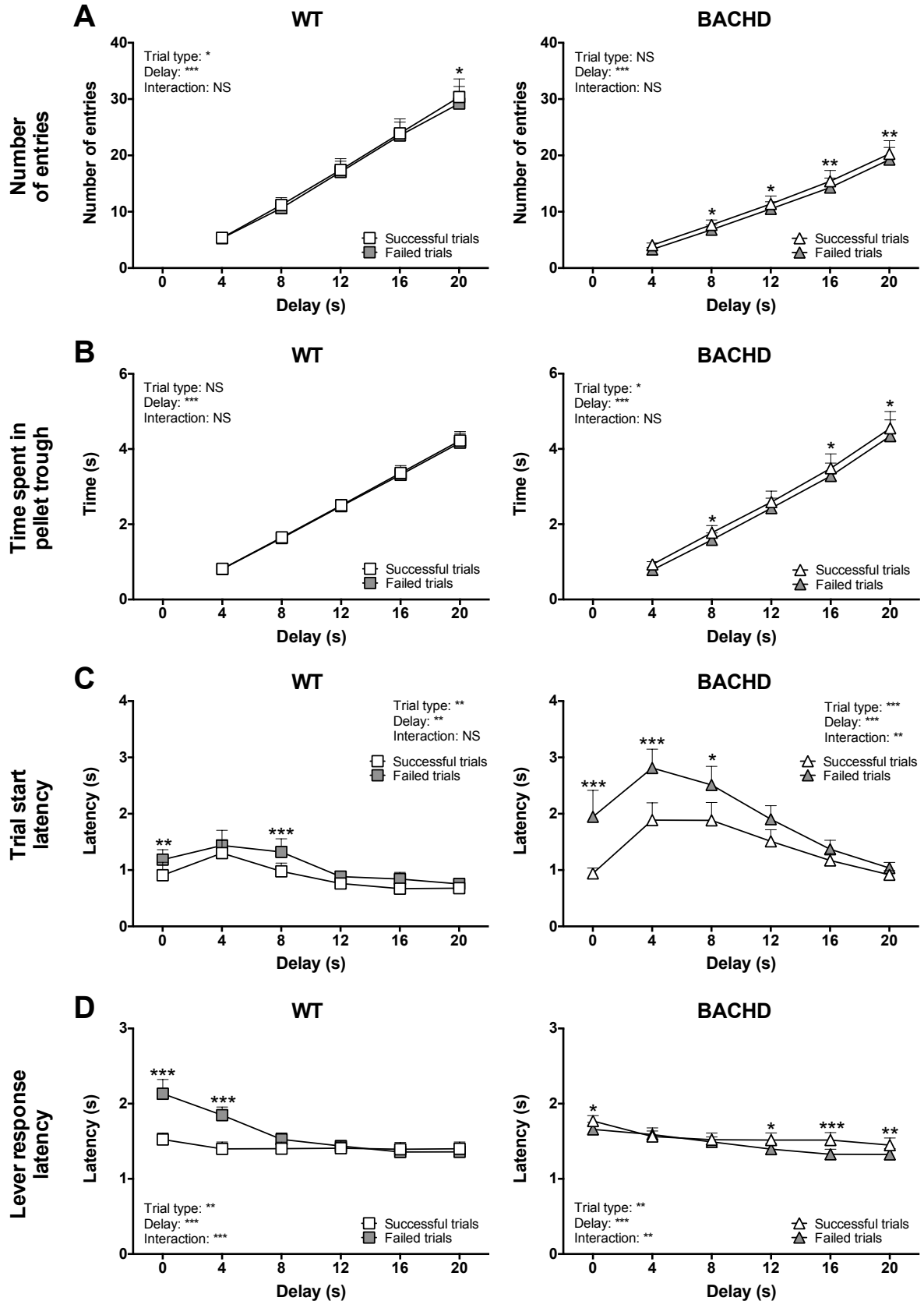
S2 Fig. Success rate per delay in the delayed alternation test during retesting
The graphs show the success rate on trial types with delays of different durations in the delayed alternation test. Each graph shows the stable baseline performance of rats maintained on the standard food restriction protocol. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. For (A), results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Trial start latency
4 months
Latency (s)
Delay (s)
Genotype: NS
Delay: ***
Interaction: NS
WT
BACHD
B
Trial start omissions
Number of omissions
Delay (s)
Genotype: NS
Delay: **
Interaction: NS
WT
BACHD
C
Retesting
Latency (s)
Delay (s)
Genotype: **
Delay: ***
Interaction: ***
WT
BACHD
D
Number of omissions
Delay (s)
Genotype: **
Delay: ***
Interaction: ***
WT
BACHD

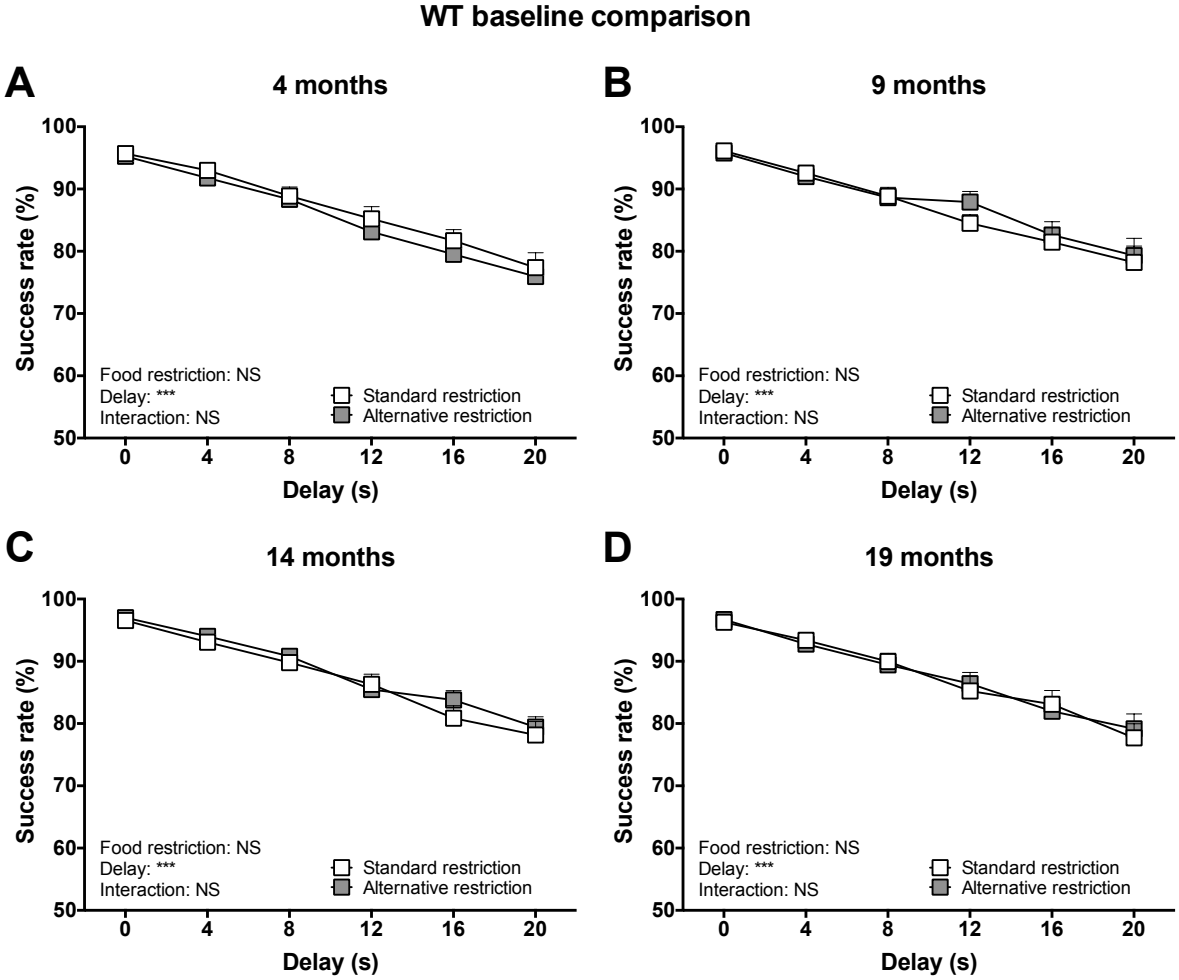
S3 Fig. Trial start latency and omissions during delayed alternation
The graphs show trial start latency and omissions during the delayed alternation protocol. (A) and (B) show the behavior at the four-month test age, while (C) and (D) show the mean performance at the three older ages. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Lever response latency
Genotype: NS
Delay: ***
Interaction: **
WT
BACHD
Latency (s)
Delay (s)
B
Pellet retrieval latency
Genotype: NS
Age: *
Interaction: NS
WT
BACHD
Latency (s)
Age (months)

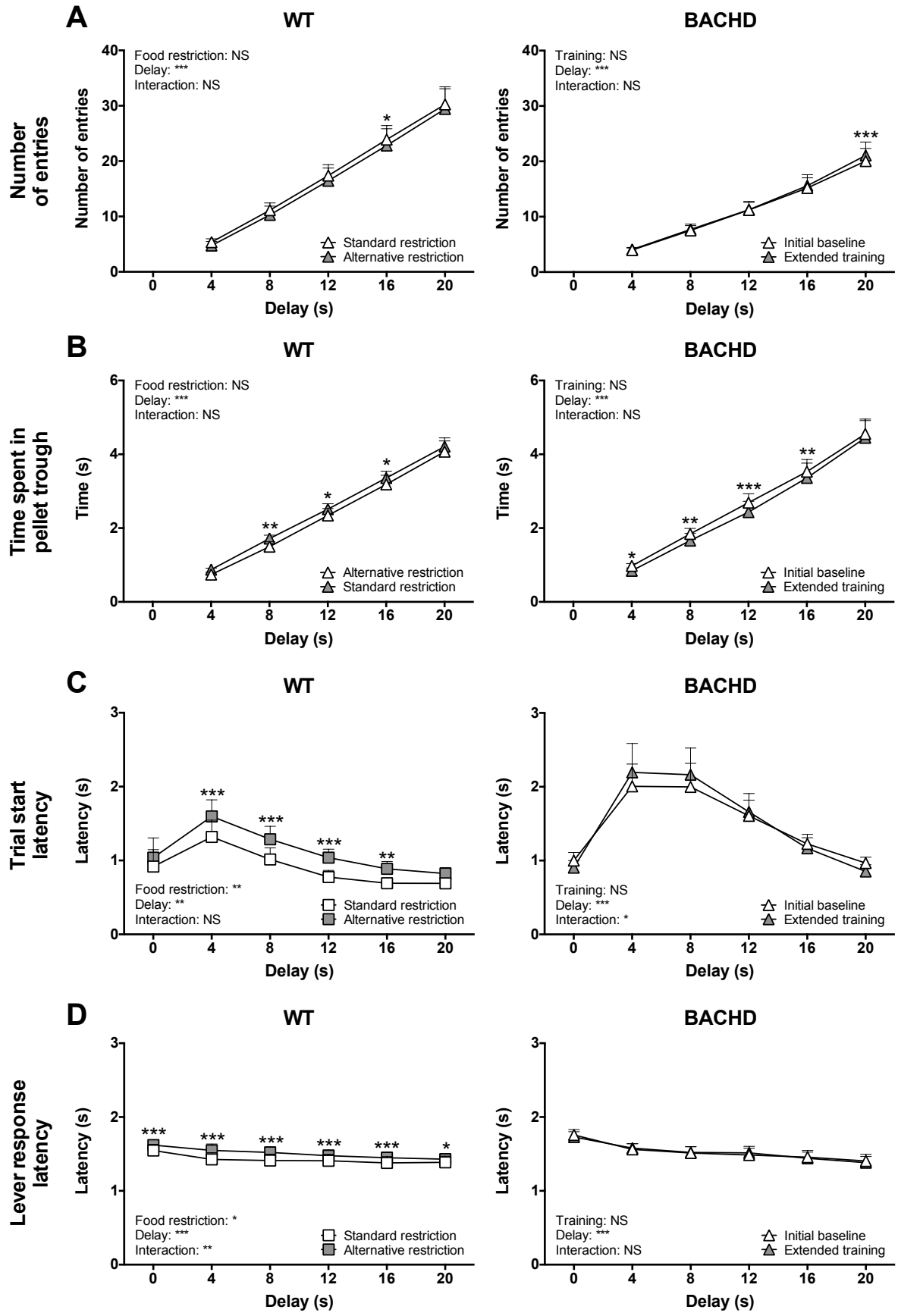
S4 Fig. Additional parameters of delayed alternation performance
The graphs show the last two parameters investigated for delayed alternation performance. (A) is based on the overall performance on all test ages, as no significant change with age was found for the parameter. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
WT
BACHD
Number of entries
Trial type: *
Delay: ***
Interaction: NS
Trial type: NS
Delay: ***
Interaction: NS
Successful trials
Failed trials
Delay (s)
B
Time spent in pellet trough
Time (s)
Trial type: NS
Delay: ***
Interaction: NS
Trial type: *
Delay: ***
Interaction: NS
C
Trial start latency
Latency (s)
Trial type: **
Delay: **
Interaction: NS
Trial type: ***
Delay: ***
Interaction: **
D
Lever response latency
Trial type: **
Delay: ***
Interaction: ***
Trial type: **
Delay: ***
Interaction: **

S5 Fig. Parameters indicating success or failure on delayed alternation
The graphs show some of the parameters of the delayed alternation protocol with performance of WT and BACHD rats separated for successful and failed trials. All graphs were constructed based on the mean performance over all test ages, as the relation to trial outcome did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

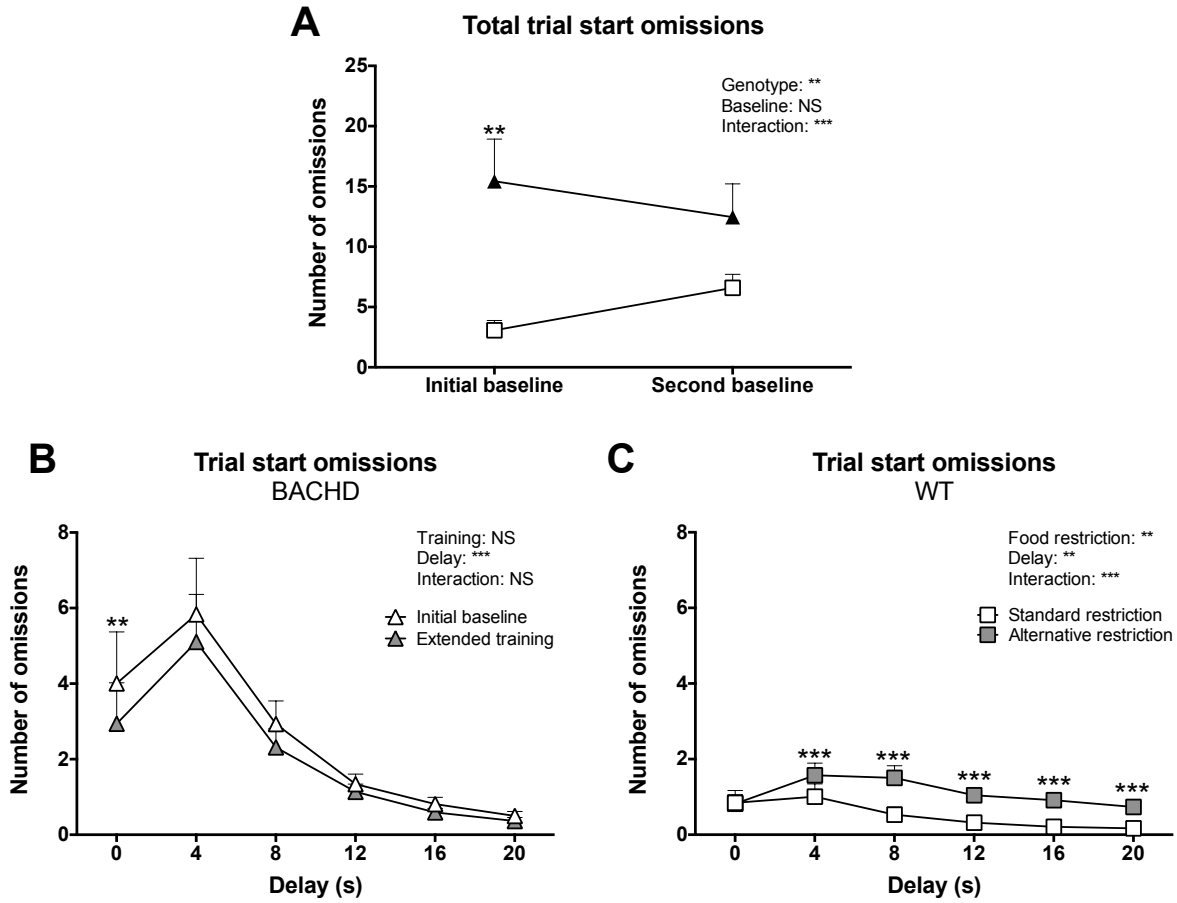

WT baseline comparison
A
4 months
B
9 months
C
14 months
D
19 months
Success rate (%)
Delay (s)
Food restriction: NS
Delay: ***
Interaction: NS
Standard restriction
Alternative restriction

S6 Fig. Effect of food restriction adjustment on success rate in delayed alternation test
The graphs show the WT rats' performance in the delayed alternation test during two different food restriction settings at the four investigated ages. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

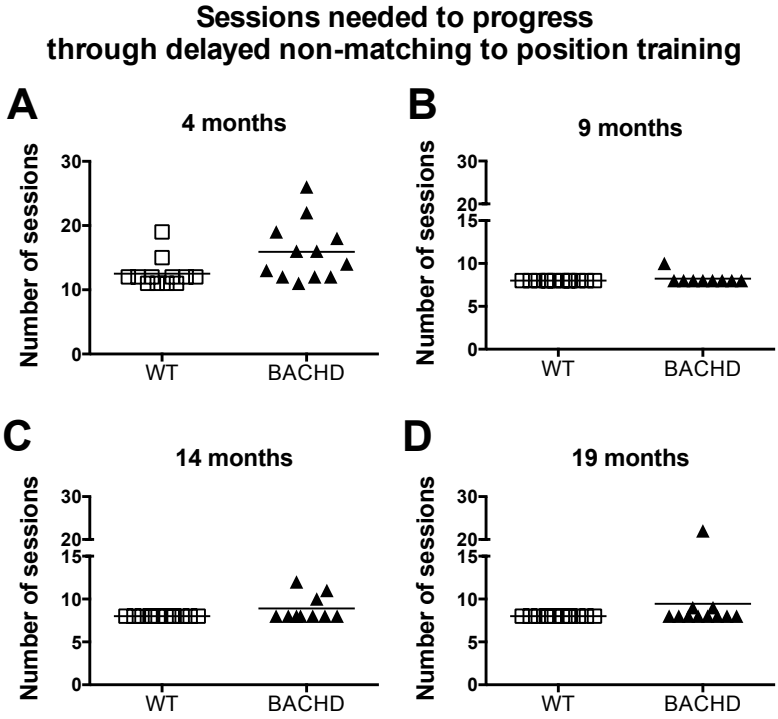

A
WT
BACHD
Number of entries
Number of entries
Food restriction: NS
Delay: ***
Interaction: NS
Training: NS
Delay: ***
Interaction: NS
Standard restriction
Alternative restriction
Initial baseline
Extended training
Delay (s)
B
Time spent in pellet trough
Time (s)
Food restriction: NS
Delay: ***
Interaction: NS
Training: NS
Delay: ***
Interaction: NS
C
Trial start latency
Latency (s)
Food restriction: **
Delay: **
Interaction: NS
Training: NS
Delay: ***
Interaction: *
D
Lever response latency
Latency (s)
Food restriction: *
Delay: ***
Interaction: **
Training: NS
Delay: ***
Interaction: NS

S7 Fig. Effect of food restriction adjustment and extended training on delayed alternation parameters

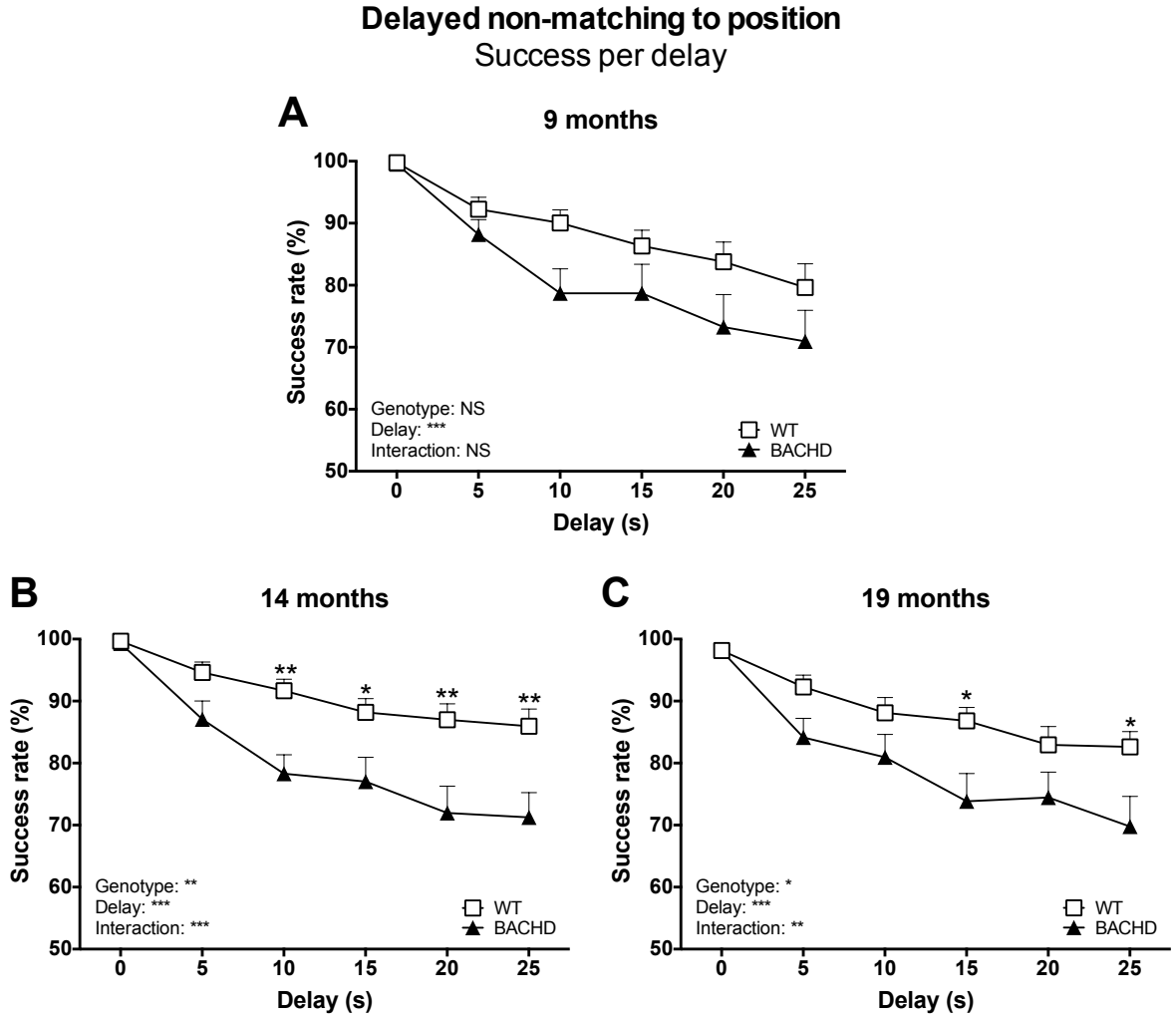
The graphs show some of the parameters of the delayed alternation protocol, comparing performance of WT and BACHD rats during their initial baseline with performance after changing food restriction protocol or given extended training, respectively. All graphs were constructed based on the mean performance over all test ages, as the effect of changing food restriction protocol or giving extended training did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant differences between baselines were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Total trial start omissions
Number of omissions
Genotype: **
Baseline: NS
Interaction: ***
**
Initial baseline
Second baseline
B
Trial start omissions
BACHD
Training: NS
Delay: ***
Interaction: NS
Initial baseline
Extended training
**
Number of omissions
Delay (s)
C
Trial start omissions
WT
Food restriction: **
Delay: **
Interaction: ***
Standard restriction
Alternative restriction
***
Number of omissions
Delay (s)

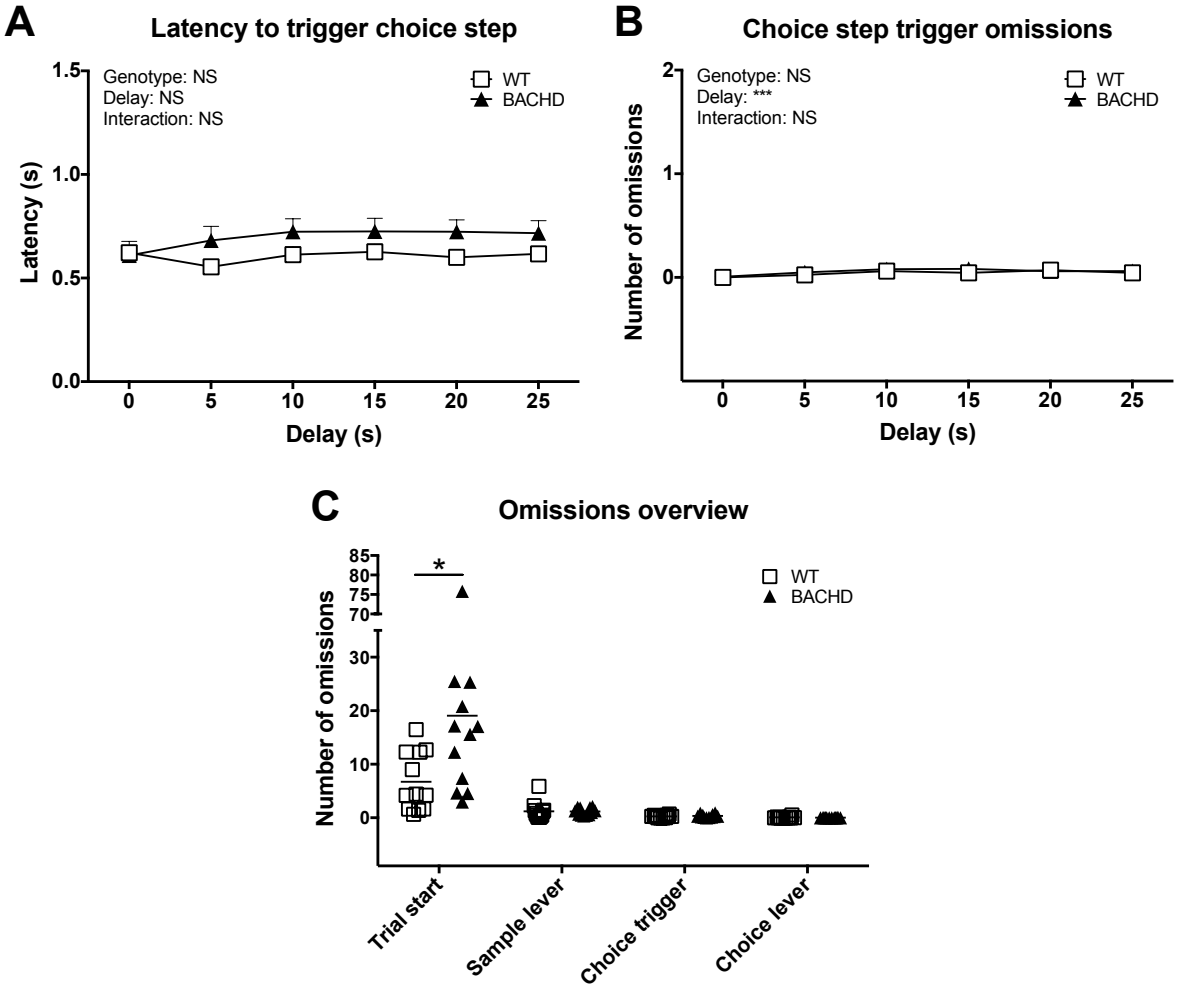
S8 Fig. Effect of food restriction adjustment on omissions during delayed alternation
The graphs show the effect of food restriction adjustment and extended training on the number of trial start omissions performed during the delayed alternation test. All graphs were constructed based on the mean performance over all test ages, as the effect of changing food restriction protocol or giving extended training did not noticeably change with age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant differences between baselines were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


Sessions needed to progress
through delayed non-matching to position training
A
4 months
B
9 months
C
14 months
D
19 months
Number of sessions
WT
BACHD

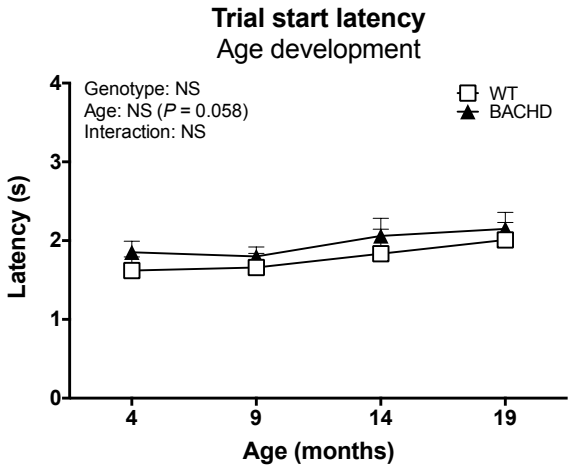
S9 Fig. Sessions required to progress through delayed non-matching to position training
The graphs show the total number of sessions required for progressing through the series of delayed non-matching to position protocols with gradually increasing delay durations, which were implemented before the training on the final delay set had started. The values were adjusted for the change in criterion that was made after the first test age. Rats that did not reach criterion on each protocol were excluded from the analysis. Plots indicate single values for individual rats. Note that the scale on the y-axis differs between (A) and the remaining graphs. Results from *t*-test or Mann-Whitney U test are indicated in case significant genotype differences were present. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


Delayed non-matching to position
Success per delay
A
9 months
Success rate (%)
Delay (s)
Genotype: NS
Delay: ***
Interaction: NS
WT
BACHD
B
14 months
Genotype: **
Delay: ***
Interaction: ***
C
19 months
Genotype: *
Delay: ***
Interaction: **

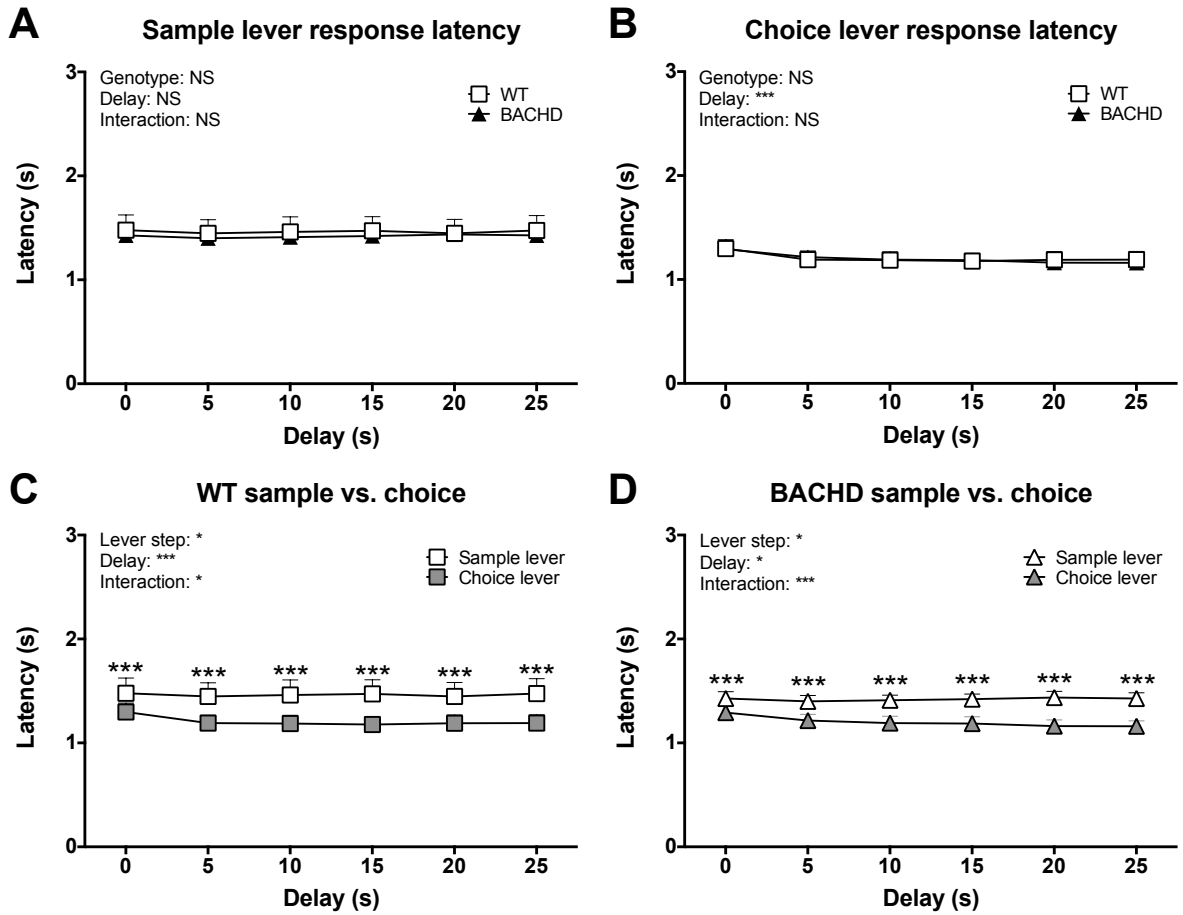
S10 Fig. Success rate per delay in the delayed non-matching to position test during retesting
The graphs show the age development of success rate on trial types with delays of different durations in the delayed non-matching test. Each graph shows the stable performance found when rats were maintained on the standard food restriction protocol. Curves display group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Latency to trigger choice step
Genotype: NS
Delay: NS
Interaction: NS
WT
BACHD
Latency (s)
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Delay (s)
B
Choice step trigger omissions
Genotype: NS
Delay: ***
Interaction: NS
WT
BACHD
Number of omissions
2
1
0
0
5
10
15
20
25
Delay (s)
C
Omissions overview
WT
BACHD
Number of omissions
85
80
75
70
30
20
10
0
*
Trial start
Sample lever
Choice trigger
Choice lever

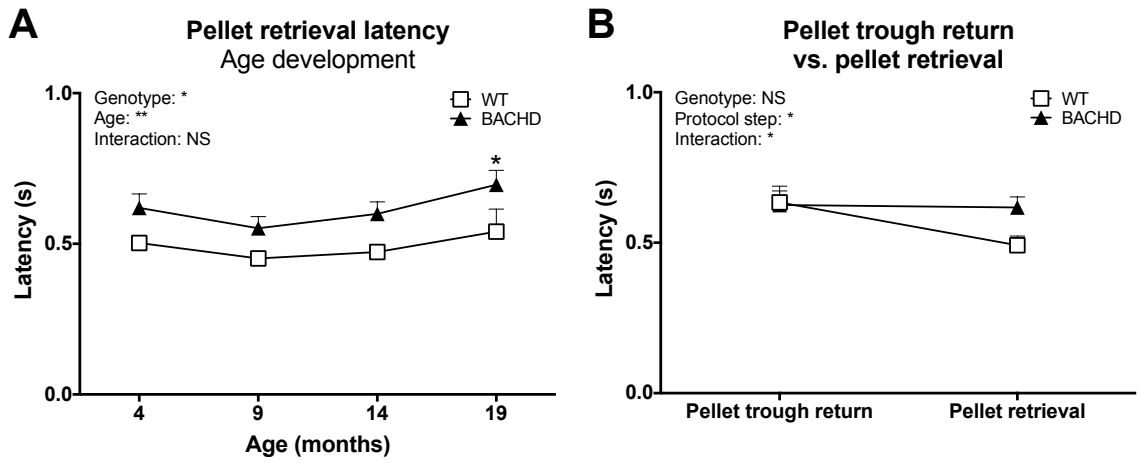
S11 Fig. Latency to trigger choice step and omissions for delayed non-matching to position
The graphs show the latency to initiate the choice step, related omissions and omissions overview during the delayed non-matching to position protocol. Graphs display the mean performance over all ages, as no significant differences in the rats' behavior at different ages was found. (A) and (B) indicate group mean plus standard error. (C) indicates the performance of individual rats. For (A) and (B), results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. For (C), results from *t*-test or Mann-Whitney U test are indicated in case the genotypes differed significantly. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


Trial start latency
Age development
Genotype: NS
Age: NS (P = 0.058)
Interaction: NS
WT
BACHD
Latency (s)
0
1
2
3
4
4
9
14
19
Age (months)

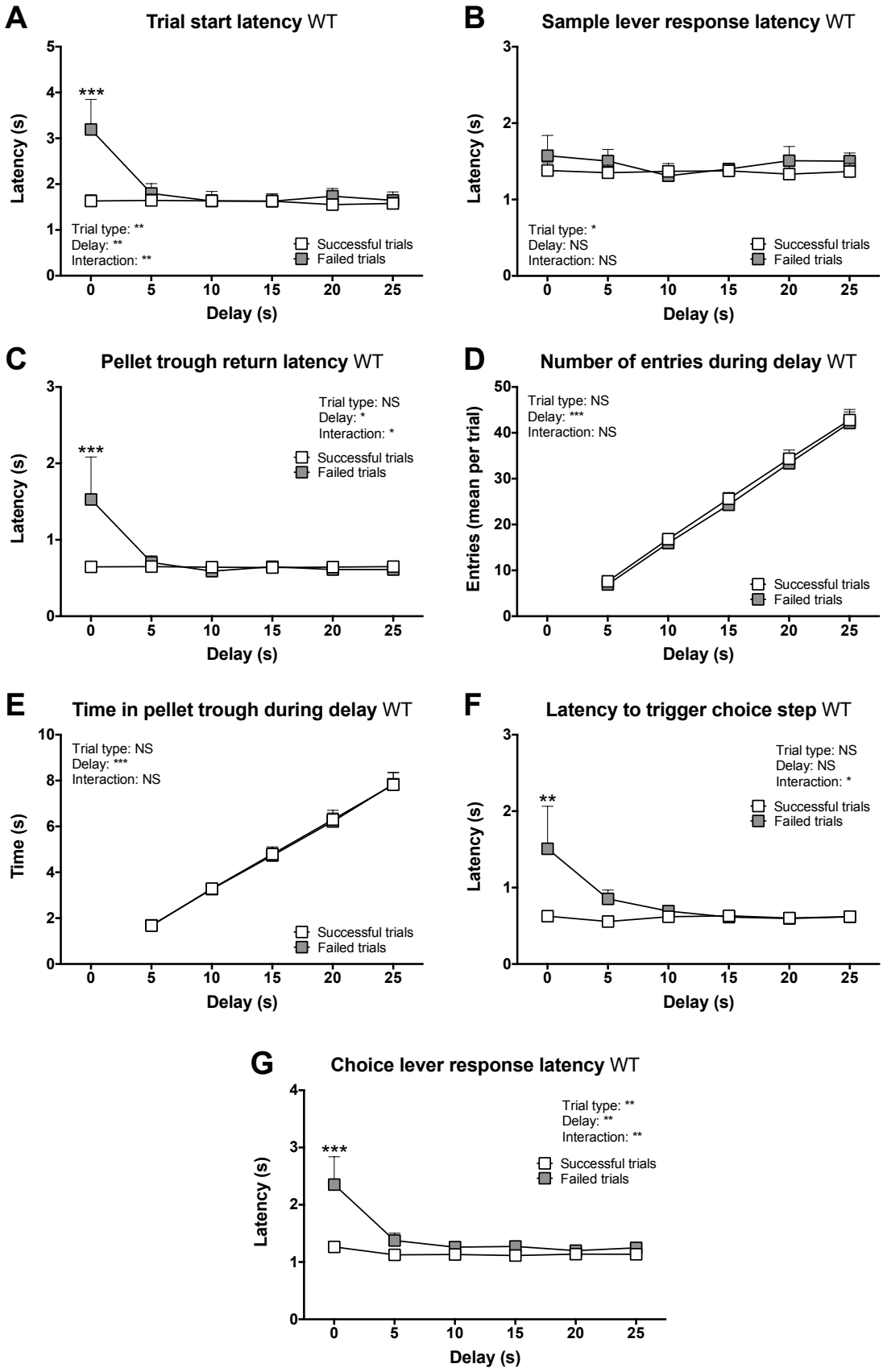
S12 Fig. Trial start latency in the delayed non-matching to position test
The graph shows the latency to initiate trials on the different test ages of the delayed non-matching to position protocol. The curve indicates group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graph, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Sample lever response latency
Genotype: NS
Delay: NS
Interaction: NS
WT
BACHD
Latency (s)
Delay (s)
B
Choice lever response latency
Genotype: NS
Delay: ***
Interaction: NS
WT
BACHD
Latency (s)
Delay (s)
C
WT sample vs. choice
Lever step: *
Delay: ***
Interaction: *
Sample lever
Choice lever
***
Latency (s)
Delay (s)
D
BACHD sample vs. choice
Lever step: *
Delay: *
Interaction: ***
Sample lever
Choice lever
***
Latency (s)
Delay (s)

S13 Fig. Lever response latencies during delayed non-matching to position
The graphs show the latencies to respond to a lever during either the sample step or the choice step of the delayed non-matching to position protocol. (A) and (B) display the comparison between WT and BACHD for both response latencies, while (C) and (D) display comparisons between the type of response latencies for both genotypes. Graphs display mean performance over all ages, as no significant differences in the rats' behavior at different ages was found. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated for data points where significant genotype differences were found. * ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$).

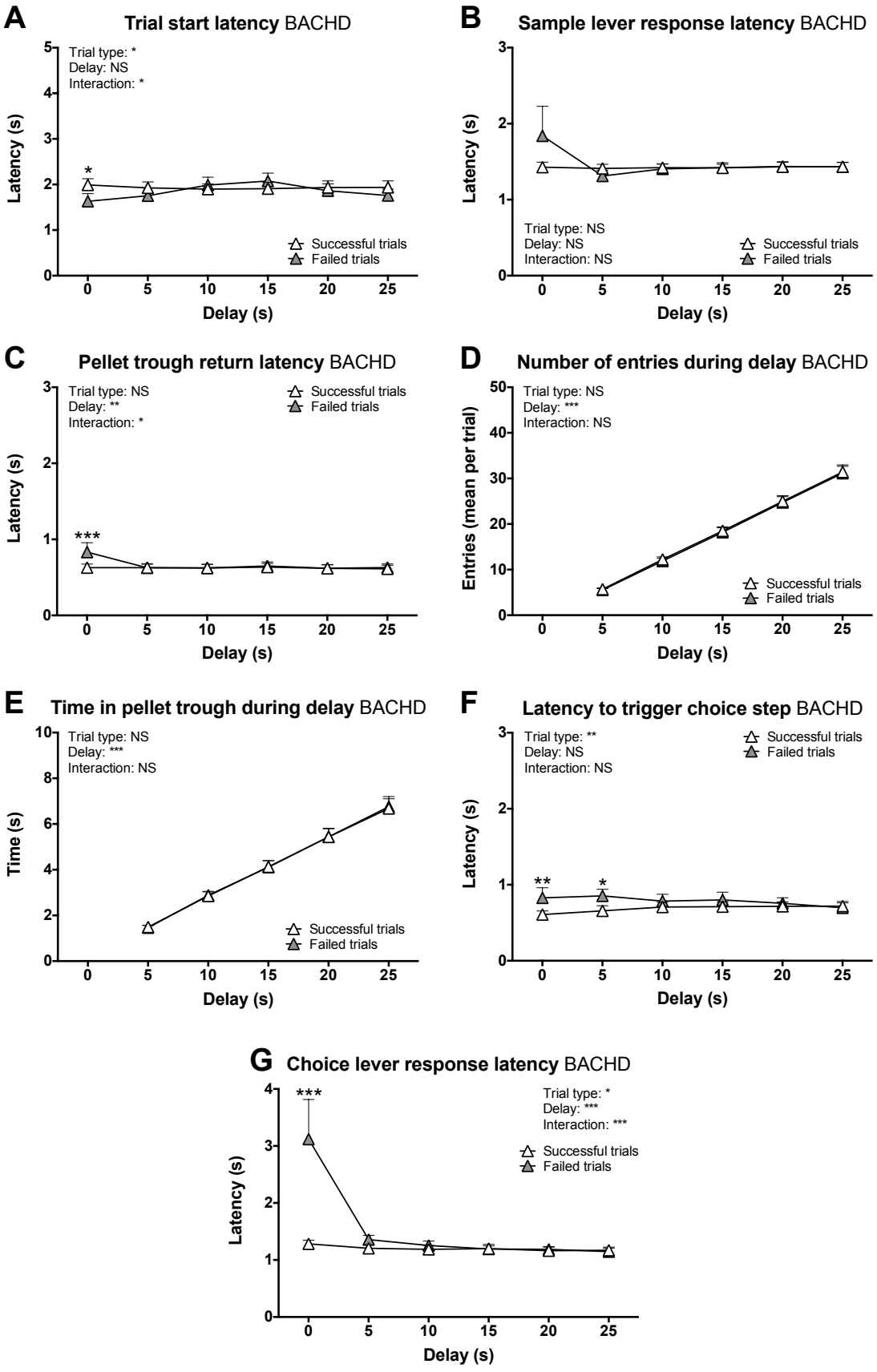

A
Pellet retrieval latency
Age development
Genotype: *
Age: **
Interaction: NS
WT
BACHD
Latency (s)
1.0
0.5
0.0
4
9
14
19
Age (months)
*
B
Pellet trough return
vs. pellet retrieval
Genotype: NS
Protocol step: *
Interaction: *
WT
BACHD
Latency (s)
1.0
0.5
0.0
Pellet trough return
Pellet retrieval

S14 Fig. Reward pellet retrieval latency during delayed non-matching to position
(A) shows the mean pellet retrieval latency of WT and BACHD rats during the delayed non-matching to position protocol at all investigated ages. (B) shows a comparison of the mean pellet retrieval latency with the mean latency to return to the pellet trough after pushing the sample lever. For this, the mean of all investigated ages and trial types were used, as the phenotypes or differences between latencies did not clearly change with age. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Trial start latency WT
Latency (s)
***
Trial type: **
Delay: **
Interaction: **
Successful trials
Failed trials
Delay (s)
B
Sample lever response latency WT
Latency (s)
Trial type: *
Delay: NS
Interaction: NS
Successful trials
Failed trials
Delay (s)
C
Pellet trough return latency WT
Latency (s)
***
Trial type: NS
Delay: *
Interaction: *
Successful trials
Failed trials
Delay (s)
D
Number of entries during delay WT
Entries (mean per trial)
Trial type: NS
Delay: ***
Interaction: NS
Successful trials
Failed trials
Delay (s)
E
Time in pellet trough during delay WT
Time (s)
Trial type: NS
Delay: ***
Interaction: NS
Successful trials
Failed trials
Delay (s)
F
Latency to trigger choice step WT
Latency (s)
**
Trial type: NS
Delay: NS
Interaction: *
Successful trials
Failed trials
Delay (s)
G
Choice lever response latency WT
Latency (s)
***
Trial type: **
Delay: **
Interaction: **
Successful trials
Failed trials
Delay (s)

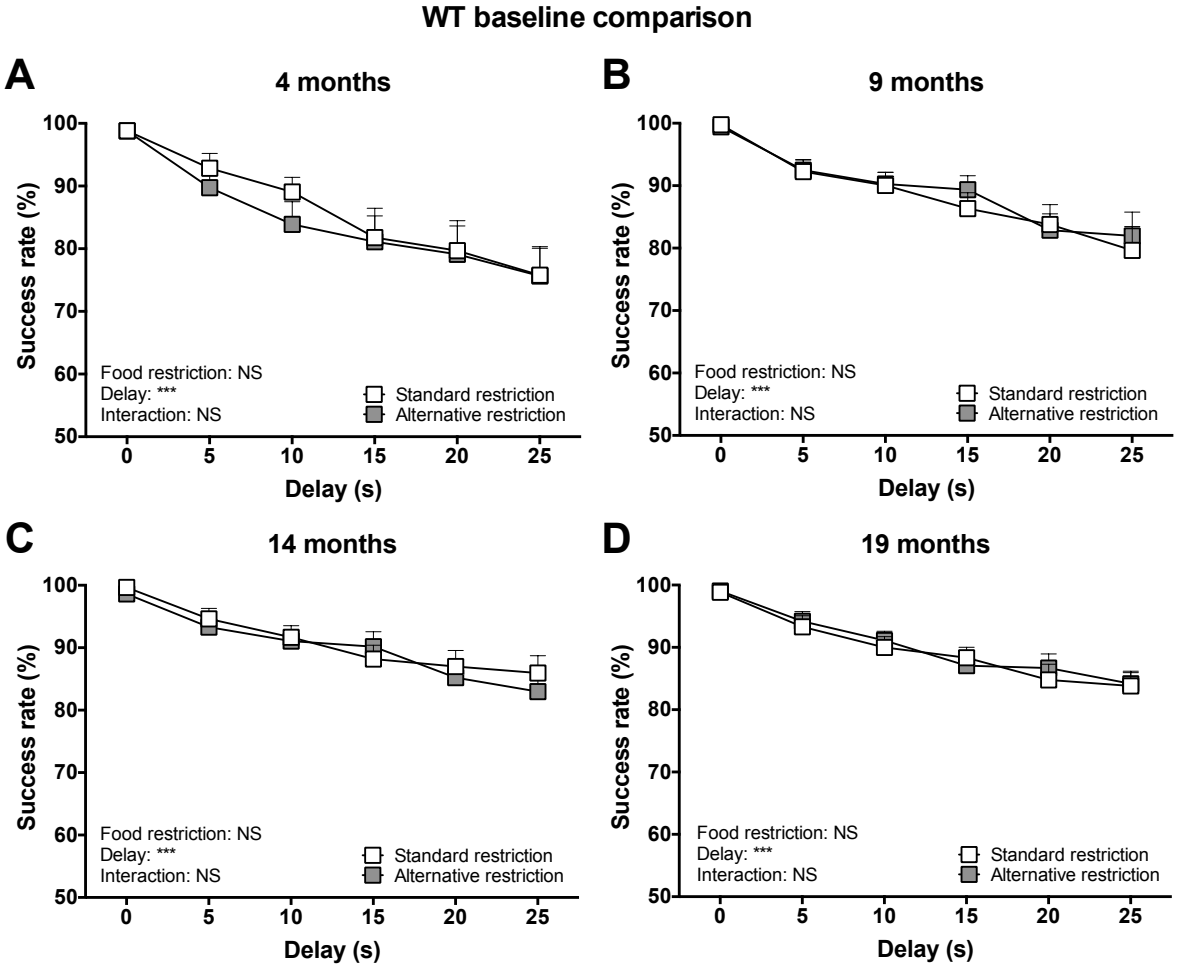
S15 Fig. Parameters indicating success or failure for WT rats in the delayed non-matching to position test

The graphs show some of the parameters of the delayed non-matching to position protocol performance of WT rats separated for successful and failed trials. All graphs were constructed using the mean performance over all ages, as the parameters' relation to trial outcome did not noticeably change between test ages. In addition, this was necessary to obtain data for failed 0-second delay trials for all rats. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

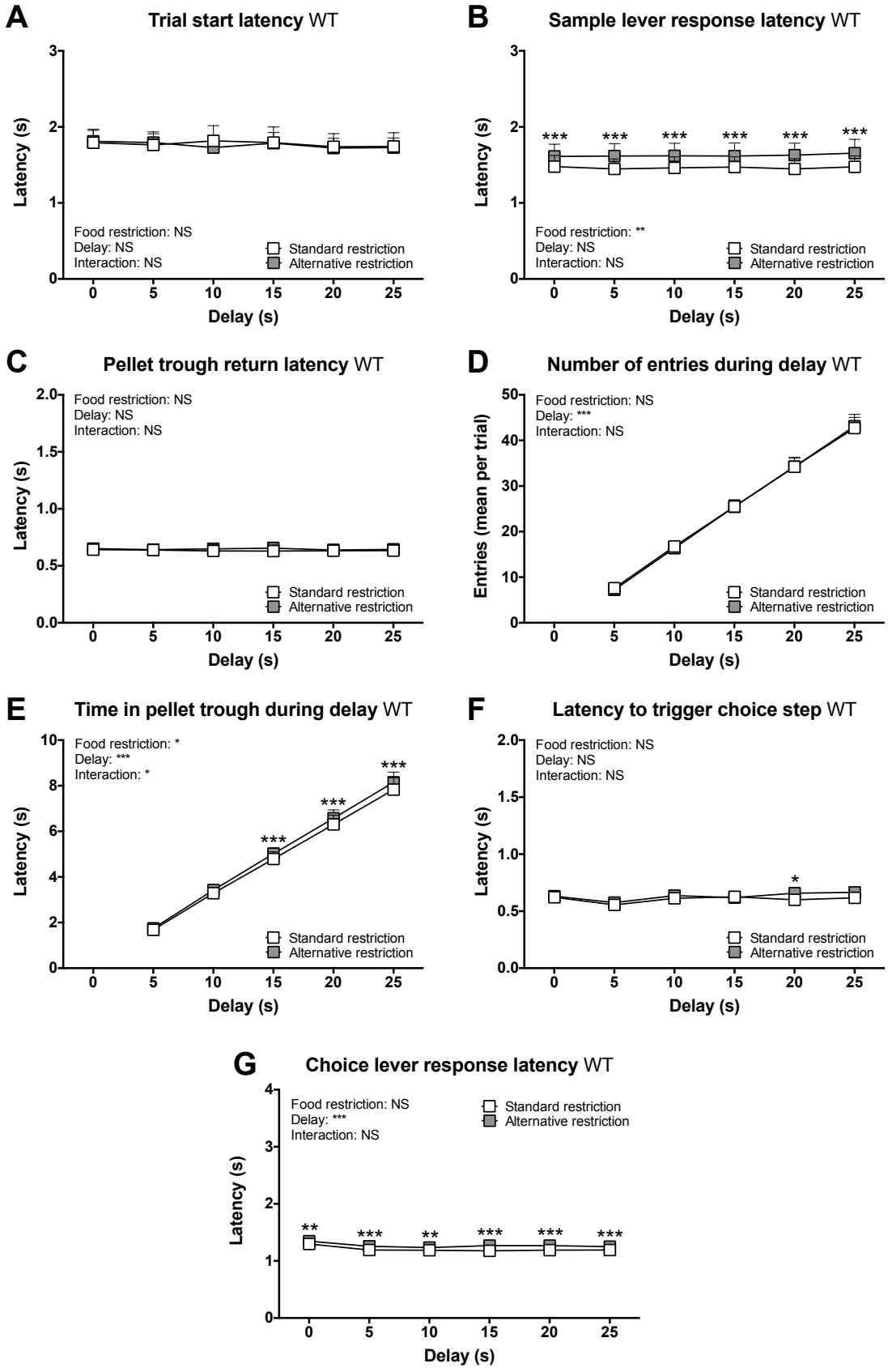

A
Trial start latency BACHD
Trial type: *
Delay: NS
Interaction: *
Latency (s)
Delay (s)
Successful trials
Failed trials
B
Sample lever response latency BACHD
Trial type: NS
Delay: NS
Interaction: NS
C
Pellet trough return latency BACHD
Trial type: NS
Delay: **
Interaction: *
D
Number of entries during delay BACHD
Trial type: NS
Delay: ***
Interaction: NS
Entries (mean per trial)
E
Time in pellet trough during delay BACHD
Trial type: NS
Delay: ***
Interaction: NS
Time (s)
F
Latency to trigger choice step BACHD
Trial type: **
Delay: NS
Interaction: NS
G
Choice lever response latency BACHD
Trial type: *
Delay: ***
Interaction: ***

S16 Fig. Parameters indicating success or failure for BACHD rats in the delayed non-matching to position test

The graphs show some of the parameters of the delayed non-matching to position protocol performance of BACHD rats separated for successful and failed trials. All graphs were constructed using the mean performance over all ages, as the parameters' relation to trial outcome did not noticeably change between test ages. In addition, this was necessary to obtain data for failed 0-second delay trials for all rats. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

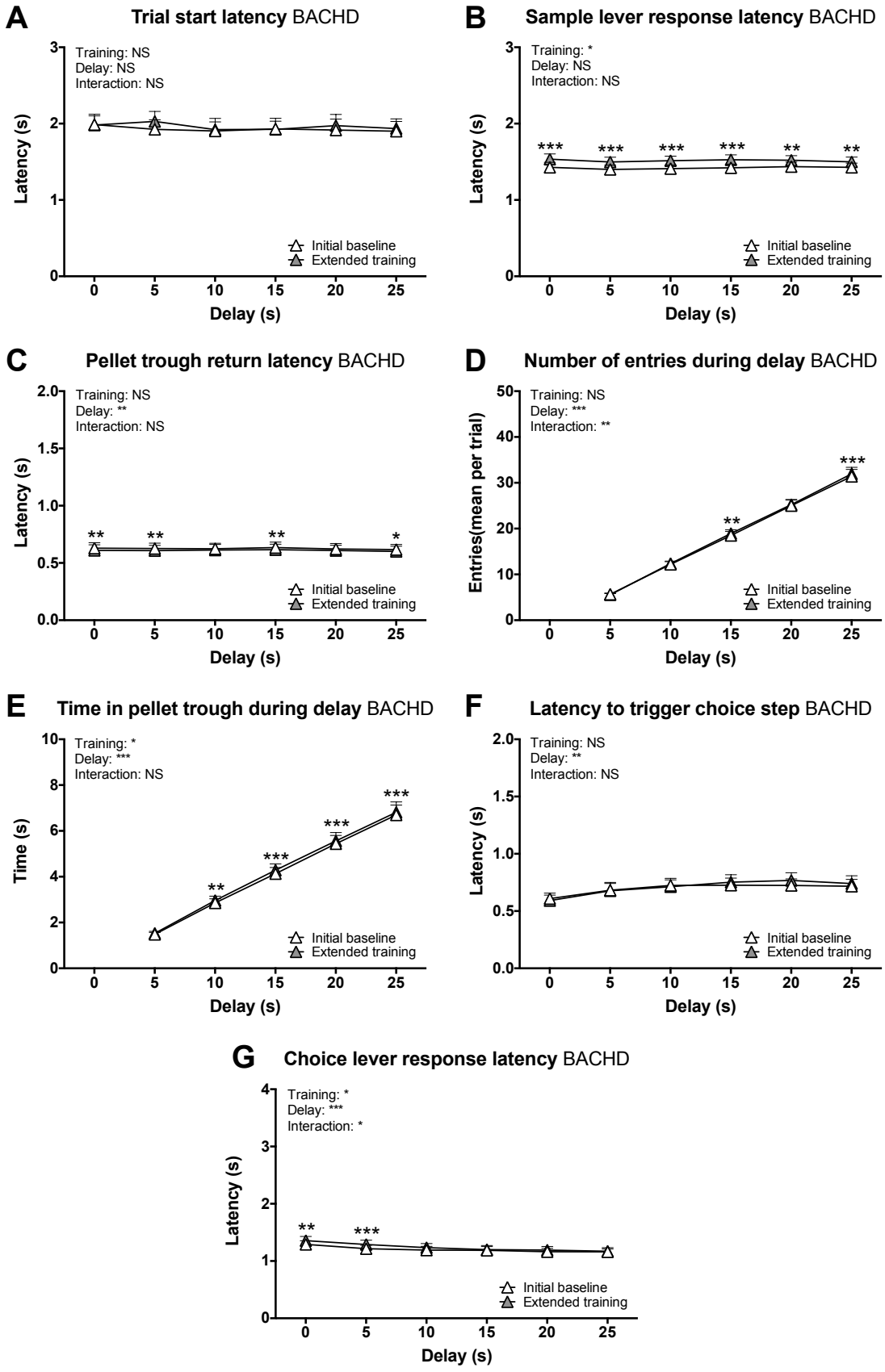

WT baseline comparison
A
4 months
B
9 months
C
14 months
D
19 months
Success rate (%)
Delay (s)
Food restriction: NS
Delay: ***
Interaction: NS
Standard restriction
Alternative restriction

S17 Fig. Effect of food restriction adjustment on success rate in delayed non-matching to position
The graphs show the WT rats' performance in the delayed non-matching to position test during two different food restriction settings at the four investigated age. Graphs indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs. Results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

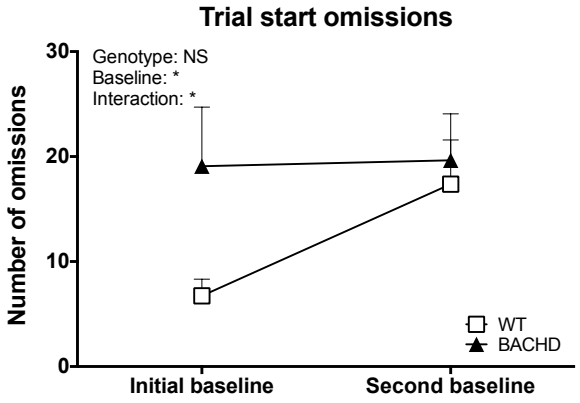

A
Trial start latency WT
Latency (s)
Food restriction: NS
Delay: NS
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)
B
Sample lever response latency WT
Latency (s)
Food restriction: **
Delay: NS
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)
C
Pellet trough return latency WT
Latency (s)
Food restriction: NS
Delay: NS
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)
D
Number of entries during delay WT
Entries (mean per trial)
Food restriction: NS
Delay: ***
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)
E
Time in pellet trough during delay WT
Latency (s)
Food restriction: *
Delay: ***
Interaction: *
Standard restriction
Alternative restriction
Delay (s)
F
Latency to trigger choice step WT
Latency (s)
Food restriction: NS
Delay: NS
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)
G
Choice lever response latency WT
Latency (s)
Food restriction: NS
Delay: ***
Interaction: NS
Standard restriction
Alternative restriction
Delay (s)

S18 Fig. Effect of food restriction adjustment of WT rats in the delayed non-matching to position test

The graphs show some of the parameters of the delayed non-matching to position protocol performance of WT rats separated for standard and alternative food restriction protocols. All graphs were constructed using the mean performance over all ages, as the parameters' relation to motivational state did not noticeably change between test ages. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


A
Trial start latency BACHD
Training: NS
Delay: NS
Interaction: NS
Latency (s)
Delay (s)
Initial baseline
Extended training
B
Sample lever response latency BACHD
Training: *
Delay: NS
Interaction: NS
C
Pellet trough return latency BACHD
Training: NS
Delay: **
Interaction: NS
D
Number of entries during delay BACHD
Training: NS
Delay: ***
Interaction: **
Entries(mean per trial)
E
Time in pellet trough during delay BACHD
Training: *
Delay: ***
Interaction: NS
Time (s)
F
Latency to trigger choice step BACHD
Training: NS
Delay: **
Interaction: NS
G
Choice lever response latency BACHD
Training: *
Delay: ***
Interaction: *

S19 Fig. Effect of extended training of BACHD rats in the delayed non-matching to position test
The graphs show some of the parameters of the delayed non-matching to position protocol performance of BACHD rats separated for the baselines after initial and extended training. All graphs were constructed using the mean performance over all ages, as the parameters' relation to the amount of training did not noticeably change between test ages. Curves indicate group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graphs, and results from post-hoc analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).


Trial start omissions
Genotype: NS
Baseline: *
Interaction: *
Number of omissions
30
20
10
0
Initial baseline
Second baseline
WT
BACHD

S20 Fig. Trial start omissions during different baselines of delayed non-matching to position
The graph shows the number of omissions during the initial baselines and after either a change in food restriction protocol or extended training on the delayed non-matching to position protocol. The graph was constructed using the mean performance over all ages, as the parameters' relation to food restriction or extended training did not noticeably change between test ages. The curve indicates group mean plus standard error. Results from two-way repeated measures ANOVA are shown inside the graph, and results from *post-hoc* analysis are indicated in case significant genotype differences were found. * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$).

Appendix I

Organ weights from Publication I

E K H Jansson, L E Clemens, O Riess, H P Nguyen

Introduction

Organ weights of male BACHD and WT rats were investigated as part of the body composition study presented in Publication I. The data was, however, not included in the publication in order to maintain focus on the overall body composition and motivational aspects of the animal model. An overview of the organ weights is thus presented in the current appendix.

Material and methods

A total of five animal groups were at different ages subjected to detailed dissection. The experimental details concerning breeding, housing and group selection are thoroughly explained in Publication I.

The dissection protocol included the following parameters: body weight, body length, head length, trunk length, tail length, weight of skin, weight of adipose tissue deposists, weight of internal organs and weight of remaining bone and muscle tissue. The weights of the following internal organs were investigated at all ages: brain, heart, lungs (combined weight), liver, kidneys (combined weight), gastrointestinal tract (including pancreas and omentum majus with attached adipose tissues) (GI tract), spleen and testicles. Thymus weights were investigated at one and three months of age. A detailed dissection of the GI tract was performed at 12 months of age. Through this, separate weights of the omentum majus with attached adipose tissue, GI tract content, actual GI tract tissues and pancreas were obtained. In addition, the weight and length of the rats' femur was investigated at 12 months.

Results

As noted, the results concerning the overall body composition phenotypes have been described and discussed in Publication I. The current appendix will focus on the weights of internal organs.

Most internal organs showed some indication of being lighter among BACHD rats, compared to WT rats (Figure I). This phenotype was strong for brain and kidneys, while being less pronounced for heart and lungs, and finally weak for liver, spleen and testicles. Thymus weight was unchanged on the investigated ages. In contrast to the other organs, the weight of the GI tract was generally increased among BACHD rats, compared to WT rats. Detailed investigation of the GI tract indicated that the omentum majus and attached adipose tissue was heavier in the BACHD rats, while the weights of GI tract content and tissues were unchanged (Figure 2). In addition, BACHD rats' pancreas was lighter than that of WT rats. Finally, the BACHD rats' femur was both shorter and lighter than that of the WT rats, although the weight per length quotient did not appear to be changed (Figure 3).

Additional analyses were performed to investigate if the apparent organ weights were proportionate to the BACHD rats' smaller body size. Thus, the weight of each organ was related to the body compartment it resided in (i.e. head length for brain weights and trunk length for all other organs). Results from this analysis indicated that the BACHD

rats' brain, heart, kidneys and testicles were disproportionally light relative to their body size (Figure 4).

Finally, it should be noted that the various differences found in organ weights did not appear to be caused by a gradual degeneration, but rather impaired growth.

Discussion

As discussed in Publication I, BACHD rats are generally smaller than WT rats. This overarching phenotype likely also explains the quite general phenotype of BACHD rats having lighter organs than WT rats. Still, specific growth deficits appear to be present for brain, heart, kidneys, pancreas and testicles, as their weights were disproportionally small compared to the BACHD rats' body size. Details concerning these phenotypes are further discussed in the main Results and discussion section of the current thesis. It should, however, be noted that specific functional analyses would be necessary to conclude whether the noted size differences are related to any organ dysfunctions. In addition, the relative weights calculated here are rather simplistic, and might not give a fair picture of the rats' physiology. More advanced calculations would be needed to better take the allometric growth of organs and tissues into account (see Shea BT, Hammer RE, Brinster RL. Growth allometry of the organs in giant transgenic mice. 1987. *Endocrinology*. 121:6:1924-1930 and Lindstedt SL, Schaeffer PJ. Use of allometry in predicting anatomical and physiological parameters of mammals. *Lab Anim.* 2002; 31:1:1-19 for further information on this topic).

As noted, the GI tract was the only organ that appeared to be heavier in BACHD rats, compared to WT rats. However, this phenotype appeared to be caused by an increased weight of the omentum majus, which contained a large amount of adipose tissue. Thus, the phenotype is in line with the overall obesity found in BACHD rats (see Publication I).

Finally, the results indicated that the BACHD rats' femurs were shorter and lighter than those of the WT rats. Importantly, the overall bone density was unchanged, suggesting that the disproportionately lower weight of bone/muscle tissues described in Publication I was primarily caused by a deficit in muscle growth. A general growth deficit of muscle tissues could also explain the disproportionately lower heart weights of the BACHD rats.

Conclusion

The BACHD rats show a general phenotype of having lighter organ weights compared to WT rats. This phenotype is likely connected to the BACHD rats' overall growth deficit, although additional investigations should be made regarding the rats' brain, muscle, kidney, pancreatic and testicular tissues, to evaluate the presence of organ specific dysfunctions.

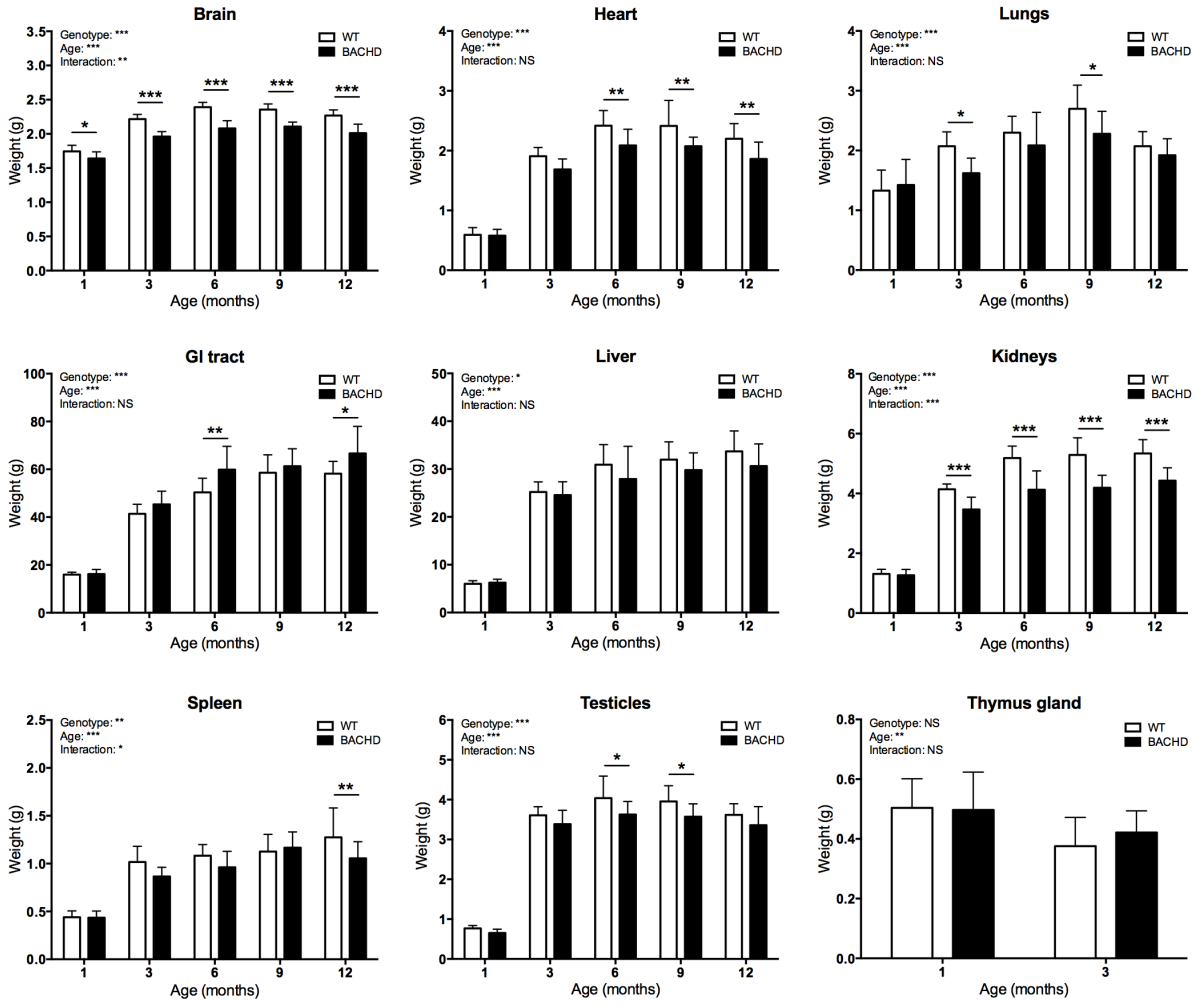


Figure 1. Organ weights in grams from animal groups described and discussed in Publication I. Group mean plus standard error is shown. Significant results from two-way ANOVA are indicated inside graphs. Data points where post-tests indicated a significant genotype effect are also noted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

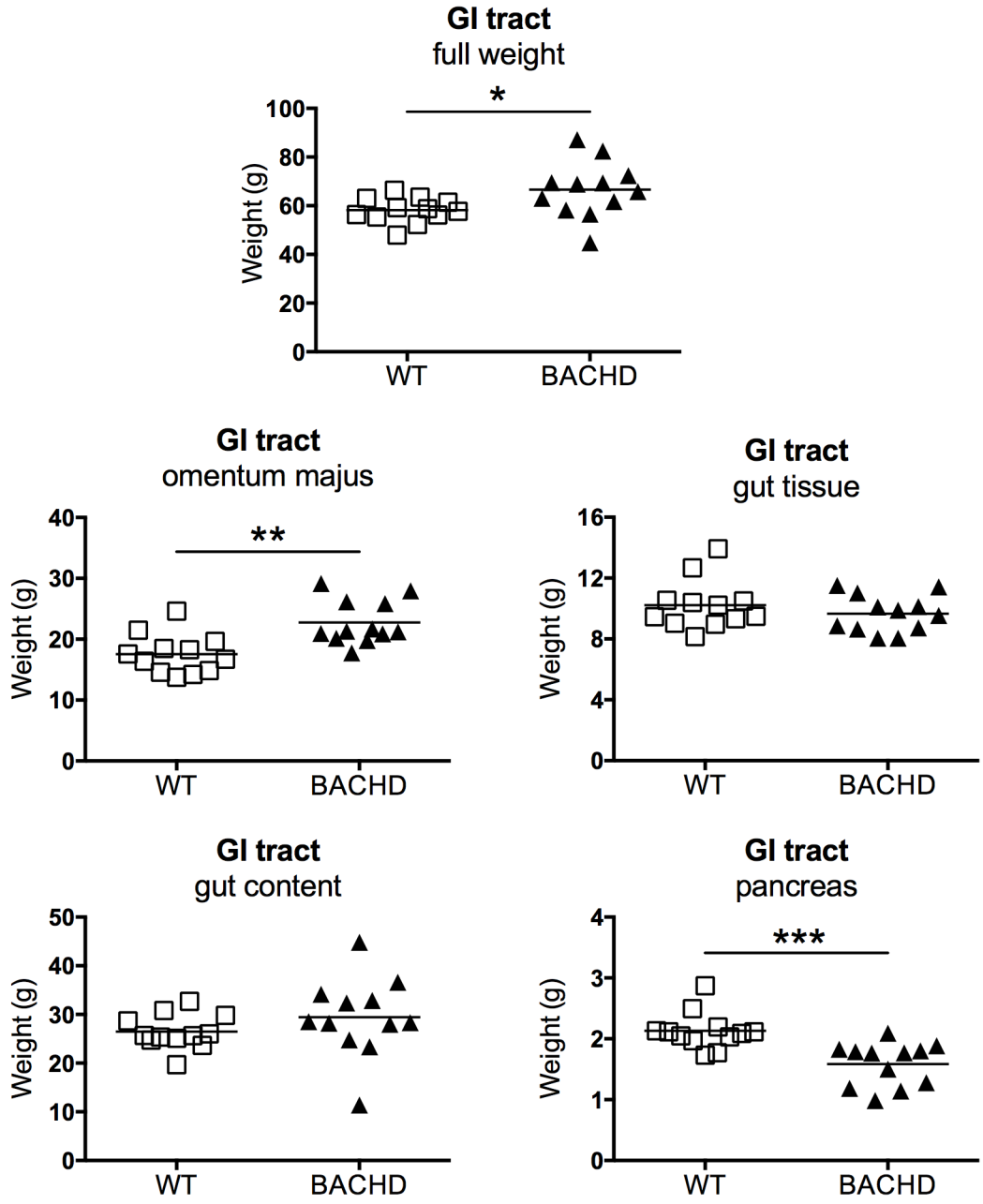


Figure 2. Detailed investigation of gastrointestinal tract components. Data was obtained from the 12 months old rats described and discussed in Publication I. Scatter plots indicate values from individual animals and group mean. Significant genotype differences obtained through Student t-test or Mann-Whitney U test are indicated in the figures (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

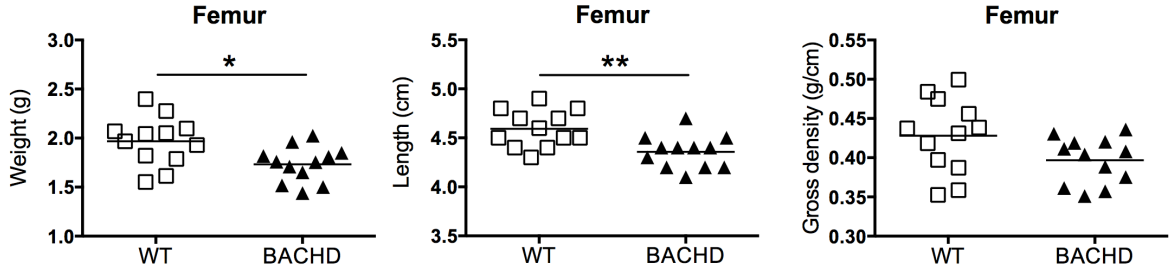


Figure 3. Specific investigation of femur parameters. Data was obtained from the 12 months old rats described and discussed in Publication I. Scatter plots indicate values from individual animals and group mean. Significant genotype differences obtained through Student t-test are indicated in the figures (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

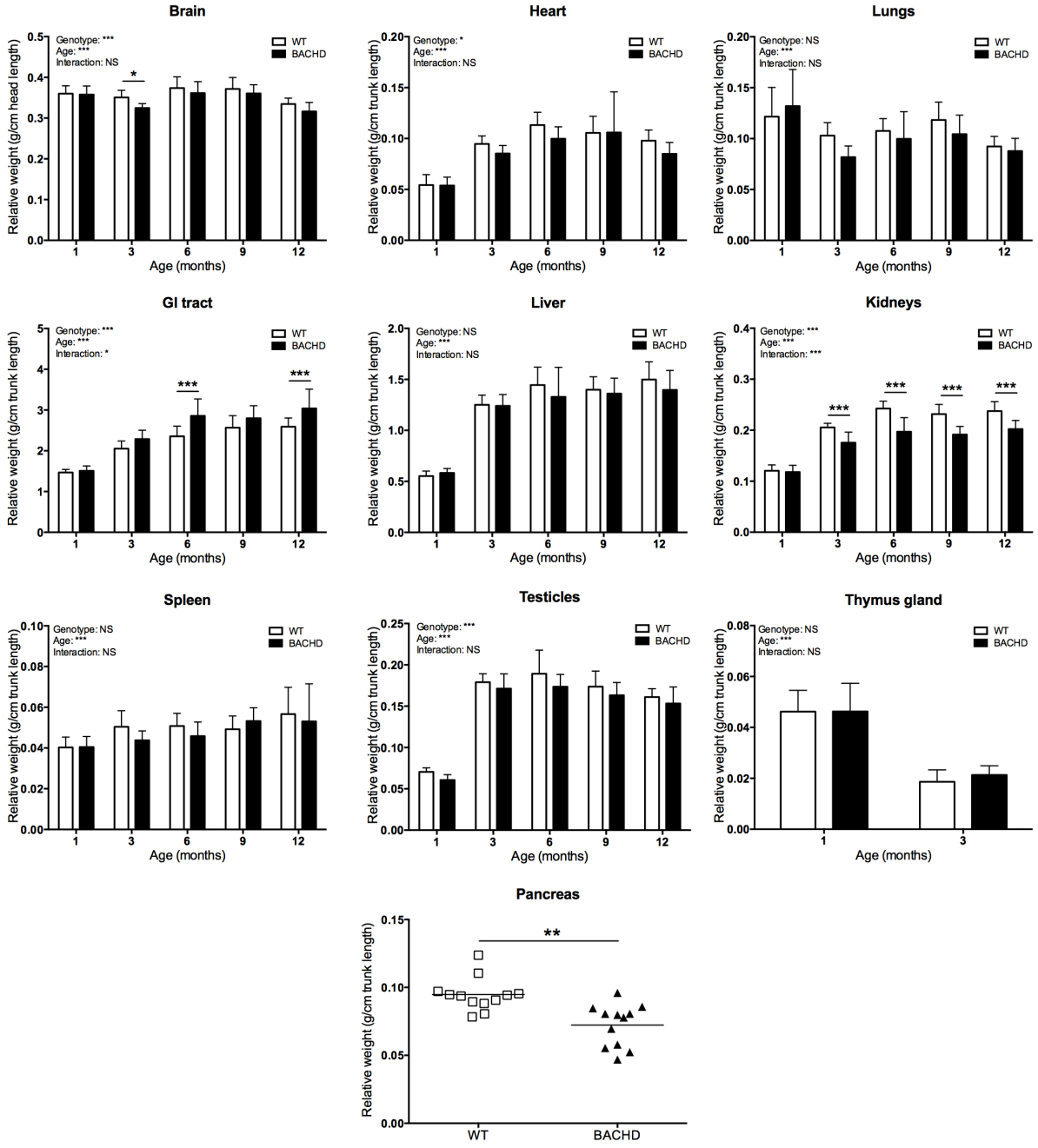


Figure 4. Organ weights in relation to size of the respective, related body compartment. Data was obtained from animal groups described and discussed in Publication I. For bar graphs, group mean plus standard error is shown. Significant results from two-way ANOVA are indicated inside graphs. Data points where post-tests indicated a significant genotype effect are also noted. For scatter plot, values from individual rats are indicated together with group mean. A significant genotype difference from Mann-Whitney U test is indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Appendix II

BACHD rats performance in a symmetrically reinforced Go/No-Go test

E K H Jansson, A Novati, L E Clemens, O Riess, H P Nguyen

Introduction

As part of a larger project to characterize the BACHD rats' performance in several inhibitory control tests they were assessed in a symmetrically reinforced Go/No-Go test. This constituted a well-structured study with control tests that are in line with the ideas presented in the current thesis.

Material and methods

Animals

A group of 24 male rats (12 hemizygous BACHD (TG5), 12 WT) were bred for the study. Housing was in line with what is presented in Publication I for the group used in the Progressive ratio test. Rats were maintained on food restriction during experiments, and given free access to food between tests. Food restriction during experiments used the alternative food restriction protocol as presented in the current thesis, where food consumption rates of WT and BACHD rats were matched to minimize motivational differences. Body weights were measured weekly between experiments to monitor general health, and daily during experiments to monitor food restriction levels.

Behavioral protocol

Rats were assessed in the Go/No-Go protocol at 2, 7, 12 and 17 months of age. The test was performed in a bank of six operant conditioning boxes, which are described in detail in Publication I. Four runs were required to assess all rats. Each run assessed three BACHD and three WT rats. Each rat was assigned to a specific conditioning chamber, although each conditioning chamber was used to assess two rats of each genotype. Training was performed during the early part of the light cycle's dark-phase, with the first run being performed roughly 30 minutes after dark-phase onset. Duration of training sessions varied as described below.

The behavioral protocol used a series of different training steps, which differed between the test ages. During the two-months test age, rats were first given two habituation sessions to the operant conditioning boxes. During these, both levers were retracted, the house light was switched on, and a total of 100 reward pellets were delivered to the pellet receptacle at intervals that were randomized between 10, 15, 20, 25, and 30 seconds. Afterwards, rats were trained to perform lever pushes on a continuous reinforcement (CRF) protocol. During this, one lever was inserted into the conditioning box, and the house light was switched on. Any lever push performed by the rats was rewarded with the delivery of a reward pellet. Rats were initially given rewards for approaching, sniffing and touching the lever, but eventually learned to reliably push it. Training continued until rats performed 100 pushes on their own within 30 minutes. When rats had reached this criterion, they were trained on a second CRF protocol, where the previously retracted lever was now available. As the previously inserted lever was now retracted, this protocol forced rats to also associate the second lever with a pellet reward. Rats were once again trained until they had performed 100 lever pushes on their own within 30 minutes. Once this had been achieved, they were run on a protocol that trained them to initiate discrete trials, and to reliably respond to both the left and right lever, when they were inserted. The sessions were composed of 100 trials,

which followed a similar pattern. The protocol started with an inter-trial interval (ITI), during which all lights were off, and both levers were retracted. Duration was randomized between 5, 7, 9, and 11 seconds. At the end of the ITI, the light in the pellet receptacle would start to shine. When the rats entered into the pellet receptacle, the light would be switched off. At the same time, both the house light and the two cue lights positioned above each lever would start to shine. In addition, one of the two levers would be inserted. The protocol was designed to that the left and right lever were presented an equal number of times in a pseudo-randomized order. At this point in training, the lever remained inserted until the rats responded to it. When rats performed a lever push, a reward pellet was delivered to the pellet receptacle and the cue lights above the levers were switched off. Retrieving the reward pellet resulted in the lever retracting, the house light being switched off, and a new ITI being started. Rats were trained on this protocol until they completed 100 trials within 30 minutes. When rats had reached this criterion, time limits were added to the protocol. Thus, the rats had to respond to the initial light signal in the pellet receptacle as wells as the inserted levers within ten seconds. If no response was made, the trials were omitted. This was trained until the rat made less than five lever response omissions during a full session. This protocol was also used as the first protocol trained when rats were reassessed at older ages. In later sections of the current appendix it will be referred to as a protocol with a series of Go trials. Once rats had learned to perform reliably on this protocol they were trained on a series of symmetrically reinforced Go/No-Go protocols (Figure 1). The aim of these protocols was to train the rats to recognize and discriminate two light cues, which signaled that they had to either perform or withhold a lever response in order to be rewarded with a food pellet. Each session was composed of 100 trials, with equal numbers of Go and No-Go trials presented in a pseudo-randomized order. For both Go and No-Go trials, the left and right lever was presented an equal number of times in a pseudo-randomized order. Each trial followed a series of similar steps. The protocol started with a ten-second ITI, where both levers were retracted, and all lights were off. At the end of the ITI, the light in the pellet receptacle would start to shine. When the rats entered the pellet receptacle, the light was switched off and one of the two light cues was presented. For Go trials, the two cue lights above the levers shone together with the house light. For No-Go trials, the house light shone alone. Both light cues shone for a total of five seconds. Afterwards, one lever was inserted and the rats had to either respond to it (on Go trials) or withhold a lever response (on No-Go trials). Successful performance was rewarded with a food pellet. During the initial protocol, Go trials were set to be six seconds long, while No-Go trials were only two seconds long. This protocol was trained until the rat showed above 80% success rate on both Go and No-Go trials on three consecutive sessions. When rats had reached this criterion, they were trained on a protocol where the No-Go trial duration had been increased to four seconds. This was trained until rats showed above 80% success rate on both Go and No-Go trials on a single session. Finally, rats were trained on a protocol where both Go and No-Go trials were six seconds long. Performance criterion was once again set to rats showing 80% success rate on both Go and No-Go trials on three consecutive sessions. At the end of this, rats had thus reliably learned to discriminate the light cues and respond

accordingly. To further evaluate their ability to withhold lever responses they were given a single test session where the duration of the No-Go trials varied in a pseudo-randomized manner between 6, 10, 14, 18, and 22 seconds.

During the final test age, the rats were given extensive training on the protocol with varied No-Go duration, to better evaluate their ability to learn to withhold lever responses. In addition, the rats' motivation to perform lever responses for food rewards was assessed in a progressive ratio test. This test protocol is described in detail in Publication I.

Behavioral parameters

Several different behavioral parameters were analyzed from the various protocols. The number of sessions needed to reach criterion on the different protocols was used as the main parameter of learning. Due to the difference in criterion (i.e. three consecutive criterion sessions or only one) the number of sessions to criterion was calculated from the first training session to the first criterion session. Additional parameters were analyzed from sessions were rats performed at criterion level, during the final Go/No-Go protocol (i.e. where both trial types were limited to six seconds). These parameters included the mean success rate on both trial types, the latency to initiate trials (i.e. latency to respond to the pellet receptacle light), the number of entries performed during cue presentation, the latency to perform a lever response on Go trials and the latency to retrieve the reward pellet on Go trials. For the protocol where the duration of No-Go trials was varied, separate success rates were calculated for each trial type. In addition, the latency to perform a lever push was analyzed for both successful Go trials, and failed No-Go trials.

Statistical methods

Behavioral data was extracted by running the log files created by the system through a series of R scripts. Statistical analysis was performed in GraphPad prism (v. 6.0) Analyses were performed with two-way repeated measures ANOVAs, using genotype as between subject factor and either age or trial type as within subject factor. Sidak's post-test was used to evaluate genotype differences on individual data points. Some BACHD rats did not manage to reach the performance criterion on the final Go/No-Go protocol (where both Go and No-Go trials were limited to six seconds). These rats were excluded from analyses of sessions to criterion and criterion session performance. The rats were still assessed on the protocol where No-Go trials varied in duration, and were included in the analysis of this step. This was done so that a comprehensive analysis of the BACHD rats' performance could still be obtained. A total of three WT rats became ill during the experiment, and were sacrificed before the final test age. Data from these rats was excluded from longitudinal analyses. Lever response latencies during the protocol with varied No-Go trial duration had occasional gaps, as rats happened to show perfect success on some subtypes of No-Go trials. The exact n for analyses is noted below. The extended training given on the protocol with varied No-Go durations consisted of an additional 24 sessions. Analysis of this step included the initial test session, giving a total of 25 sessions, which were analyzed in five blocks of five sessions each. The progressive ratio training consisted of 12 sessions, the first three of which were excluded to construct the stable performance baseline.

Results

Although there were some discreet performance differences between BACHD and WT rats during the initial habituation and lever training protocols these will not be discussed here. The noted phenotypes have not been consistently seen across experiments and are likely of little importance. Still, it should be noted that both WT and BACHD rats learned to reliably respond to the levers, and with few exceptions required more than one or two sessions to reach criterion on each initial protocol.

During the Go/No-Go training, BACHD rats were found to reliably require more training sessions before reaching criterion on the first protocol (i.e. when Go trials were 6 seconds long and No-Go trials were 2 seconds long), but not the protocols that followed (Figure 2). As noted, three BACHD rats did not reliably reach criterion during the final protocol, despite being given extensive training. One rat failed to reach criterion during bot the 12 and 17-month test age. One rat failed to reach criterion during the 17-month test age. The last rat failed to reach criterion during the 12-month test age, but managed during the 17-month test age. Regardless, all rats were excluded from the analysis shown in Figure 2, giving an n of 12 for WT and 10 for BACHD rats.

Among the rats that did reach criterion, there was no difference in overall success rates between WT and BACHD rats for either trial type (Figure 3). Still, BACHD rats were found to be slightly slower at initiating trials, responding to the inserted lever during Go trials, and retrieving the reward pellets. Although the rats' specific performance changed with age, the noted phenotypes remained arguably stable. It is interesting to note that the slowed trial start and lever response among BACHD rats was not seen during the protocol that only contained Go trials (i.e. the protocol trained before introducing two-second long No-Go trials) (Figure 4) (data from 7 months of age was excluded, as not all rats received the training on that occasion).

When the duration of No-Go trials was extended and randomized, both BACHD and WT rats showed a drop in success rate with longer No-Go durations (Figure 5). No difference was found between genotypes regarding their overall success rate, or the latency to respond to the inserted lever. As noted, some rats had to be excluded from the analysis of response latencies due to missing data values. Thus, the number of included animals at a given age varied between 5-9 and 11-10 for WT and BACHD rats respectively.

When rats were given extended training on the protocol with varied No-Go durations, there were no apparent differences in the WT and BACHD rats' abilities to learn to withhold responses (Figure 6). The progressive ratio test also indicated that WT and BACHD rats were equally motivated to perform lever pushes for a food reward (Figure 7).

Discussion

As noted, the BACHD rats showed consistent difficulties with initially learning to withhold lever responses, as indicated by the significantly higher number of sessions required to reach criterion on the Go/No-Go protocol that used two-second long No-Go trials. However, once they had learned this initial response withholding they showed no deficit in withholding responses for longer durations of time. This was evident in several steps of the test, including the sessions to criterion for the other Go/No-Go protocols (i.e. with four- and six-second long No-Go trials), their performance on the protocols with

varied No-Go durations, as well as their gradual improvement in that protocol through extended training.

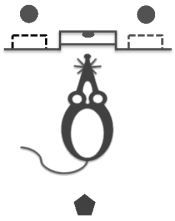
BACHD rats also showed slowed trial starts, lever response latencies, and pellet retrieval latencies. Interestingly, these phenotypes appeared to be unique to the Go/No-Go protocol, as they were not present in the earlier protocols that presented the rats with only Go trials. Thus, the phenotypes are unlikely to be related to motoric impairments, but could rather be due to slight differences in motivation and/or attention (trial start and pellet retrieval) and cognitive processing speed (lever response latency).

As the current test did not rely on establishing a stable baseline (such as the progressive ratio, delayed alternation and delayed non-matching to position protocols), the control tests used in Publication III were not suitable for evaluating the Go/No-Go readouts' dependency on motivational factors. Instead, we sought to establish food restriction levels that were likely to result in comparable motivation. To evaluate if this had worked, we assessed the rats' performance in a progressive ratio test at the end of the study. As noted, WT and BACHD rats showed similar motivation to perform lever pushes for a food reward, and it is thus unlikely that the phenotypes noted above were due to motivational differences between WT and BACHD rats.

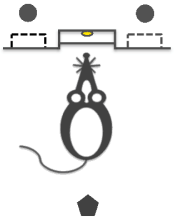
Although the slowed learning to withhold lever responses seen among BACHD rats could be due to a discreet inhibitory control deficit, one has to consider the possibility that the BACHD rats might have impaired attention, visual abilities or general learning impairments. Further behavioral characterization is needed to reach conclusions on this matter.

Conclusion

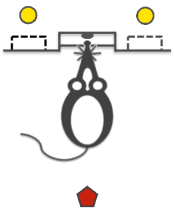
BACHD rats show a consistent, slight impairment when learning to withhold lever responses in the current Go/No-Go protocol. This could indicate a discreet impairment in response inhibition control, although other cognitive and visual aspects should also be considered. Regardless, the impairment is not strong enough to result in an overall impaired response inhibition among BACHD rats.

1. 

Inter-trial interval
Levers are retracted and all lights are switched off for 10 seconds.

2. 

Start of trial
The light in the pellet receptacle starts to shine.

3a. 

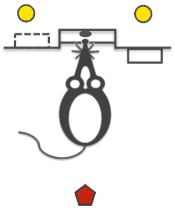
Go trial, cue presentation
When the rat responds to the receptacle cue light with a nosepoke, the cue light switches off. The lights that signal a Go trial (both lever cue lights plus the house light) shine for 5 seconds.

The protocol presents an equal number of Go and No-Go trials in a pseudo-randomized fashion.

3b. 

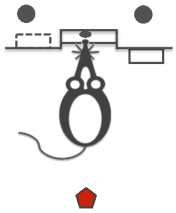
No-Go trial, cue presentation
When the rat responds to the receptacle cue light, the cue light switches off. The light that signal a No-Go trial (the house light) shines for 5 seconds.

The protocol presents an equal number of Go and No-Go trials in a pseudo-randomized fashion.

4a. 

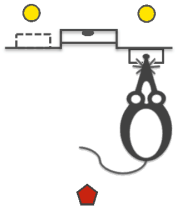
Go trial, lever insertion
After presenting the light cue for 5 seconds, one lever is inserted. The lever remains inserted for 6 seconds and the light cues continue to be presented during this time.

The protocol presents the left and right lever on an equal number of trials in a pseudo-randomized fashion.

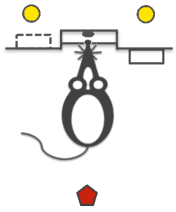
4b. 

No-Go trial, lever insertion
After presenting the light cue for 5 seconds, one lever is inserted. The lever remains inserted for 6 seconds and the light cue continues to be presented during this time.

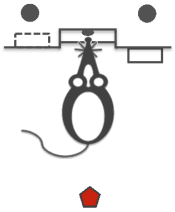
The protocol presents the left and right lever on an equal number of trials in a pseudo-randomized fashion.

5a. 

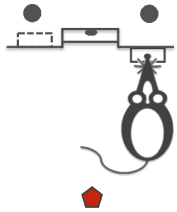
Go trial, correct response
Responsing to the inserted lever within 6 seconds results in a food pellet being delivered.

5b. 

Go trial, incorrect response
Withholding a lever response for the 6 seconds the lever is presented results in the termination of the trial (no reward is delivered).

5c. 

No-Go trial, correct response
Withholding a lever response for the 6 seconds the lever is presented results in a food pellet being delivered.

5d. 

No-Go trial, incorrect response
Responsing to the inserted lever within 6 seconds results in the termination of the trial (no reward is delivered).

6. **Continuation of protocol**
After a finished trial, the protocol loops back to a new inter-trial interval (step 1).

Figure 1. Schematic of the symmetrically reinforced Go/No-Go protocol. The different steps of the protocol are noted. Components of the Skinner box are indicated with circles for lever cue lights, and a pentagon for the house light.

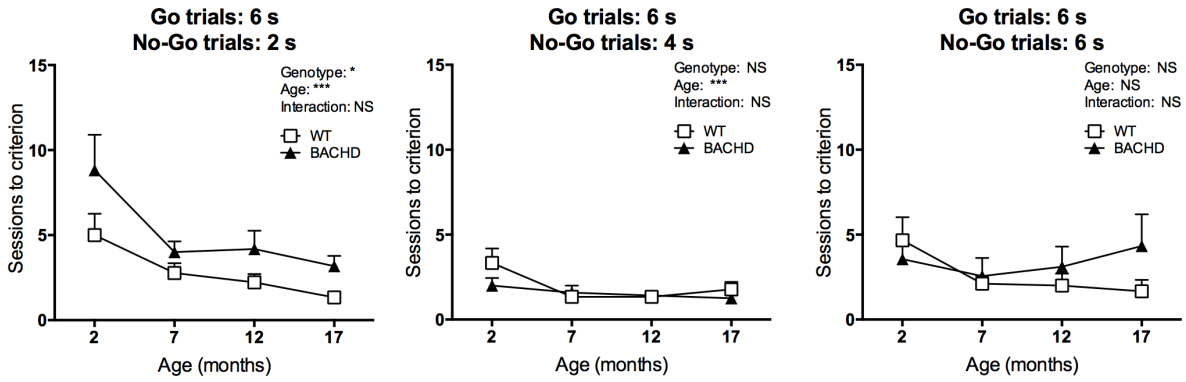


Figure 2. Number of training sessions needed before rats reached criterion performance. Curves indicate group mean plus standard error for each test age and Go/No-Go protocol. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

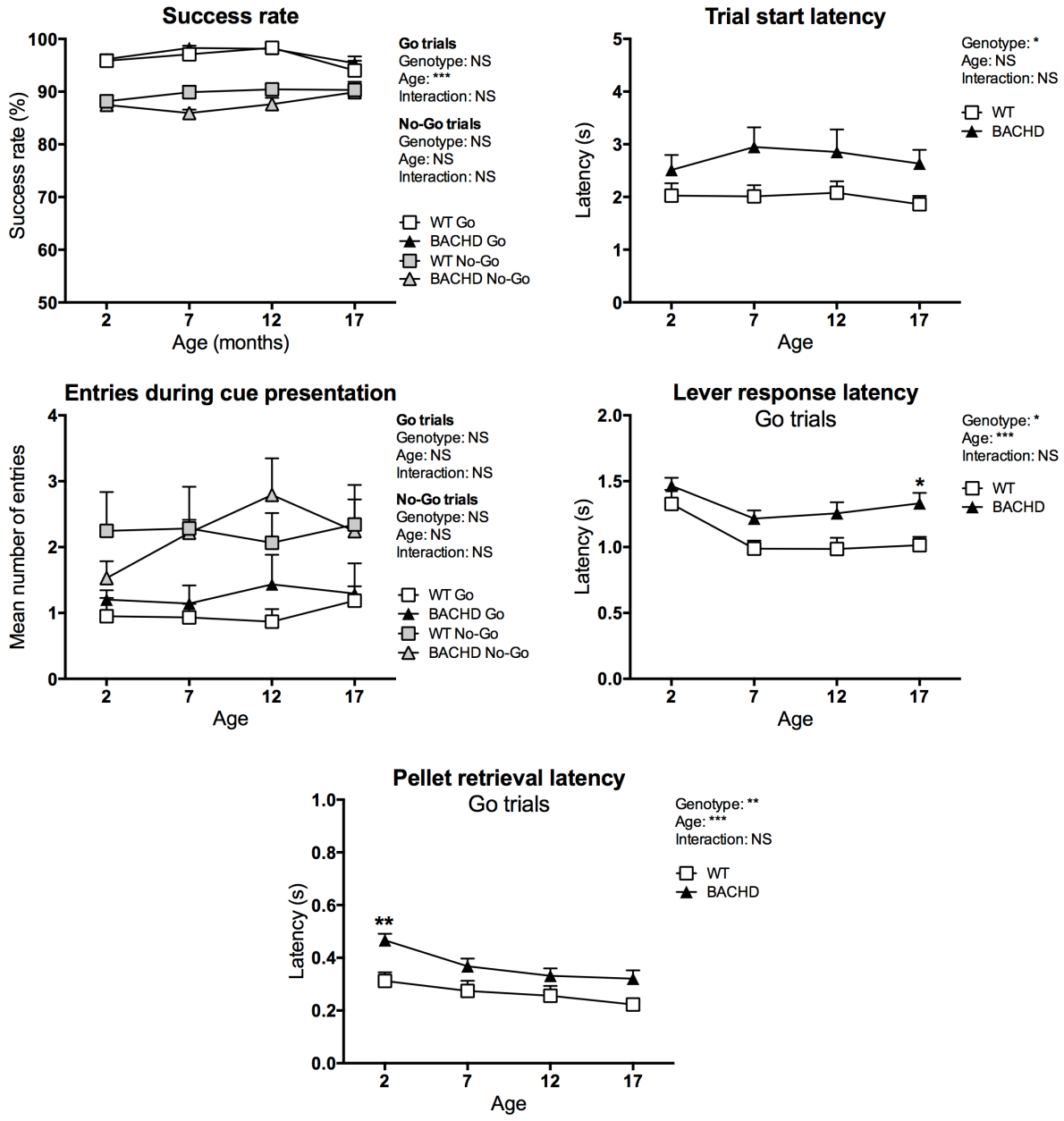


Figure 3. Selected parameters from criterion performance during the Go/No-Go protocol, where both trial types were six seconds long. Curves indicate group mean plus standard error for each test age. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

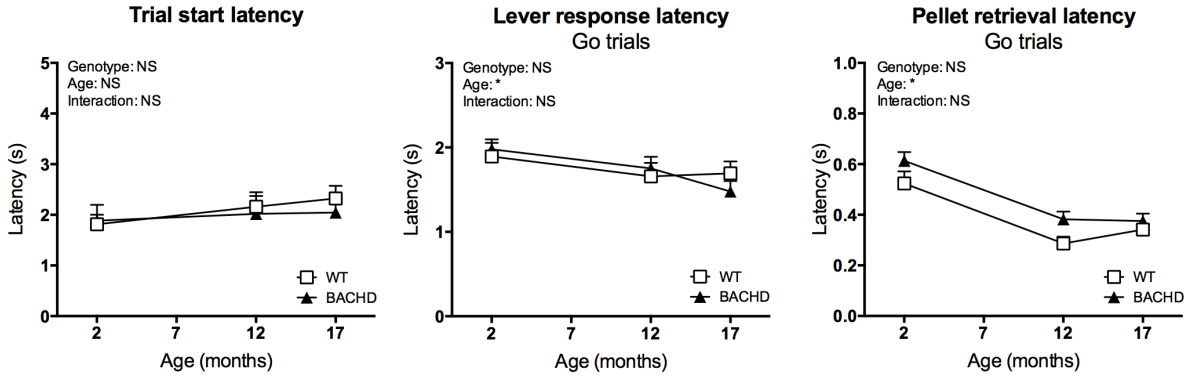


Figure 4. Selected parameters from the final lever training step, where rats were presented with a series of Go trials. Curves indicate group mean plus standard error for each test age and Go/No-Go protocol. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

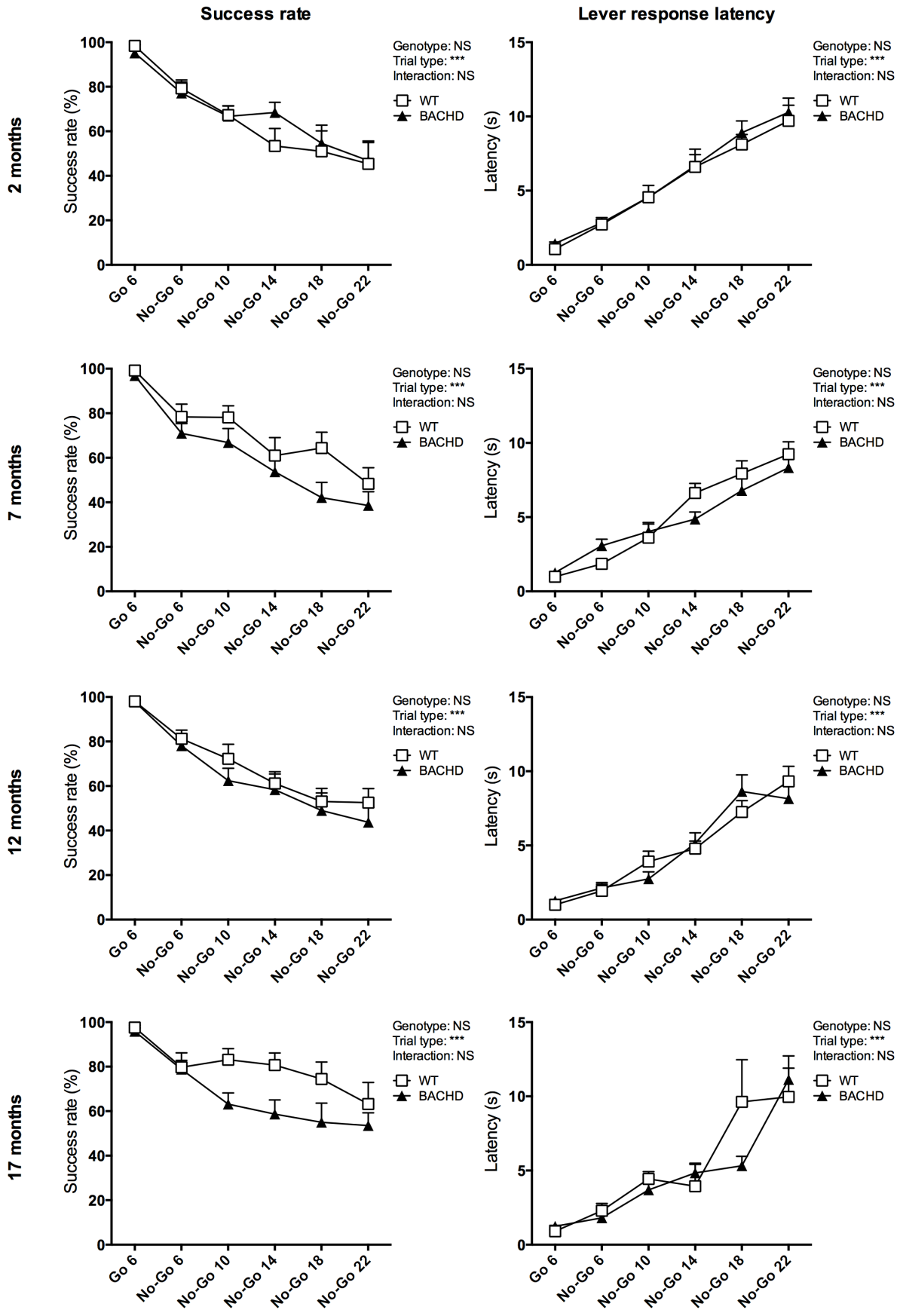

Success rate
Lever response latency
2 months
7 months
12 months
17 months
Success rate (%)
Latency (s)
Genotype: NS
Trial type: ***
Interaction: NS
WT
BACHD
Go 6
No-Go 6
No-Go 10
No-Go 14
No-Go 18
No-Go 22

Figure 5. Success rate and lever response latencies for all trial types during the Go/No-Go test protocol, where the duration of No-Go trials varied. Lever response latencies refer to successful Go trials and non-successful No-Go trials. The number that follows Go or No-Go trial refers to the trial duration. Curves indicate group mean plus standard error. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

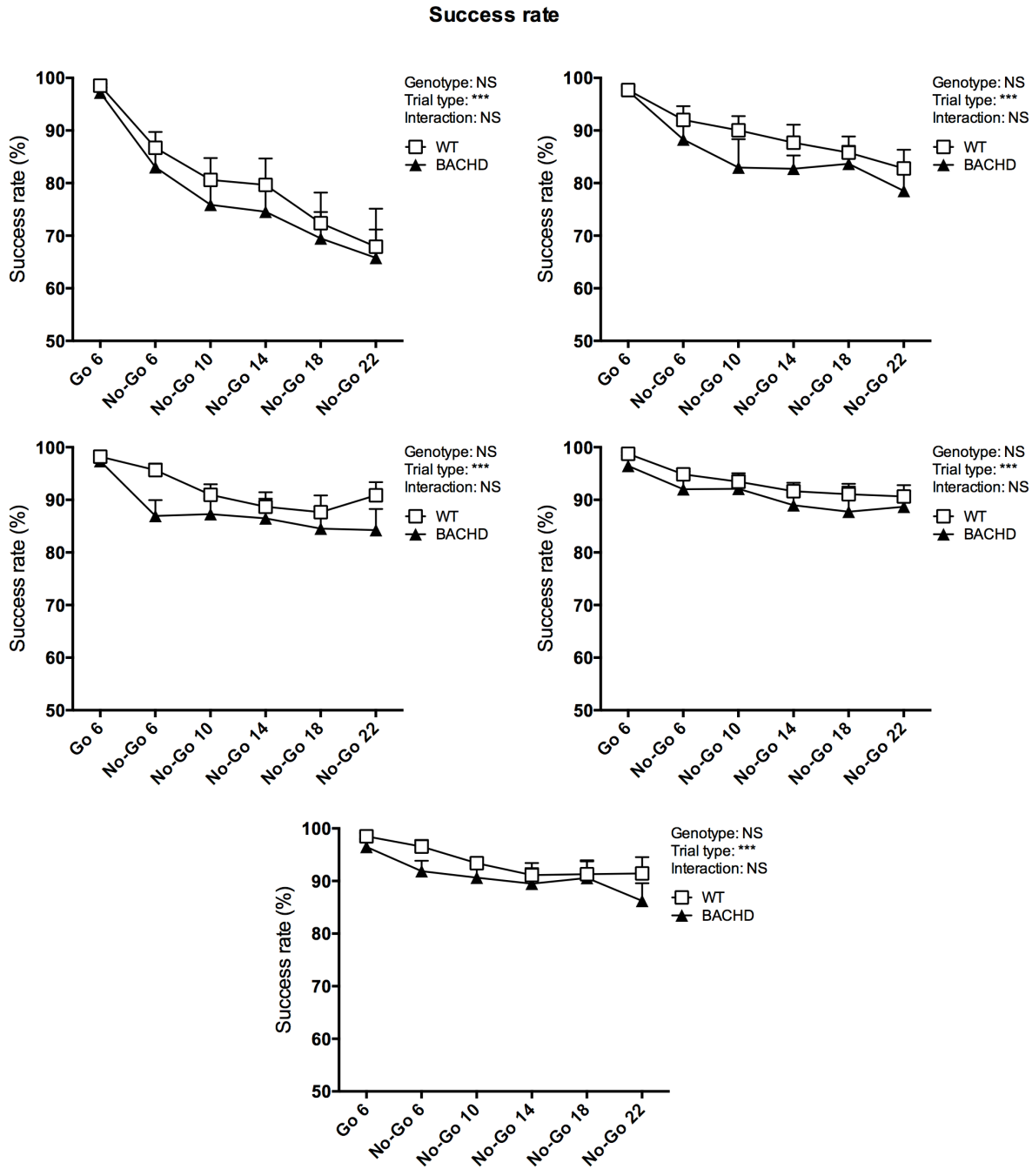


Figure 6. Change in success rate through extensive training on the Go/No-Go protocol, where the duration of No-Go trials varied. Graphs show mean success rates on blocks of five consecutive sessions. Curves indicate group mean plus standard error. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

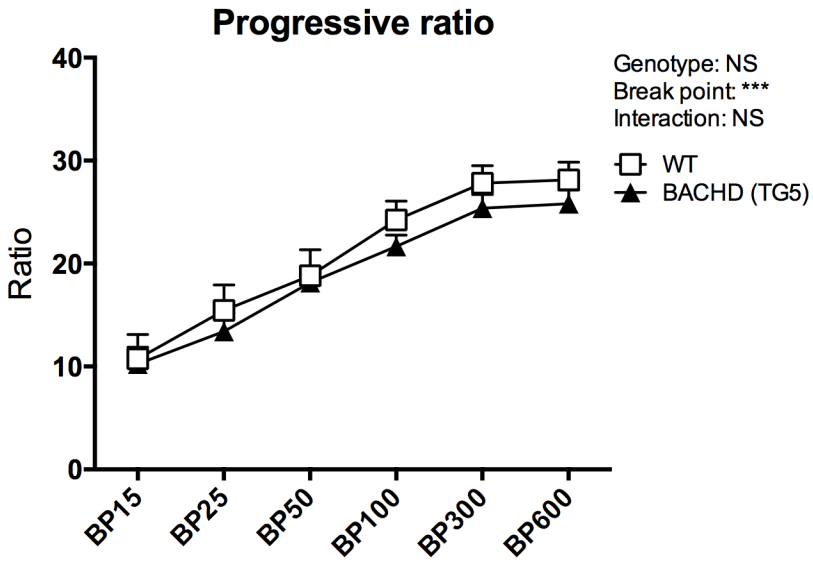


Figure 7. Performance on a progressive ratio control test during the 17-month test age. A series of break points were assessed. Curves indicate group mean plus standard error. Results from repeated measures two-way ANOVAs are indicated inside each graph. Results from post-hoc analysis are indicated for data points, where significant genotype differences were found (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Appendix III

BACHD rat performance in two DRL protocols

E K H Jansson, L Yu-Taeger, O Riess, H P Nguyen

Introduction

As part of a larger project to characterize the BACHD rats' performance in several inhibitory control tests they were assessed in two different protocols based on a differential reinforcement of low-rates (DRL) schedule.

Material and methods

Animals

The first study used a total of 36 male rats (13 hemizygous BACHD (TG5) rats, 11 hemizygous BACHD (TG9) rats, 12 WT rats). The rats were housed in mixed groups, so that each cage contained one rat of each genotype, although due to the uneven genotype distribution there were two cages where this was not the case. Other aspects of housing condition were in line with what is presented in Publication I for the group used in the Progressive ratio test. Rats were maintained on food restriction during experiments, and given free access to food between tests. Food restriction during experiments aimed at restricting rats to 85% of their free-feeding body weights. Body weights were measured weekly between experiments to monitor general health, and daily during experiments to monitor food restriction levels.

The second study used a total of 24 male rats (12 hemizygous BACHD (TG5) rats, 12 WT rats). The rats were housed in genotype-matched groups of three rats per cage. Other aspects of housing conditions were in line with what is presented in Publication I for the group used in the Progressive ratio test. Rats were maintained on food restriction during experiments, and given free access to food between tests. Food restriction during experiments used the alternative food restriction protocol, which aimed at matching the rats' apparent hunger levels. Body weights were measured weekly between experiments to monitor general health, and daily during experiments to monitor food restriction levels.

Behavioral protocol

The two studies used different operant conditioning systems. The first study used an older system manufactured by TSE systems (259900-SK-RAT-LA/1, TSE-systems GmbH, Bad Homburg, Germany). The operant conditioning boxes measured 48.5x38.5x22 cm (length x width x height). They contained a red house light and a pellet receptacle trough throughout all behavioral protocols. During some protocols, a single lever was placed next to the pellet trough, 10 cm above the cage floor and protruding 4 cm from the wall. A tri-colored cue light was positioned over the lever. The system did not use isolation boxes. The second study used the same operant conditioning boxes as described in Publication I. In both studies, a total of six boxes were used. Thus, six and four runs were needed to assess all rats in the first and second study respectively. Each run of the first study assessed two rats of each genotype, while each run of the second study assessed three rats per genotype. Each given rat was assigned to a specific operant chamber, while each chamber was used to assess equal numbers of rats from the different genotypes. Rats were assessed during the dark phase, with the first run starting approximately 30 minutes after dark phase onset.

The two studies used slightly different training protocols.

In the first study, two months old rats were first given two 30-minute habituation sessions, where no lever was present, the house light switched on, and a single reward pellet was delivered to the pellet receptacle with 30-second intervals. Afterwards, rats were trained on a continuous reinforcement (CRF) protocol. During this, a single lever was present inside the conditioning chamber, and each push resulted in a pellet being delivered. A green cue light situated above the lever, and the red house light, shone throughout the sessions. The system did not allow for manual pellet deliveries, meaning that rats had to learn to push the lever on their own. Still, a paste made of mashed reward pellets was placed on top of the lever during initial CRF sessions to promote lever investigation. The rats were given CRF sessions until they managed to obtain 100 reward pellets within 30 minutes on seven consecutive sessions. When rats had reached this criterion they were trained on the DRL protocol. In this protocol, the lever was initially active, as indicated by the green cue light. Responding to the lever thus resulted in the delivery of a reward pellet, although it also resulted in the start of a five-second long time-out phase, which was indicated by the cue light being switched off. During the time-out, lever responses were not rewarded but only served to restart the time-out phase. Thus, rats had to withhold lever responses for at least five seconds in order for the lever to become active again, which was indicated by the green cue light being switched on (see Figure 1). It should be noted that it is uncommon to include cue lights in DRL protocols. The main reason why cue lights were used in the current study was that the ultimate aim was to reverse the cue light in order to study reversal learning. However, that part of the study did not reveal any interesting phenotypes, and will not be described here. Each DRL session lasted 30 minutes, regardless of the rats' performance. Rats were given a total of seven DRL sessions. When data had been gathered for the two months old rats they were put back on free feeding. The rats were then reassessed at about six months of age. The retesting followed the same protocols, although training started with the CRF protocol, and DRL training lasted only six sessions.

In the second study, two months old rats were first given two habituation sessions in the operant conditioning boxes. During these, both levers were retracted, the house light was switched on, and a total of 100 reward pellets were delivered to the pellet receptacle at intervals that were randomized between 10, 15, 20, 25, and 30 seconds. Afterwards, rats were trained to perform lever pushes on a CRF protocol. During these protocols, one lever was inserted into the conditioning box and both the cue light above the lever and the house light were switched on. Any lever push performed by the rats was rewarded with the delivery of a reward pellet. Rats were initially given rewards for approaching, sniffing and touching the lever, but eventually learned to reliably push it. Training continued until rats performed 100 pushes on their own, within 30 minutes, on three consecutive sessions. Afterwards, training stopped and the rats were maintained on free feeding conditions until they were six months old. At that age, the rats were first retrained on a single CRF session. Afterwards, the rats were run for 15 sessions on a

DRL protocol. The structure of this was similar to the protocol used for the first study. Thus, one lever was inserted into the operant chamber at the start of the session. At this time, the lever was reinforced, as indicated by the cue lights that shone over both lever panels. Pushing the lever resulted in a pellet being delivered, and a time-out phase being started, indicated by the cue light being switched off. The duration of the time-out varied in a pseudo-randomized manner between 0, 5, 10, 15 and 20 seconds. Pushing the lever during this time was not rewarded, but resulted in the time-out being restarted. Thus, the rats had to withhold lever responses for the full duration of the time-out. The cue lights were switched on at the end of the time-out phase, indicating that the lever was once again active (see Figure 2). Sessions ended after 30 minutes, or when rats had obtained 100 pellets.

Behavioral parameters
Only the main readouts have been analyzed from the two studies. This included the time needed to complete 100 lever pushes during CRF sessions, and the overall success rate during DRL sessions. For the second study, additional analysis was made to obtain separate success rates for trials with different time-out durations.

Statistical methods
Behavioral data was extracted by running the log files created by the systems through a series of R scripts. Statistical analysis was performed in GraphPad prism (v6.0). Analyses were performed with two-way repeated measures ANOVAs, using genotype as between subject factor and either age or trial type as within subject factor. Different post-test were used to evaluate genotype differences on individual data points when two (Sidak's) or more (Dunnett's) groups were included. One-way ANOVAs with Dunnett's post-test were used for analysis of baseline performance during CRF and DRL sessions of the first study. Finally, analysis of performance during CRF retraining on the second study was done using student t-test.

Results
In the first study, BACHD rats were found to be slower than WT rats in completing 100 lever pushes during the CRF protocol (Figure 3). This phenotype was present both during the two- and six-months test age, although it was slightly more pronounced during the second test. BACHD rats were also found to perform significantly worse than WT rats during the DRL protocol at both test ages (Figure 4). Once again, the phenotype appeared to be more pronounced during the second test age, although this was likely due to rats not really reaching a stable performance during the first test age. There were no clear differences between BACHD rats of the TG5 and TG9 line, although there was a trend indicating that TG9 rats were more impaired.

In the second study, BACHD rats were found to only initially be slower than WT rats in completing 100 lever pushes during the CRF protocol (Figure 5). This phenotype disappeared with training, and was also not present when the rats were retrained at six months of age. There were no differences in DRL performance between WT and BACHD rats, despite trials using up to 20-second long time-outs (Figure 6).

Discussion
As noted, BACHD rats were found to be slower than WT rats at completing the CRF protocol during the first study, but not the second. This is comparable to results from

other studies, where we have found that BACHD rats on occasion are slower than WT rats at completing 100 lever pushes. When present, the phenotype appears to be caused by BACHD rats being slower at returning to the lever, after retrieving the reward pellet, rather than a change in other motor-timing parameters. A detailed analysis of performance during the first study could not be performed due to the limited data being stored in the log files of that system. However, as the phenotype has been found to not be fully reproducible or stable it is of little interest.

In the first study, BACHD rats were found to show a stably impaired performance during the DRL protocol, indicating that they had problems withholding lever responses. However, due to the design of the study the exact nature of the phenotype was unclear. Specifically, as rats were given extensive CRF training prior to the DRL training, it is possible that their apparent impairment was due to them having difficulties with adjusting their behavior, rather than an underlying impairment in general response inhibition. The second study aimed at further evaluating this, by only using a very brief CRF training. As noted, BACHD rats were not impaired during this test, despite experiencing time-outs that were up to 20 seconds long. The results thus supported the hypothesis that the initial impairment was based on a difficulty of adjusting an established behavior, rather than a general response inhibition impairment. Still, the results could also be explained by differences in selective attention and visual acuity. It is therefore also important to note that the cue lights used in the first study were more discreet than the ones used in the second study. This could explain why BACHD rats showed impaired performance in the first, but not the second study. Additional characterization tests would be needed to further investigate this.

Conclusion

BACHD rats do not appear to show a generally impaired performance when withholding lever responses in the DRL test. However, impaired performance might be present when the rats need to adjust their behaviors, and apply inhibitory control to a previously learned behavior.

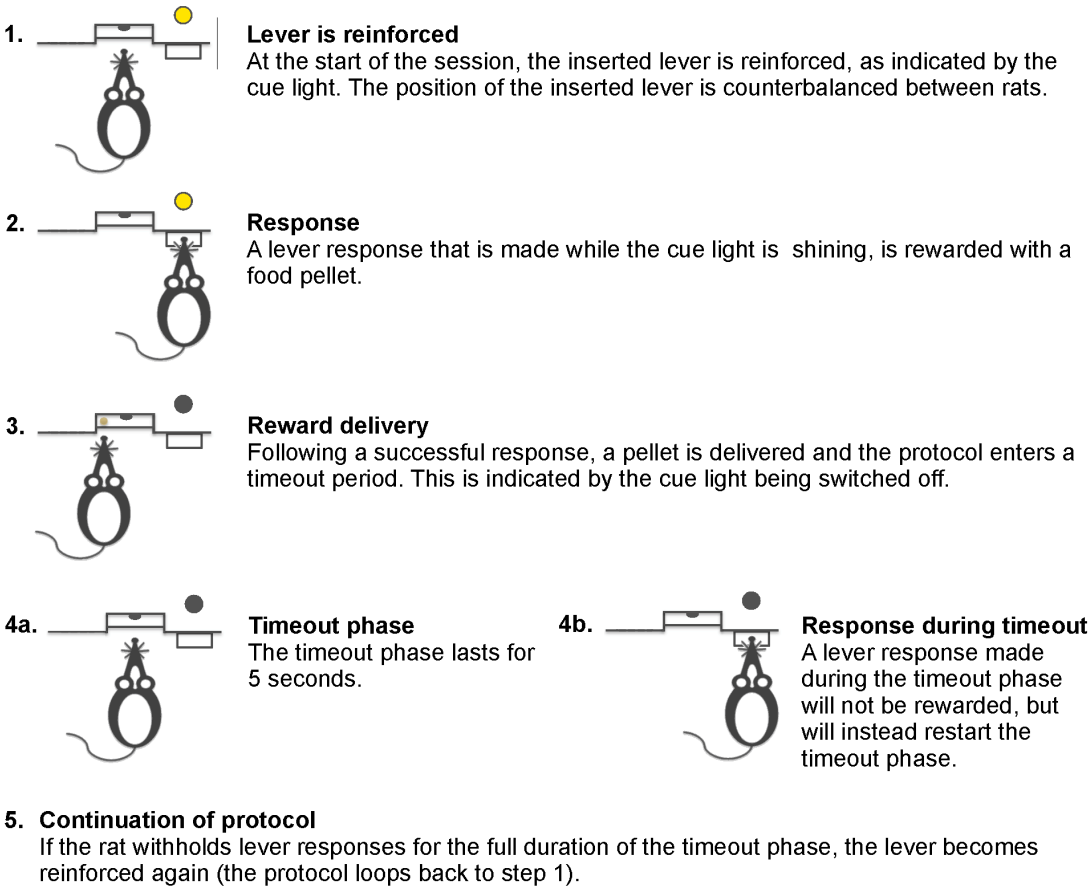


Figure 1. Schematic of the first DRL protocol. The different steps of the protocol are noted. Components of the Skinner box are indicated with circles for lever cue lights.

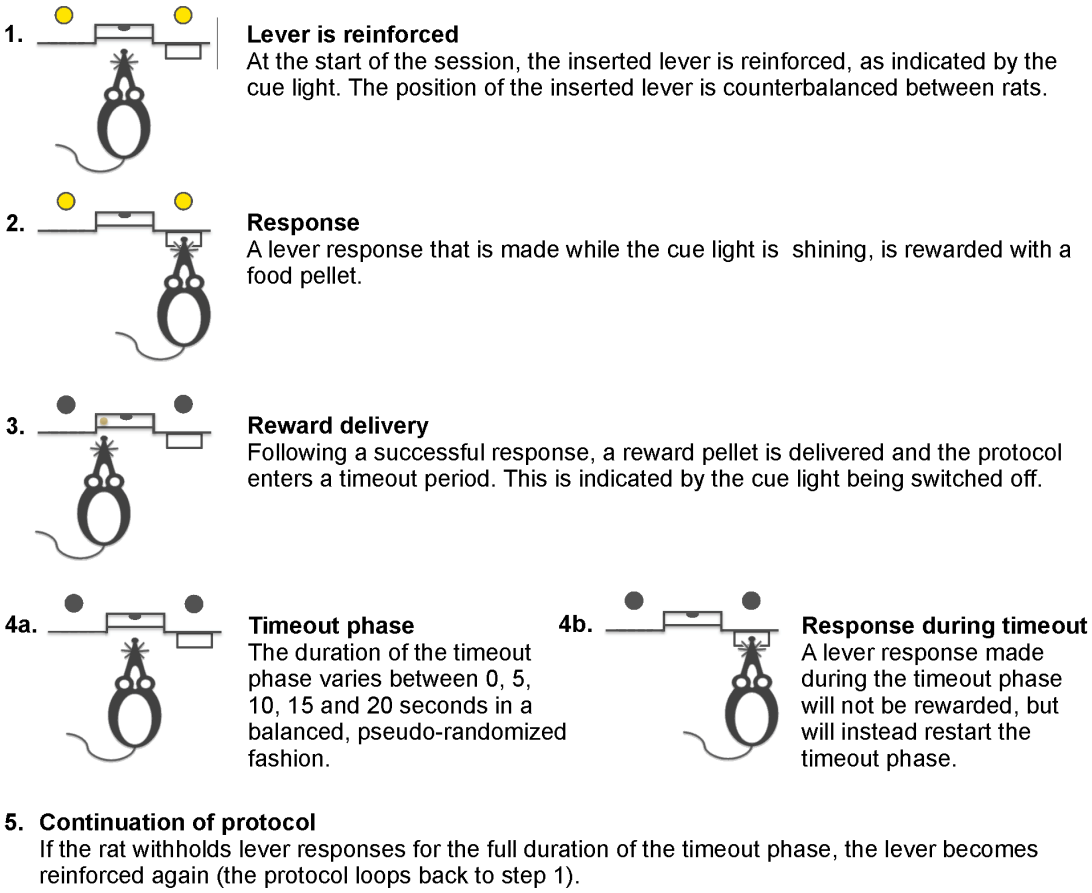


Figure 2. Schematic of the second DRL protocol. The different steps of the protocol are noted. Components of the Skinner box are indicated with circles for lever cue lights.

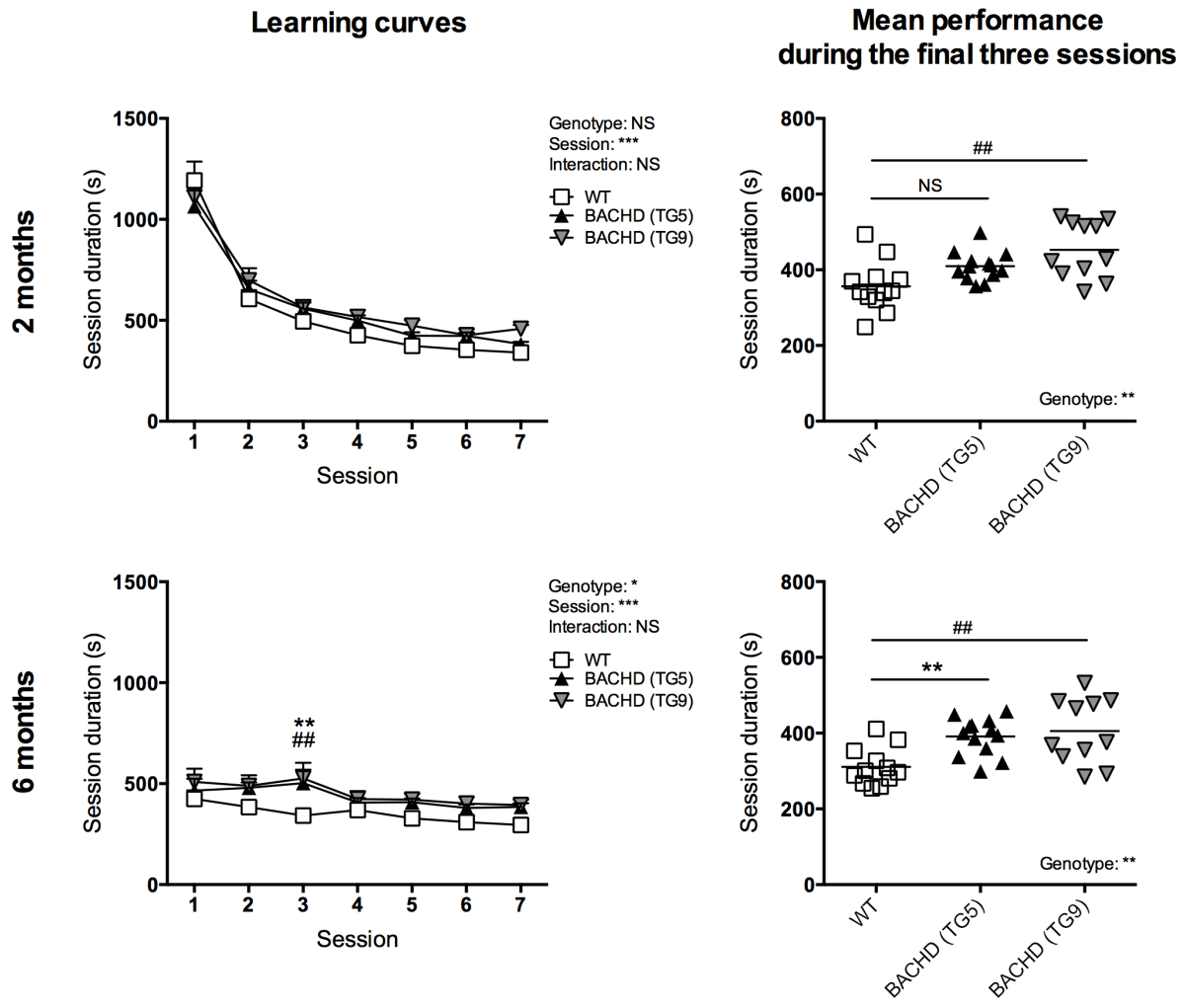


Figure 3. Performance on the CRF protocol during the first study. Graphs indicate the time needed to perform 100 lever pushes. Curves indicate group mean plus standard error for each individual session, while scatter plots indicate mean performance of individual rats during the final three sessions. For curves, results from two-way ANOVA are indicated inside graphs. Data points, where post-tests indicated a significant difference between WT and BACHD rats are also indicated, with * for WT and TG5 comparisons and # for WT and TG9 comparisons. For scatter plots, results from one-way ANOVA are indicated inside graphs. Post-tests that detected a significant difference between WT and BACHD rats are also indicated, with * for WT and TG5 comparisons and # for WT and TG9 comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

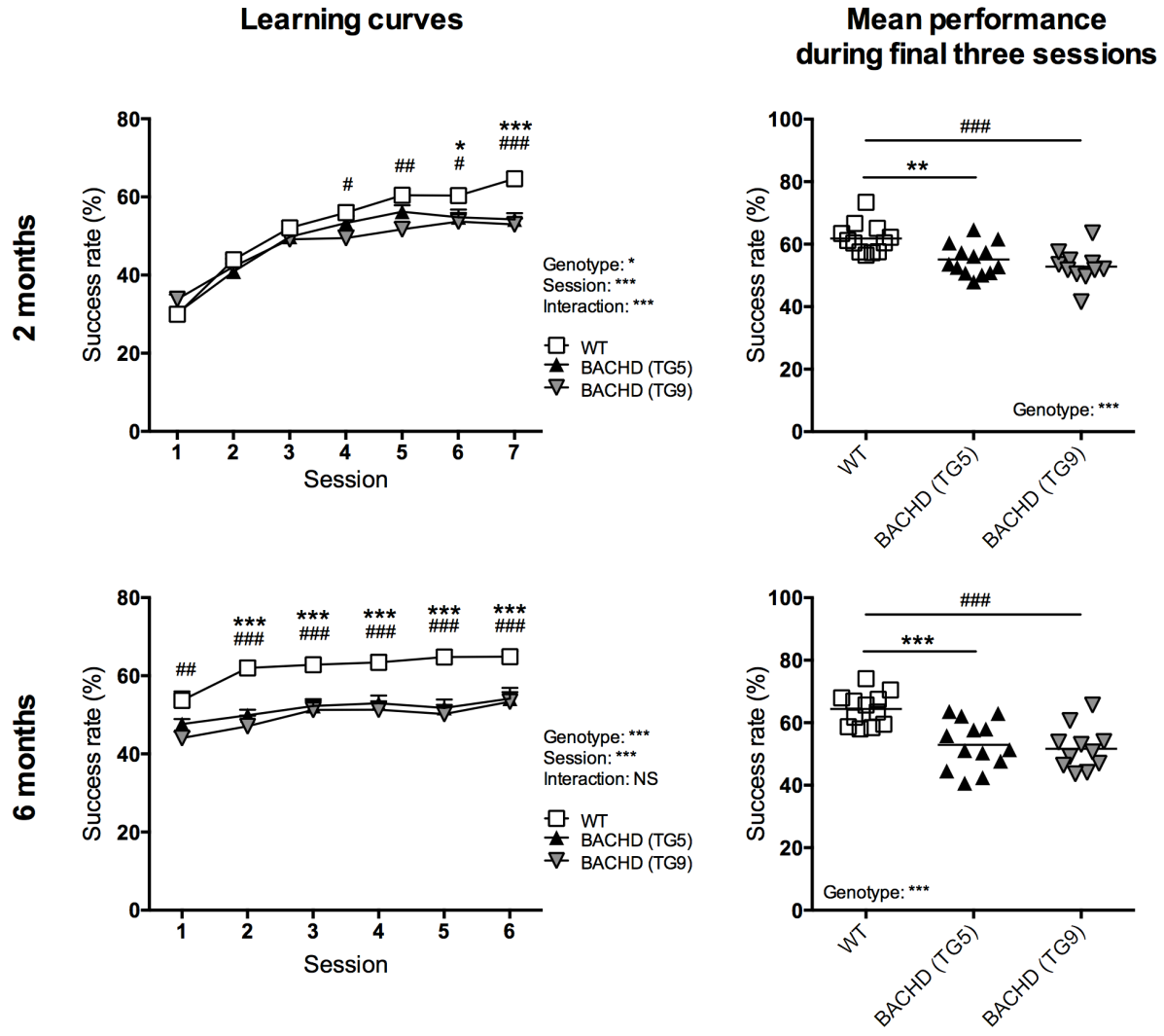


Figure 4. Performance on the DRL protocol during the first study. Graphs indicate the percentage of successful responses. Curves indicate group mean plus standard error for each individual session, while scatter plots indicate mean performance of individual rats during the final three sessions. For curves, results from two-way ANOVA are indicated inside graphs. Data points, where post-tests indicated a significant difference between WT and BACHD rats are also indicated, with * for WT and TG5 comparisons and # for WT and TG9 comparisons. For scatter plots, results from one-way ANOVA are indicated inside graphs. Post-tests that detected a significant difference between WT and BACHD rats are also indicated, with * for WT and TG5 comparisons and # for WT: and TG9 comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

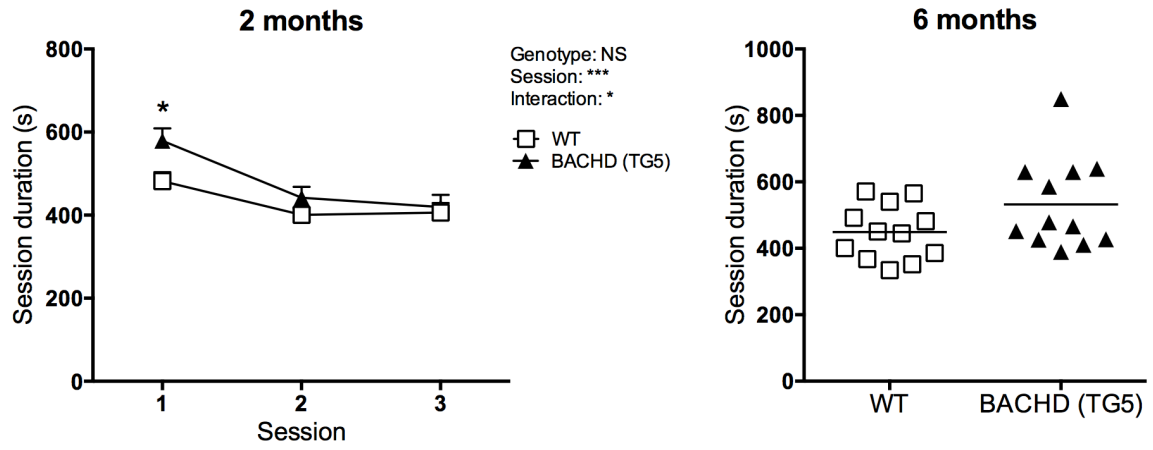


Figure 5. Performance on the CRF protocol during the second study. Graphs indicate the time needed to perform 100 lever pushes. The curve indicates group mean plus standard error for each individual session run at two months of age, while the scatter plot indicates performance of individual rats during the single session run at six months of age. For the curve, results from two-way ANOVA are indicated inside the graph. Data points, where post-tests detected a significant difference between WT and BACHD rats are also indicated. For scatter plots, results from Student t-test is indicated inside graphs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

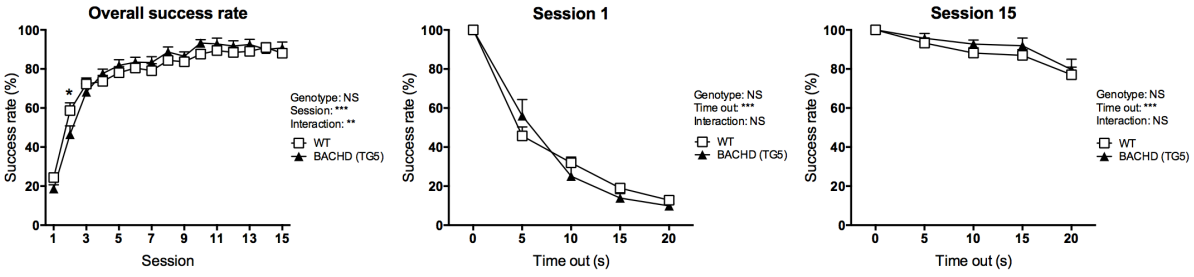


Figure 6. Performance on the DRL protocol during the second study. Graphs indicate the percentage of successful responses. Curves indicate group mean plus standard error. The graph on the left shows the overall mean success rate over the 15 sessions that were run, while the middle and right graph show the mean success rate on trials with different time-out durations on the first and last session, respectively. Results from two-way ANOVA are indicated inside graphs. Data points, where post-tests detected a significant difference between WT and BACHD rats are also indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).