Autism as a quantitative phenomenon

beyond single genes and dichotomous outcomes

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Autism as a quantitative phenomenon beyond single genes and dichotomous outcomes

Autisme als een kwantitatief fenomeen
voorbij afzonderlijke genen en dichotome uitkomstmaten

(met een samenvatting in het Nederlands)

Proefschrift

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General introduction

Modelling autistic features in mice

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ABSTRACT

Animal studies provide a unique opportunity to study the consequences of genetic variation at the behavioral level. Over the past decade, human genetic studies have identified hundreds of risk genes for autism spectrum disorder (ASD). These findings can lead to understanding on how genetic variation contributes to individual differences in neurodevelopmental phenotypes, such as social interaction and stereotyped behavior in people with ASD. To develop rational therapeutic interventions, systematic animal model studies are needed to understand the relationships between genetic variation, pathogenic processes and the expression of autistic behaviors. Genetic and non-genetic animal model strategies are here reviewed in their propensity to study the underpinnings of behavioral trait variation. We conclude that the value of genetic animal models for ASD may be enhanced by characterizing behavioral traits across developmental stages. Finally, we propose that integration of reverse and forward genetic approaches may be essential to increase translational value of animal models for neurodevelopmental disorders.

INTRODUCTION

Clinical Definition of ASD

Autism spectrum disorder (ASD) is the name for a group of neurodevelopmental conditions that are clinically defined by impairments in social interaction and communication and by restricted, repetitive and stereotyped behavior. Most autistic behaviors become manifest in the first years of life when the development of brain circuits becomes influenced by sensory experience. The autistic spectrum is highly heterogeneous with respect to severity, timing and variability of symptom manifestation (Fountain et al., 2012). Some patients show predominantly stereotyped behaviors, whereas others may mainly suffer from deficits in social behavioral development. In addition, patients can have co-morbidities, such as epilepsy, motor abnormalities and sleep problems.

Prevalence

The prevalence of autism has been increasing since first studied, when about 4.1 of every 10,000 individuals were diagnosed with a form of ASD (Lotter, 1966). This increase is partly a result of changes in diagnostic routines as well as improved awareness and recognition, but an increase in unidentified risk factors cannot be ruled out (Weintraub, 2011). The median worldwide ASD prevalence is currently estimated at 1–2% based on recent surveys (Baron-Cohen et al., 2009; Blumberg et al., 2013; Kim et al., 2011; Mattila et al., 2011).

Treatment

At this moment, therapeutic options are mostly restricted to behavioral interventions, which have proven successful in a subset of patients, especially when applied in early stages (Kasari et al., 2014; Rogers et al., 2014). Clinical biomarkers to guide treatment and patient selection are lacking, although tracking eye movements and electroencephalogram (EEG) have shown promise (Jeste et al., 2015; Jones and Klin, 2013). Further understanding of neurobiological abnormalities in individual patients is essential to guide patient selection for rational treatments.

Risk Factors

Epidemiological studies have identified various risk factors that contribute to ASD. Advanced paternal or maternal reproductive age is probably the most accepted and consistent risk factor (Hultman et al., 2011; Sandin et al., 2012). Additionally, gestational factors that could affect neurodevelopment, such as complications during pregnancy and exposure to teratogenic agents, have been suggested to

increase risk of ASD (Canetta et al., 2014; Christensen et al., 2013; Gardener et al., 2009, 2011; Hultman et al., 2011; Sandin et al., 2012; Volk et al., 2013). Many diverse conditions reflecting general compromises to perinatal and neonatal health are also associated with increased risk (Gardener et al., 2011). Recently, prevalence of ASD has also been related to urbanization and to parents who work in the sector of information technology (Roelfsema et al., 2012). Most cases are probably the result of interactions between genetic and non-genetic risk factors.

Genetics

Twin studies have suggested that ASD has high heritability (more than 80%) (Ronald and Hoekstra, 2011). This heritability occurs in the context of environmental risks and gene–environment interplay, since monozygotic twin rates never reach 100% concordance. The genetic architecture of ASD is beginning to be elucidated, and the amount of ASD risk genes is now estimated to range between 400 and 1000 (Geschwind and State, 2015; de la Torre-Ubieta et al., 2016). A key insight into the genetic basis of ASD came from the recognition of rare medical genetic syndromes due to single genetic disorders with high penetrance. Each of these clinical syndromes is found in less than 1% of patients with ASD; however, collectively they are estimated to be found in ~5% of the total ASD population (de la Torre-Ubieta et al., 2016).

Many ASD risk genes have been identified based on rare inherited and de novo variants (de la Torre-Ubieta et al., 2016). While contributing de novo variants are found in 10–20% of cases, about 50% of ASD liability can be attributed to common genetic variation (Gaugler et al., 2014; Klei et al., 2012; De Rubeis et al., 2014). Most genetic risk factors for ASD are also found in the general population, influencing a continuum of behavioral and developmental traits (Robinson et al., 2016). The rapid progress of genetics has fueled the development of animal model systems to enable the identification of common molecular and cellular pathways in ASD. Homologs exist for most genes and a reasonable degree of functional conservation is generally assumed (Langen et al., 2011a).

ANIMAL MODELS BASED ON ENVIRONMENTAL AND GENETIC FINDINGS

Studies into ASD have been complicated by a lack of known causal factors, poor access to brain tissue and the broad clinical definition of the disorder. A logical scientific progression has become to develop animal models in order to overcome some of these limitations.

Lesion Studies

Initial modelling approaches in nonhuman primates emphasized social and communicative deficits following neuroanatomical lesions. Rhesus monkeys with neonatal lesions to the medial temporal lobe developed severe cognitive and socio-emotional deficits, including abnormal social interaction, absence of facial and body expressions and stereotypic behaviors (Bachevalier, 1994, 1996). These monkeys were characterized by memory impairments in certain types of learning tasks, which may be similar to low-functioning patients with ASD. Further investigation indicated that monkeys with neonatal damage only to the amygdaloid complex were less impaired in memory function, while developing socio-emotional deficits similar to combined amygdalo-hippocampal lesions (Bachevalier, 1994).

Fetal Valproate Syndrome

Another line of research emerged when increased frequency of ASD symptoms was observed in children exposed to valproic acid (VPA) during pregnancy (Christianson et al., 1994; Roullet et al., 2013). Prenatal treatment of Wistar outbred rats with VPA was found to result in behavioral abnormalities such as reduced frequency of social interactions, increased latency to social contact and reduced exploratory activity, as well as repetitive behavior and stereotypic-like movements (Schneider and Przewlocki, 2005). Prenatal VPA-treated animals are still being considered as an approach to model ASD pathology in rodents (see Roullet et al. 2013 for a review).

Genetic Disorders

Emphasis shifted to genetic models when genetic knowledge increased and molecular technologies for engineering rodent models expanded. In the 1990s, genetic causes of neurological syndromes strongly associated with ASD were being discovered, including causes of fragile X syndrome, tuberous sclerosis and Rett syndrome (Amir et al., 1999; European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997; Verkerk et al., 1991). Subsequently, mouse models of these human genetic disorders were developed, and brought new possibilities to study ASD patho-mechanisms in animal models (Moy et al., 2006). For instance, fragile X syndrome was found to be caused by a CGG-repeat expansion in the *FMR1* gene in 1991, and associated with ASD in 35% of cases (Hagerman, 2006; Verkerk et al., 1991). Subsequently, *Fmr1* knockout mice were found to develop patho-mechanistic hallmarks such as increased spine density, immaturity of dendritic spines and abnormalities in protein synthesis-dependent synaptic plasticity (Huber et al., 2002; Kazdoba et al., 2014). Further studies revealed that *Fmr1* knockout mice also develop abnormalities in behavioral and sensorimotor

traits, including deficits related to the core symptoms of ASD such as abnormal social interaction and increased perseverance during reversal learning (Bernardet and Crusio, 2006; Kazdoba et al., 2014).

These genetic and non-genetic examples of animal models (neonatal medial temporal lobe lesions in monkeys, prenatal exposure to VPA in rats, and modelling genetic disorders associated with ASD in mice) greatly improved our understanding of pervasive developmental disorders, but they also have their limitations.

STRENGTHS AND LIMITATIONS OF ANIMAL MODELS

Historically, three main validity criteria have been developed to estimate the strength of animal models, namely, construct, predictive and face validity (Nestler and Hyman, 2010). The use of animal models for ASD is complicated by the large number of implicated genetic and environmental risk factors, and neurobiological and ethological differences between species.

Construct Validity

Construct validity is used to estimate whether the model is based on the same pathogenic process that causes the human disease. For instance, lesions to the neonatal medial temporal lobe in monkeys were considered useful, because this brain region was also associated with autism in the human population (Bachevalier, 1994, 1996).

Construct validity of the VPA animal model for ASD is based on the observation that in humans, exposure during the first trimester of pregnancy leads to a sevenfold increase in the incidence of autistic symptoms (Roullet et al., 2013). To model this etiology, rodents can be exposed to VPA during corresponding time windows. The relevance of this construct is supported by the observation that VPA use during pregnancy is common across countries (Roullet et al., 2013), but may be limited for people with ASD that were not exposed to VPA.

Construct validity of genetic animal models for ASD is largely based on evidence from human genetic studies ("genetic validity"). In addition, *Fmr1* gene knockout mice also display molecular and neuropathological hallmarks that have been observed in human *post-mortem* brain tissue of fragile X patients (Kazdoba et al., 2014). The existence of Mendelian disorders associated with ASD enables the analysis of large-effect size mutations associated with the disorder, in contrast to mental illnesses such as schizophrenia. However, large-effect size mutations are rare (each seen in less than 1% of cases), and their patho-mechanistic relevance to

other forms of ASD remains to be estimated based on further evidence (Baudouin et al., 2012). At this moment, many different genetic animal models for ASD have been studied (Abrahams et al., 2013), each claiming construct validity for a subset of the total ASD population.

Predictive Validity

The predictive validity of animal models is estimated based on the similarities in response to treatments that are known to prevent or reverse symptoms in the human disease. Predictive validity is difficult to determine for ASD animal models, given the current lack of rational treatments for ASD core symptoms. Risperidone was the first drug approved by the US Food and Drug Administration (FDA) to treat irritability associated with ASD (McDougle et al., 2008). In the *Cntnap2* gene knockout mouse model for ASD, risperidone was shown to rescue hyperactivity, repetitive grooming and perseveration in a T-maze, while having no effect on social interaction deficits (Peñagarikano et al., 2011). Further studies in *Cntnap2* knockout mice have been performed, suggesting that social outcomes could be improved by acute administration of oxytocin (Brunner et al., 2015; Penagarikano et al., 2015). Rational treatments for ASD core symptoms are yet to be established, rendering the use of predictive validity infeasible to estimate the strength of an ASD animal model. The chloride reducing agent bumetanide is an interesting candidate in this respect and has shown efficacy for ASD in a number of different studies (Bruining et al., 2015a; Lemonnier et al., 2012).

Face Validity

Face validity is used to estimate similarities in symptoms between the animal model and the human disease. For example, behavioral deficits in *Fmr1* knockout mice have similarities to the behavioral abnormalities seen in fragile X syndrome patients. Face validity in animal models of ASD is a topic of debate, given the behavioral and evolutionary differences between humans and animal models (Hyman, 2014; Liu et al., 2016). ASD is a neurodevelopmental condition characterized by multifactorial etiology and behavioral functioning as the ultimate clinical endpoint. Perhaps somewhat underestimated is the difficulty of comparing mouse to human behavior. Mice and humans have a shared evolutionary path, except for the last 60 million years. The sharing of biological processes between mice and humans enables the use of mouse models to study human diseases (Schughart et al., 2013). On the other hand, mice and humans differ in their behavioral manifestation, raising the question which aspects of neurobiology and behavior are comparable across species. Some behavioral phenotypes are frequently observed in genetic mouse models for ASD

(Kazdoba et al., 2015) and therefore deemed relevant. Detailed behavioral assays to probe core features of ASD have been developed (for review, see (Kas et al., 2014)); however, the feasibility to achieve such direct levels of face validity remains challenging.

EVOLUTIONARY PERSPECTIVE OF INHERITED BEHAVIORS

An alternative strategy to overcome evolutionary distances is to focus on naturally occurring behaviors that are important to the survival of an organism and its species. The hypothesis would be that the neurobiological mechanisms regulating these behavioral strategies are conserved across species. Environmental pressure can lead to evolution of distinct inherited behavioral traits, similar to traits related to bodily structure and form (Lorenz, 1958). A recent study into behavioral evolution concerned differences in burrowing behavior in two sister species of *Peromyscus* mice (Weber et al., 2013). Old field mice (*Peromyscus polionotus*) mostly inhabit open fields and construct unique burrows characterized by a long entrance tunnel and secondary tunnel that may serve as an escape to predators such as snakes. These mice have recently diverged from deer mice (*Peromyscus maniculatus*), which inhabit prairie and forest habitats and build small and single-tunnel burrows. Burrow length and the presence of an escape tunnel were studied under controlled laboratory conditions, and genetic analyses in offspring hybrids indicated four genetic loci that independently affected either burrow length or the presence of an escape tunnel. These results indicate that 'extended' and complex behaviors can evolve through evolutionary mechanisms of behavioral adaptation (Weber et al., 2013). Indeed, survival of a species in its environment depends on the proper behavioral responses to external cues.

Communication and Social Behavior

Social interaction and communication are essential for an animal to find the best available reproduction partners and to deal with threats such as natural predators and attacks from conspecifics (Kas et al., 2007). Social cues in rodents differ from those in humans and have a differential effect on how they socially engage. For instance, mice guide their behavioral responses on the basis of olfactory signals from the environment (Keverne, 2002; Silverman et al., 2010), where humans largely depend on verbal language and visual cues. Mice display vocal communications, although it is unknown to what extent these signals in mice exert a communicative function as does language in humans.

Different assays have been proposed to study aspects of social behaviors in mice (Silverman et al., 2010). For example, social approach is most studied in the three-chambered apparatus, through comparison of the amount of exploration of a novel conspecific placed in a wired cage versus a wired cage without conspecific (Nadler et al., 2004). Perhaps more naturalistic, reciprocal social interactions can be assessed in freely moving animals by scoring the amount of social behaviors such as nose-to-nose sniffing, anogenital sniffing and allogrooming (Silverman et al., 2010). These readouts can be used to evaluate short-term and long-term social recognition, by comparing the preference for a novel mouse over a familiar conspecific (Bruining et al., 2015b; Pearson et al., 2010). Abnormalities in social approach and recognition have been described in a variety of genetic mouse models for ASD, including *Cntnap2*, *Nlgn1*, *Nlgn4*, *Pten*, *Shank2* and *Shank3* mutants (Kazdoba et al., 2015).

New approaches to study social group dynamics over time are also being implemented. For example, in a recent study, automated longitudinal tracking of animal colonies has been used to study social dominance in BTBR T+tf/J mice in a semi-natural environment (Weissbrod et al., 2013). In this setup, integrated radio-frequency identification (RFID) tagging and video data enabled the analysis of locomotor behaviors in multiple socially interacting animals. Commonly observed social interactions, such as avoidance, being avoided, chasing and being chased, could be detected, and quantification of social dominance indicated reduced social hierarchy in groups of BTBR T+tf/J mice compared to control animals (Weissbrod et al., 2013).

Stereotyped and Restricted Behaviors

Another behavioral domain that is effected in ASD relates to restricted, stereotyped and repetitive behaviors and resistance to change. Fixed and repeatedly performed action patterns form a vital part of typical development and normal functioning across species (Langen et al., 2011a). Repetition is essential to the survival of lower species such as invertebrates, birds and rodents, and occurs in higher animals as part of normal behavior to acquire skilled acts through practice (Langen et al., 2011b; Turner, 1999). In typically developing young children, repetitive behaviors decrease over time (Langen et al., 2011a). In individuals with ASD, daily life is severely impaired by high levels of atypical stereotyped and repetitive behaviors and inappropriate adherence to interests and routines (Fountain et al., 2012; Langen et al., 2011b). Caregivers commonly report that these behaviors are among the most challenging aspects of ASD (Boyd et al. 2012), and the severity of repetitive behavior symptoms at preschool age has been linked with poor outcomes (Troyb et al. 2015)

Abnormal repetitive and stereotypic behaviors are also seen in other species, for example, following environmental distress. This may be expressed as pacing in birds, rocking and self-injurious behavior in monkeys and repetitive jumping, somersaulting and excessive grooming in mice. Some species of mice also display repetitive behaviors under typical laboratory conditions. For instance, deer mice (*Peromyscus maniculatus*) display high levels of spontaneous stereotypic vertical jumping and backward somersaulting when reared in standard rodent cages (Tanimura et al., 2008, 2011). Interestingly, deer mice display natural variation in the expression of stereotypic behaviors, perhaps similar to continuous variation in autistic traits in the human population. In addition, high rates of stereotypies were correlated with poor performance in a T-maze reversal learning task and associated with decreased activity of the indirect basal ganglia pathway (Tanimura et al., 2008, 2011). Indeed, cognitive inflexibility and adherence to routines have also been associated with repetitive behaviors in ASD (Langen et al., 2011a).

Repetitive behaviors in genetic mouse models for ASD are predominantly assessed by the analysis of self-grooming (Silverman et al., 2010). For example, increased levels of self-grooming have been described in many genetic models, including in *Shank1*, *Shank2 and Shank3* gene knockout mice (Kalueff et al., 2016). In addition, repetitive and perseverative behavior is assessed in the marble-burying assay based on the number of marbles buried underneath the bedding material within a defined period of time (Thomas et al., 2009). Cognitive flexibility has been assessed in genetic mouse models of ASD using a variety of reversal learning paradigms (Kas et al., 2014). For instance, choice learning and reversal may be based on olfactory, textural, visual or spatial differences between cues associated with a reward (Bissonette and Powell, 2012; Brigman et al., 2013; Molenhuis et al., 2014). Additional assays are being developed to assess other relevant aspects related to inflexibility and repetitive behaviors, such as repetitive locomotor patterns, restricted interests and resistance to change (Bonasera et al., 2008; Karvat and Kimchi, 2013; Pearson et al., 2011). These assays may be used to further understand the impact of ASD-related genetic variation on the development of repetitive behaviors in ASD. However, it is currently unknown how the expression of stereotyped and repetitive behaviors in rodents relates to those observed in ASD.

Behavioral Development

Regardless of *what* to measure, another issue is *when* to measure. A striking aspect of ASD studies is that the majority of studies characterize genetic models at adult ages, despite the fact that ASD is regarded as a disorder of neurodevelopment. ASD is heterogeneous with respect to symptom manifestation, and the trajectories

of repetitive and social behavior can be highly variable over time (Fountain et al., 2012). Mouse models offer unique opportunities to study behavioral development, because mice reach their adult stage at around 8 weeks after birth.

Genetic background and genotype-phenotype relationships

Genotype–phenotype relationships are typically investigated in a single mouse inbred strain genetic background. However, a disadvantage of this approach is that the behavioral expression of a genetic disorder may very well depend on the genetic background composition of the individual (Sittig et al., 2016).

For example, expression of ASD-related behaviors in *Fmr1* knockout mice depends on the genetic background strain in which the mutation was tested (Pietropaolo et al., 2011). Deficits in social interaction and sensory hyper-responsiveness were observed on both C57BL/6 and FVB backgrounds, while aggressive tendencies and expression of repetitive behaviors were only observed in *Fmr1* knockout mice with the C57BL/6 background.

In another study, female mice with heterozygous *Fmr1* knockout in the C57BL/6J background were bred with males from six different inbred strains (A/J, DBA/2J, FVB/NJ, 129S1/SvImJ, C57BL/6J and CD-1) (Spencer et al., 2011). Across hybrids, motor stereotypies and increased marble burying were only observed in *Fmr1* knockouts with the DBA/2J background. Moreover, social interaction was only effected by deletion of *Fmr1* in the DBA/2J or C57BL/6J hybrid background, and passive social behaviors were only decreased in hybrids derived from the 129S1/SvImJ inbred strain. Genetic background effects on genotype–phenotype relationships were also suggested in other genetic models for ASD, including in *Nlgn3* (Chadman et al., 2008; Tabuchi et al., 2007) and *Shank3* (Mei et al., 2016; Peca et al., 2011) mutants.

Some phenotypes may be more "resistant" to genetic background effects than others. For example, sensory hyper-responsivity in *Fmr1* knockout mice is commonly observed across genetic backgrounds, which may indicate that this phenotype is more closely related to the genetic dysfunction. These examples indicate that genetic background and the choice of phenotypes are critical to the study of genotype–phenotype relationships in the context of ASD.

FORWARD GENETIC APPROACHES IN MICE

Current approaches to study ASD-related behaviors in mouse models are practically limited by the number of genes that can be studied and the amount of behavioral outcome measures that are assessed. Would it be possible to screen all ASD-

implicated human genetic mutations through an extensive behavioral testing battery, and across a wide variety of genetic backgrounds?

Indeed, human genetic studies in ASD has indicated an overwhelming diversity in the genetic landscape, with between 400 and 1000 genes estimated to be involved (Geschwind and State, 2015). Rare variants have a large contribution to disease risk on an individual level, but their relevance to individuals with a different mutation, or common genetic susceptibility, may be limited. Common genetic variation may play a role in many cases but likely has a low contribution on an individual level. Understanding how all these genetic variants contribute to the expression of autistic behaviors and co-morbidities is a great challenge.

Given practical limits to the number of genes and genetic backgrounds to be studied via reverse genetic approaches in mice, systematic analysis of genotype-phenotype relationships is challenging. Another strategy to study the relation between genotypes and the expression of ASD-related behavioral phenotypes in mice would be to start with behavioral expression in mice and systematically investigate the impact of natural genetic variation, including in genes related to ASD.

Continuous variation in ASD-related behavioral traits

Autistic traits are continuously distributed in the general human population, and influenced by common and rare genetic variation implicated in ASD (Gaugler et al., 2014; de la Torre-Ubieta et al., 2016; Robinson et al., 2016). The distribution of autistic traits and associated genetic variation in the general human population suggests the possibility to study ASD-related traits in animal populations, by making use of natural genetic variation (Kas et al., 2009).

Behavioral variation is commonly observed across different inbred and outbred strains of mice. For example, the FVB/NJ strain expresses very high levels of social interaction, while A/J and BTBR T+tf/J strains are notorious for low sociability (Bolivar et al., 2007). Strain differences have also been observed in mouse exploratory behaviors and reversal learning paradigms (Moy et al., 2008). Sociability and reversal learning were found to dissociate across strains (Moy et al., 2007), which may parallel similar "fractionation" of ASD-related traits across humans (Ronald and Hoekstra, 2011).

These innate behavioral differences can be used to study behavioral phenotypes in mice as a quantitative trait to identify natural genetic variants contributing to continuous trait variation. For example, multiple quantitative trait loci (QTLs) for sociability and juvenile social interaction were identified using an F2 intercross of the BTBR T+tf/J and C57BL/6 inbred strains (Jones-Davis et al., 2013). The strongest

detected QTL explained 10% of behavioral phenotypic variation, suggesting that social behavior abnormalities in BTBR T+tf/J mice are caused by a variety of genetic loci, each explaining part of the continuous behavioral trait variation between BTBR T+tf/J and C57BL/6 inbred strains.

In another study, genetic mapping of social recognition memory using chromosome substitution strains (CSS) revealed specific involvement of the *Pcdh9* gene in social recognition memory and sensory cortex development. This gene had previously been associated with ASD, indicating that social recognition in mice is influenced by natural genetic variation with relevance to ASD in humans (Bruining et al., 2015b).

Modeling the quantitative nature of autistic traits

A powerful tool to further dissect the genetic and neurobiological underpinning of behavioral trait variation is through the use of genetic reference populations. For example, the BXD panel consists of a large collection of mouse recombinant inbred lines with a "mosaic" genetic structure derived from C57BL/6J and DBA/2J founder strains, and captures about 10% of the known natural genetic variations in mice (Peirce et al., 2004). Forward genetic analysis in 51 BXD inbred lines led to the discovery of a reversal learning QTL on chromosome 10, and subsequent analysis of available brain mRNA levels identified that expression levels of *Syn3*, *Nt5dc3*and *Hcfc2* correlated with the reversal learning phenotype (Laughlin et al., 2011). Reversal learning in these lines was also correlated with expression of ventral midbrain dopamine D2 receptors, which had previously been reported in the same BXD inbred lines (Jones et al., 1999; Klanker et al., 2013; den Ouden et al., 2013).

The Collaborative Cross (CC) is a promising novel genetic reference population that consists of mouse recombinant inbred lines derived from eight founder strains. The CC population captures 90% of the known genetic variation in mice, which is equal to about two times the number of common genetic variants in the human population (Churchill et al., 2004; Gralinski et al., 2015; Keane et al., 2011; Schughart et al., 2013). In incipient CC lines, investigation of general behavioral traits indicated wide-ranging variation (Aylor et al., 2011). Moreover, the CC population was found to have strong power to identify quantitative trait loci underlying complex traits (Durrant et al., 2011). Three of the eight founder strains of the CC population were wild-derived. Indeed, the presence of wild-derived genetic variation gives unprecedented opportunities to study the genetic basis of fitness-driven behavioral responses, as classical laboratory strains and reference populations were obtained after decades of human-driven artificial selection, inbreeding and adaptation to captivity (Chalfin et al., 2014; Chesler, 2014).

Hundreds of genes contribute to ASD-liability, and the additive effect of common genetic variation plays a major role. In mouse populations, natural genetic variants may contribute to variation in the expression of ASD-related behaviors. Given the homology between human and mouse genes, and the myriad of genes involved in ASD, it is likely that genes involved in ASD in humans overlap with genes influencing ASD-related behaviors in mice. However, the extent of this overlap remains to be resolved.

Forward genetic approaches in genetic reference populations such as the CC population allow to investigate the natural genetic diversity underlying autistic-like behavioral trait variation. Ultimately, integration of both reverse and forward genetic approaches (Williams and Auwerx 2015) will help to define relevant phenotypic readouts for ASD-related features in preclinical animal studies.

CONCLUSIONS

Behavioral genetic studies in rodents can highly contribute to the understanding of behavioral trait variation relevant to autism spectrum disorder (ASD). In this chapter, the value of both forward and reverse genetic behavioral approaches has been addressed. Generally speaking, reverse genetic strategies investigate the impact of candidate human risk genes on behavioral phenotypes using genetically modified rodents with a fixed genetic background. In contrast, forward genetic strategies make use of genetic background variation to identify natural genetic variants contributing to behavioral trait variation in the mouse population. These approaches allow for systematic studies under controlled genetic and environmental conditions. However, the challenge is the selection of behavioral phenotypes and testing paradigms in view of translatability to the human ASD core features of social interaction deficits and stereotyped and restricted behavioral expression.

Thus far, a wide variety of behavioral testing paradigms have been proposed to capture face validity for ASD-related behavioral characteristics (Kas et al., 2014; Silverman et al., 2010). Interestingly, the application of these testing paradigms in reverse genetic approaches has revealed behavioral deficits in a wide variety of mouse line mutants for human ASD risk genes. For example, *Fmr1* gene knockout mice showed aberrant social and repetitive behaviors, such as social recognition deficits and stereotyped behaviors in a marble-burying task. The outcome of these studies was variable and depended on the genetic background on which the gene knockout was generated. To address controlled genetic background variability in a systematic manner, newly generated mouse genetic reference populations, such

as the Collaborative Cross have been generated. Applying these forward genetic strategies to a select set of phenotypes will provide insights in behavioral trait variation relevant to ASD.

Here, we propose that the selection of phenotypes that contribute to survival of a species may be a way forward to identify behavioral trait variation relevant to ASD. For example, by focusing on essential behavioral strategies, such as social recognition, candidate genes that contribute these phenotypes (e.g. the *Pcdh9* gene) have recently been identified. Subsequent studies using a reverse genetic approach with a *Pcdh9* mutant mouse line indicated a possible link between essential social behavioral phenotypes, sensory cortex development and sensory information processes (Bruining et al., 2015b). In this way, translational biological processes that are relevant to ASD may be identified for further functional studies, and their impact can be systematically studied across genetic backgrounds.

Aims and outline

The value of preclinical animal studies into ASD can be increased by improving behavioral phenotyping strategies as well as by better modelling of genetic risk constellations. So far, most animal models are based on single rare genetic mutations tested in only one genetic background, while there is increasing evidence that findings from a single background are often not generalizable to other genetic backgrounds (Sittig et al., 2016). Secondly, most animal studies have focused on independent traits measured at adult age, while ASD is developmental disorder that emerges early in life (Fountain et al., 2012). Therefore, in this thesis I have aimed to:

1. Establish a longitudinal test battery for the characterization of mouse behavioral and cognitive domains across development.
2. Characterize these longitudinal trajectories in mice in a genetic model for ASD and developmental retardation.
3. Improve behavioral phenotyping strategies for animal models for ASD by assessing the impact of environmental novelty on behavioral testing.
4. Establish animal model approaches that take into account the quantitative and polygenic nature of ASD-like traits in the human population.

The work presented in this thesis aims to bridge the gap between preclinical animal studies and the heterogeneous human population. To obtain translational animal models for ASD, it is of vital importance to expand mouse phenotyping strategies across developmental stages and to take into account genetic background variability in a systematic and reproducible manner. Without these improvements, treatments to improve the core symptoms of ASD will remain elusive.

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Chapter 1

Enhancing the value of psychiatric mouse models; differential expression of developmental behavioral and cognitive profiles in four inbred strains of mice

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ABSTRACT

Background

The behavioral characterization of animal models of psychiatric disorders is often based upon independent traits measured at adult age. To model the neurodevelopmental aspects of psychiatric pathogenesis, we introduce a novel approach for a developmental behavioral analysis in mice.

Methods

C57BL/6J (C57) mice were used as a reference strain and compared with 129S1/SvImJ (129Sv), BTBR T+tf/J (BTBR) and A/J (AJ) strains as marker strains for aberrant development. Mice were assessed at pre-adolescence (4 weeks), adolescence (6 weeks), early adulthood (8 weeks) and in adulthood (10–12 weeks) on a series of behavioral tasks measuring general health, neurological reflexes, locomotor activity, anxiety, short- and long-term memory and cognitive flexibility.

Results

Developmental delays in short-term object memory were associated with either a hypo-reactive profile in 129Sv mice or a hyper-reactive profile in BTBR mice. Furthermore, BTBR mice showed persistent high levels of repetitive grooming behavior during all developmental stages that was associated with the adult expression of cognitive rigidity. In addition, strain differences in development were observed in puberty onset, touch escape, and body position.

Conclusions

These data showed that this longitudinal testing battery provides sufficient behavioral and cognitive resolution during different development stages and offers the opportunity to address the behavioral developmental trajectory in genetic mouse models for neurodevelopmental disorders. Furthermore, the data revealed that the assessment of multiple behavioral and cognitive domains at different developmental stages is critical to determine confounding factors (e.g., impaired motor behavior) that may interfere with the behavioral testing performance in mouse models for brain disorders.

INTRODUCTION

The causes of most psychiatric disorders are still unknown. One of the major obstacle in finding etiologies is the clinical and genetic heterogeneity of these disorders (Bruining et al., 2010; Geschwind and Levitt, 2007; Kas et al., 2007; Laporte et al., 2008). It is assumed that the onset of most psychiatric disorders is already in early development (Paus et al., 2008), but is has proven difficult to track the onset and early stages of these disorders (Wittchen et al., 2011). In this context, studies using animal models can contribute considerably to the understanding of the development of psychiatric disorders as they allow systematic studies of phenotype expression in a controlled genetic background and environment (Kalueff et al., 2008; Kas et al., 2011; Tecott and Nestler, 2004).

Most studies using these models predominantly focus on adult ages and surpass earlier windows of development that may already be affected. One study considered the importance of social development and focused on aspects directly related to social deficits (Ricceri et al., 2007). Taking into account the diversity of social and non-social impairments, as well as the cognitive performance deficits in neurodevelopmental disorders, extension of a longitudinal phenotype battery approach for these heterogeneous disorders is needed.

Recently, several new strategies have been proposed to improve and refine phenotype assessments in psychiatric research, such as the endophenotype-approach (Gould and Gottesman, 2006) and cross-species trait genetics (Kas et al., 2007). Another suggested approach is the domain-interplay concept (Kalueff et al., 2008) that advocates a more integrated continuum with common genetic and environmental determinants. This concept is based on assessing the interplay between distinct behavioral domains in relation to genetic susceptibility but has thus far not been thoroughly tested.

To extend the use of mouse models into a wider developmental window, we measured both basal motor and neurological development as well as the emergence of cognitive abilities starting from 4 weeks of age in relation to later behavioral outcome. We systematically investigated these in four genetically defined inbred strains of mice from early adolescence into adulthood in order to detect distinct behavioral profiles and the way they change over time in relation to genetic background. Mouse strains C57BL/6J, 129S1/SvImJ, BTBR T+tf/J and A/J inbred mouse strains were chosen based on known behavioral contrast in the absence of gross abnormalities in general health. C57BL/6J mice were selected as they are commonly used as a reference or control strain, as they show sufficient exploratory motivation and do not show extremes in the affective domain and

perform well on cognitive tasks (Brigman et al., 2012; Crawley, 1999; Rogers et al., 1999; Ryan et al., 2010). By contrast, 129S1/SvImJ and A/J are known for their low exploratory drive and mild cognitive impairments. For example, all 129 derived strains carry a natural mutation in the Disc1 gene (Clapcote and Roder, 2006) that is associated with impaired working memory performance in this strain (Kvajo et al., 2008). Furthermore, BTBR T+tf/J mice are currently in favor as a model for neurodevelopmental disorders as they show several features related to autism and fragile-X syndrome, such as impaired social interaction, cognitive deficits and high levels of repetitive behaviors (Amodeo et al., 2012; McFarlane et al., 2008; Pearson et al., 2011).

METHODS

Animals

Breeding pairs of C57BL/J (C57), 129S1/SvImJ (129Sv),BTBR T+tf/J (BTBR) and A/J (AJ) strains were originally purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and further bred at the University Medical Center Utrecht, The Netherlands. Male mice were weaned at postnatal day 28 (P28, 4 weeks of age), ear punched for identification purposes and housed with litter mates in groups of 2–4 mice per cage.

General measures

Litter size and the onset of puberty were measured during weaning on P28 (4 weeks of age), body weight was measured at three different time points during adolescence (4, 6 and 8 weeks of age) and adulthood (between 10 and 12 weeks of age). Onset of puberty was determined by assessing the progression of balano-preputial separation (BPS) (Deboer and Li, 2011; Zhou et al., 2007) and scored as either 0 (no separation), 1 (separation but not full) or 2 (full separation).

Extended SHIRPA screen (eSH)

The extended SHIRPA screen used in the present study was a modified version of the standard SHIRPA primary screen that is widely used to assess basic sensorimotoric functions and various reflexes in mice (Lalonde et al., 2005; Rogers et al., 1999). Behavior during the test was recorded with an overhead camera. Mice are placed in a viewing jar to assess body position, tremor, palpebral closure, coat appearance, whiskers, lacrimation, defecation. Consequently the mice are transferred to a novel arena to observe: transfer arousal, locomotor activity, gait, tail elevation, startle response, touch escape. Further measures include: positional passivity, skin color,

trunk curl, limb grasping, pinna reflex, corneal reflex, contact righting reflex, evidence of biting and vocalization. A table of SHIRPA measures and scoring system can be found in the Supplementary material (Table S1).

During the arena-phase of the SHIRPA screen, additional measures were taken, to extend the number of behavioral domains that could be measured. Integrating multiple measures in a single test was done to avoid extensive and repeated testing in adolescent mice that may have interfered with the normal development of the mice due to stress-related factors.

Additional measures to the SHIRPA screen were taken during the novel arena-phase and included: (a) the automated recording of locomotor activity by video-tracking (EthoVision, Noldus Information Technology, Wageningen, The Netherlands), (b) the duration of self-grooming as a measure for repetitive behavior (Silverman et al., 2010a, 2010b) and (c) novel object discrimination as a measure of short-term memory. For novel object recognition the following procedure was used (Laarakker et al., 2008): mice were exposed to two objects of different materials but of similar size, while in the novel arena. One of the objects was familiar as mice were habituated to a similar object in their home cage for at least 48 h prior to testing. The other object was novel. Time spent exploring each of the objects was recorded and discrimination capacity was calculated as the duration of exploration of the novel object (D_n) divided by the sum of the time spent exploring both the novel (D_n) and familiar (D_f) objects: r =D_n/(D_n+D_f). A ratio value above 0.5 indicated a successful discrimination between novel and familiar object. Familiar objects and position of the objects in the arena were counterbalanced across subjects. The size of the objects was adjusted to the body size of the animal: smaller objects at 4 and 6 weeks of age, larger objects at 8 weeks of age and for adults. Objects were a dice and screw (4 weeks of age), a square Duplo building block and domino piece (6 weeks of age), a small round one-shot glass and a square ceramic egg cup (8 weeks of age), a round small glass with print and a round ceramic egg cup (adults). In a pilot study, no baseline preference was found for one of the objects of a pair when naïve mice were allowed to explore the objects for 5 min. Self-grooming behavior and object exploration were recorded manually using The Observer XT software (Noldus Information Technology, Wageningen, The Netherlands).

Open field (OF)

Spontaneous locomotor activity in a novel environment was measured by exposing mice to an open field test (see Supplementary materials).

Elevated plus maze (EPM)

Anxiety-related behavior was assessed in the elevated plus maze test that makes use of the natural tendency of rodents to avoid open spaces (see Supplementary materials).

Object discrimination (OD)

Short- and long-term memory in a non-social context was measured during the novel object discrimination task. The task measures whether mice are able to discriminate between a novel and a familiar object based on the innate preference of mice for novel over familiar stimuli. First, mice were exposed to an empty, transparent testing cage with 2 identical objects for 5 min (T0). After an intertrial interval (ITI) of 1 h (short-term memory, T1), mice were retested with 1 of the objects is replaced with a novel one. This procedure was repeated after an ITI of 24 h to test long-term memory (T24), but with a different novel object. Familiar objects and the location of the objects in the cage were counterbalanced across animals. Objects used were a round tall glass with print, a round metal bottle and a round plastic bottle. Mice were unable to mount the objects. The time spent exploring the objects was recorded manually using The Observer 4.0 (Noldus Information Technology, Wageningen, The Netherlands). Discrimination capacity was calculated as the preference for the novel versus the familiar object following the formula: tN/(tN+tF), where tN is the time spent exploring the novel object and tF is the time spent exploring the familiar object. A ratio value above 0.5 indicated a successful discrimination between novel and familiar object.

Passive contact with the object (e.g. when the animal was sitting immobile but close to the object) was not scored as object exploration. Data from subjects with a total object exploration time of less than 5 s, or that explored one of the objects for less than 2 s, were excluded from the analysis. This was true for 1 129Sv mouse for both the T1 and T24 interval.

Social discrimination (SD)

Short- and long-term memory in a social context was measured during the social discrimination task. First, mice were allowed to habituate to a clean transparent mouse cage with bedding for 5 min. Then, they were exposed to an unfamiliar male stimulus animal of the A/J inbred strain for 2 min (T0). After an ITI of 5 min during which the mice stayed in the testing cages, mice were retested and exposed to both the familiar animal of T0 and a novel, unfamiliar stimulus animal for 2 min (short-term memory, T1). This was repeated 24 h later to test long-term memory (T24) with a different novel animal. The time spent exploring the stimulus animals was manually recorded using The Observer XT (Noldus Information Technology, Wageningen, The Netherlands).

Discrimination capacity was calculated as the preference for the novel versus the familiar animal following the formula: tN/(tN+tF), where tN is the time spent exploring the novel animal and tF is the time spent exploring the familiar animal. A ratio value above 0.5 indicated a successful discrimination between novel and familiar animal. Aggressive behaviors and or fighting resulted in experiments to be ceased as these behaviors interfere with reliable measurements of social discrimination capacity.

Home cage screen (HC)

Baseline levels of locomotor activity and food/water intake were measured during the home cage screen (Kas et al., 2008). Mice were housed individually in automated home cages (PhenoTyper and EthoVision 3.0, Noldus IT, Wageningen, NL) for four consecutive days. See Supplementary materials for more details.

Set shifting and reversal task (SSR)

Mice were tested on a variant of the reversal/set-shifting task developed by Brown and colleagues for rats (Birrell and Brown, 2000) and adapted for mice (Bissonette et al., 2008; Colacicco et al., 2002; Garner et al., 2006). The task was performed according to (Bissonette et al., 2008) with minor modifications. Mice were required to learn the location of a hidden food reward in one of two food cups that are placed in a testing cage. The reward was hidden under a scented digging medium. Odor and digging medium represented two different dimensions, mice had to learn to focus on the relevant dimension and ignore the other dimension during the different learning phases. Different combinations of odors and digging media were used. The task consisted of 8 sub-tasks: simple discrimination, compound discrimination, intradimensional shifts I–IV, intra-dimensional shift IV reversal, extradimensional shift. During simple discrimination, mice had to associate either a medium or odor to the reward, the second dimension was kept constant for both cups. During compound discrimination, both dimensions were different, mice had to learn to ignore the non-relevant dimension and focus on the previously learned association between a certain medium or odor and the reward. During the four intradimensional shifts, the relevant dimension did not change, but the specific pairings within the dimension were changed in such a way that the animal had to learn a new association in every task. During the reversal task, the cues stayed the same, except that the previously rewarded medium or odor was no longer reinforced. Thus, mice had to switch to choose the previously not rewarded cue. During the extradimensional shift, mice had to learn to ignore the previously relevant dimension, as this was no longer associated with the reward. Thus, if an animal had started with odor as the relevant dimension, it now had to switch to medium. Each phase lasted about

8–30 trials, depending on the learning rate. Criterion for acquisition of each sub-task was set at 8 out of 10 correct trials. Mice were excluded after not digging for 10 consecutive 3 min trials. The total task duration was 3 days. Three days prior to this task, mice were housed solitary and food restricted up to 85% of their ad libitum body weight to ensure motivation. The latency to find the reward, the number of trials to reach criterion and the number of errors were recorded for each of the sub-tasks.

Experimental procedure

All mice were tested consecutively from early adolescence until adulthood during 4 different time points. A schematic figure of the different behavioral measurements taken across time is depicted in Fig. 1. During adolescence at 4, 6 and 8 weeks of age, mice were tested on the eSH screen only. As adults (between 10 and 12 weeks of age), mice were exposed to a larger battery of tests and divided over 2 separate batches to avoid a too intensive testing schedule for each individual animal. Details on the order of behavioral tasks performed per time point and batch are presented in Table S2. As breeding was routinely performed under normal light conditions in the animal facility, adolescent mice were tested under a normal light-dark cycle (white lights on from 7.00–19.00). At 8 weeks of age, after the third eSH test, animals were transported to a room with a reversed light-dark cycle (white lights on from 19.00 to 7.00) where they were left to habituate to these conditions for a minimum of 2 weeks until further testing.

Statistical analysis

Strain differences in task parameters were determined using one-way ANOVA (owANOVA). For repeated measurements, a repeated measures ANOVA (rmANOVA) was performed with 'time' as within-subjects factor and 'strain' as between-subjects factor. In case of a significant *F*-value, post-hoc comparisons were performed using a Scheffé test. Discrimination ratios in the eSH, OD and SD tasks were tested against chance level (a ratio of 0.5) within strain for each interval (T1 and T24) using a one-sample *T*-test. The puberty and SHIRPA scores were not normally distributed and therefore compared using a non-parametrical Kruskal–Wallis test with Mann–Whitney *U* tests for post-hoc comparisons. Significance was set at $p<0.05$. Values of 3×SD above or below the mean were treated as statistical outliers and excluded from further analysis. This was true for 2 mice during the eSH task, 3 mice during the OD task and 1 mouse during the SD task. SPSS 20.0 for Windows was used for analyses.

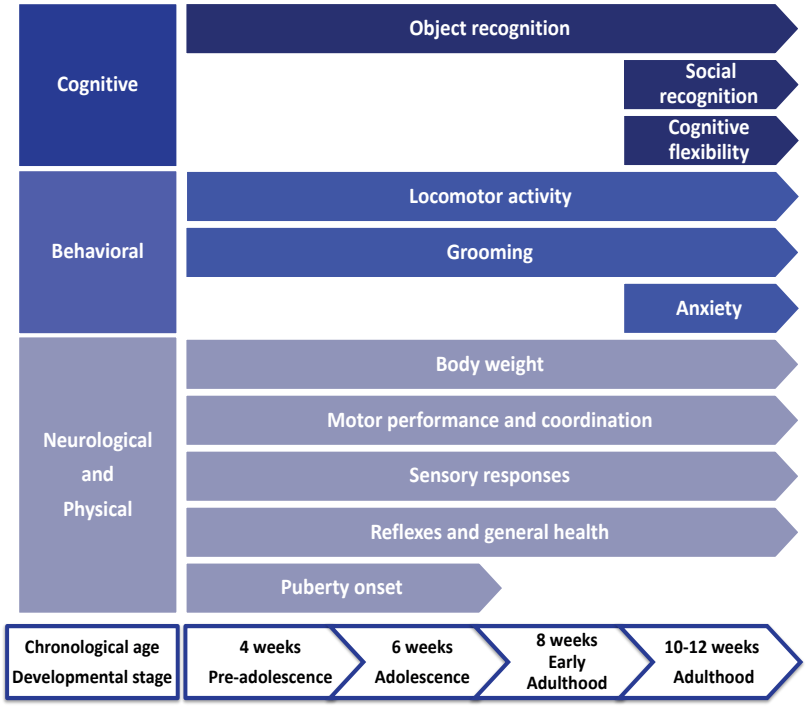

Figure 1. Schematic figure of the different phases in behavioral testing.

RESULTS

Litter size, puberty onset, body weight

Litter size of BTBR mice (9.20±0.58) was largest, followed by 129Sv (7.33±098), C57 (5.00±0.53) and AJ mice (4.80±0.61) ($F_{(3,26)}$=8.206, p=0.001). Strain differences were apparent in the progression of balano-preputial separation (Kruskall–Wallis test, p<0.001). BTBR and AJ mice showed full balano-preputial separation at P28 (median score=2), while puberty onset was slightly delayed in C57 mice (median score=1.5). 129Sv showed least progression in BPS (median score=1). By 6 weeks of age, all mice showed full BPS (data not shown).

Body weight followed a similar trajectory in all strains (rmANOVA, strain×time: $F_{(9,207)}$=0.809, p=0.609, NS) reflected by a steady increase from P28 until adulthood (Fig. 2A). However, BTBR mice showed in general a higher body weight than C57, 129Sv and AJ mice that was already apparent at 4 weeks of age (rmANOVA, strain: $F_{(3,69)}$=72.044, p<0.001, post-hoc between strains p<0.01).

Development of behavior throughout adolescence

In the SHIRPA screen, we did not see any significant changes over time (except for the coat appearance in BTBR mice, see below), thus average scores per strain across time points are presented (Supplementary Table S3). As anticipated, no strains were found to have any gross abnormalities or neurological deficits. Strain differences were however noted in body position, coat appearance and touch escape. 129Sv and AJ mice were less active while in the viewing jar than BTBR and C57 mice (Kruskal–Wallis test $p<0.001$, Mann–Whitney *U*, 129Sv and AJ vs. BTBR and C57 $p<0.005$). 129Sv mice did not respond to touch, while C57, BTBR and AJ mice did (Kruskal–Wallis test $p<0.001$, Mann–Whitney *U*, 129Sv vs. AJ, BTBR and C57 $p<0.005$).

Developmental trajectories of locomotor activity clearly differentiated between strains (rmANOVA, strain×time: $F_{(9,114)}=8.366$, $p<0.001$; Fig. 2B). C57 mice showed an increase in activity levels between 4 and 6 weeks of age after which it stabilized. 129Sv and A/J mice had low levels of activity throughout development. In 129Sv mice, activity levels continued to increase through adolescence until adulthood, indicating a delayed development of activity compared to C57 mice. BTBR mice showed a reversed trajectory as activity levels started at very high levels at 4 weeks of age and decreased over time, indicating a hyperactive profile in young adolescent BTBRs.

Pronounced strain differences were observed in the development of repetitive behavior, measured as the time spent on self-grooming in the novel arena, across ages (rmANOVA, strain×time: $F_{(9,93)}=2.025$, $p<0.045$; Fig. 2C). BTBR mice, well-known for their high-levels of self-grooming, in fact do not differ in self-grooming levels from C57 mice at the age of 4 weeks. However, whereas C57 mice show a steady decline in repetitive behavior over time, self-grooming in BTBR mice did not decrease and even showed a slight increase between 4 and 6 weeks of age. 129Sv mice displayed overall low levels of self-grooming and no change was observed over time (rmANOVA, time: $F_{(3,27)}=0.771$, $p=0.520$, NS).

Object memory was evident in C57 mice from 6 weeks of age, whereas 129Sv and BTBR showed a delay in the development of object memory as discrimination capacity in these strains was not significantly different from chance levels until 8 weeks of age (Fig. 2D). AJ mice lacked discrimination capacity throughout development.

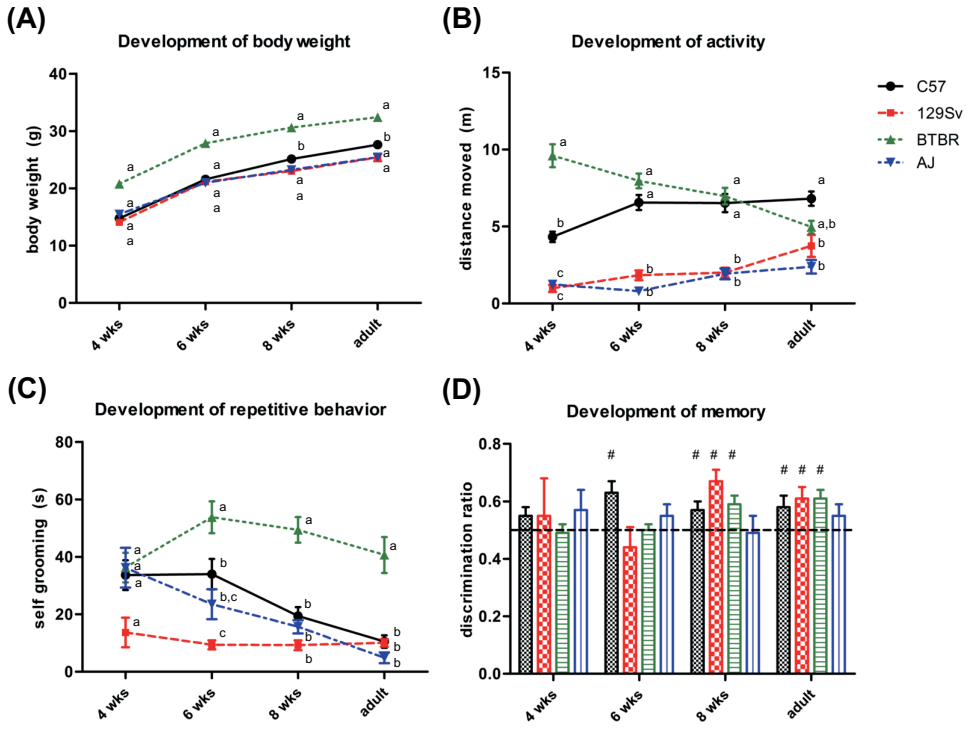

Figure 2. Developmental trajectories from early adolescence until adulthood in male C57, 129Sv, BTBR and AJ mice for (**A**) body weight, (**B**) locomotor activity, (**C**) repetitive behavior and (**D**) object memory. X-axis represents the different time-points of measurements. Per time-point the mean±S.E.M. per strain are presented. Different characters represent significantly different strain means (post-hoc Scheffé, p<0.05). In panel D # represents within-strain significant difference from 0.5 (one-sample T-test).

Adult behavior

Locomotor activity differentiated between inbred strains under both novelty (owANOVA, $F_{(3,49)}$=69.4, p<0.0001) and baseline conditions (owANOVA,$F_{(3,37)}$=8.900, p<0.001) (Fig. 3A). Notably, whereas BTBR mice showed high levels of novelty-induced locomotion in the open field test, baseline activity during the home cage screen were relatively low, suggesting that activity levels in this strain is context-dependent. Strain differences were found for the time spent on the open arm of the elevated plus maze (owANOVA, $F_{(3,48)}$=25.589, p<0.0005). BTBR mice spent a large amount of time on the open arm, whereas AJ spent very little time on the open arm, compared to C57 and 129Sv mice (Fig. 3B). These results were consistent with

other EPM measures such as the number of open arm entries and percentage of time spent on the open arm (Supplementary Table S4). These trends were paralleled with strain differences in total distance moved (owANOVA, $F_{(3,48)}$=86.114, p<0.0005). C57 mice showed superior performance on both the object and social discrimination task as they were capable of successful discrimination of the novel stimulus on both the short- and long-term tests (Fig. 3C and D). Long-term memory was impaired in 129Sv mice on both the object and social task, indicating a memory deficit independent of context. In BTBR mice however, long-term memory was specifically impaired in social discrimination but left intact in object discrimination. In contrast, AJ mice lacked both short- and long-term object discrimination, while social memory was intact.

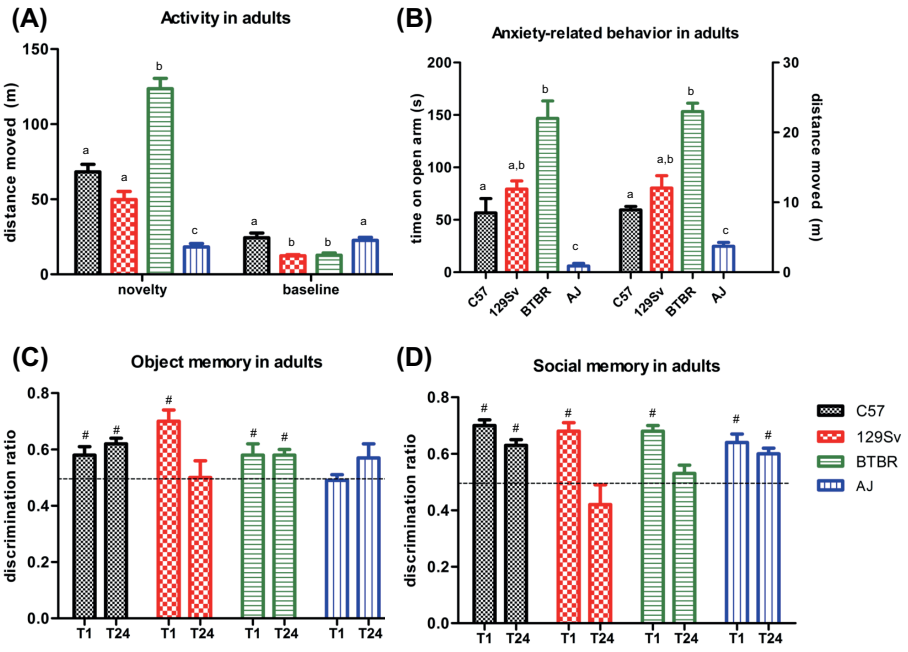

Figure 3. Strain differences during adulthood in (**A**) locomotor activity, (**B**) anxiety-related behavior, (**C**) short- and long-term object memory and (**D**) short- and long-term social memory. Means±S.E.M. per strain are presented. Different characters represent significantly different strain means (post-hoc Scheffé, p<0.05). In panels C and D, # represents within-strain significant difference from 0.5 (one-sample T-test).

Cognitive flexibility

Adult mice were assessed for cognitive flexibility on the set-shifting/reversal task that measured both simple discrimination capacities as well as the ability to shift within and between dimensions and to adjust behavior in response to reversed contingencies (Fig. 4). AJ mice were excluded from this task as they made 10 consecutive no-digs during habituation, simple or compound discrimination. Consistent with their enhanced suppression of novelty-induced locomotor behavior (Fig. 3A), A/J mice showed a significantly reduced amounts of no-digs due to reduced exploratory behavior. C57, 129Sv and BTBR mice performed equally well on simple, compound discrimination and intra- and extra-dimensional shifts (owANOVA, $p>0.100$, NS). However in the reversal learning task, BTBR mice made significantly more errors before criterion was reached (rm-ANOVA, $F_{(14,161)}=1.754$, $p=0.050$, post-hoc Scheffé, BTBR vs. C57 and 129Sv, $p<0.05$; Fig. 4) and needed more trials to reach criterion (rm-ANOVA, $F_{(14,161)}=2.068$, $p=0.016$, post-hoc Scheffé, BTBR vs. C57 and 129Sv, $p<0.05$; data not shown) than did C57 and 129Sv mice.

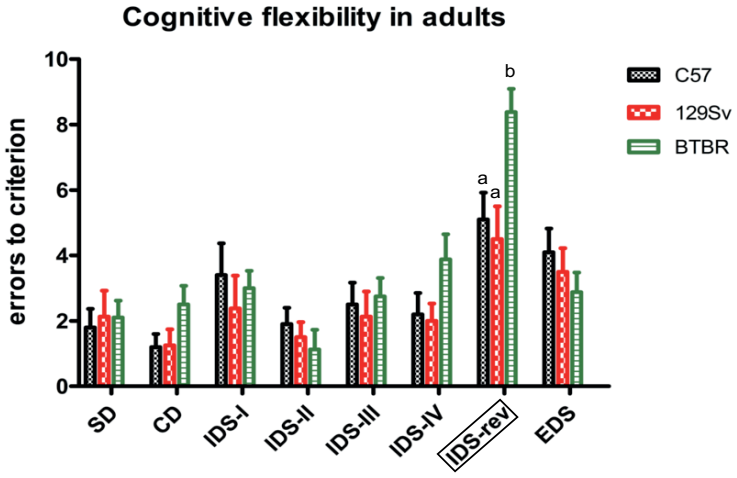

Figure 4. Strain differences in cognitive flexibility in adult mice on set shifting/reversal task. Y-axis represents the number of errors made before criterion was reached. X-axis represents the different sub-tasks: SD=simple discrimination, CD=compound discrimination, IDS I–IV=intradimensional shift I–IV, IDS-reversal=reversal of intradimensional shift IV, EDS=extradimensional shift. Different characters represent significantly different strain means (post-hoc Scheffé, $p<0.05$).

DISCUSSION

In the present study we systematically investigated multiple behavioral, neurological and cognitive domains in four inbred strains of mice from pre-adolescence into adulthood. This approach revealed distinct developmental profiles across the different genetic background strains. Furthermore, assessment of temporal changes in cognitive, behavioral and neurological parameters elucidated atypical developmental trajectories in, for instance, repetitive behavior, touch escape and puberty onset. Implementation of a longitudinal testing battery provides a basis for studying multi-dimensional behavioral and cognitive profiles across developmental stages in mouse inbred strains and, subsequently, in genetically modified mouse lines. In addition, the present data revealed that the assessment of multiple behavioral and cognitive domains at different developmental stages is critical to determine confounding factors that may interfere with the behavioral testing performance. For example, A/J mice expressed a strong suppression of novelty-induced locomotor activity, a behavioral component that strongly affects their outcome in a set-shifting task. Furthermore, they offer the possibility to relate morphological changes specific neural circuits at disease-related relevant developmental stages to parallel changes in behavioral and cognitive performance. These studies are eventually necessary to increase our understanding of the dynamic relationship between genetic, phenotypic and environmental heterogeneity observed in neuro-developmental disorders, such as ADHD and autism.

Strains are characterized by atypical developmental trajectories

In the present study, three behavioral and cognitive profiles could be distinguished, i.e. reactivity, behavioral flexibility and cognitive performance, that were differentially expressed between the tested strains. These differences were also accompanied by deficits across adolescent development.

Reactivity: Pronounced differences were found between strains in how they respond to external stimuli such as touch and novel environments. In general, 129Sv and AJ mice showed a hypo-reactive profile during development, reflected by overall low levels of locomotor activity and self-grooming and a diminished response to touch. BTBR on the other hand displayed an atypical trajectory of activity across adolescence, with hyperactivity during pre-adolescence that attenuated over time. In adulthood, open field and elevated plus maze data revealed a hyper responsiveness to novelty in BTBR mice compared to adult 129Sv, C57 and AJ mice, combined with a reduction in anxiety. The hyper responsiveness

is in line with previous reports on high locomotor activity in an open field and increased defensiveness upon predator exposure in BTBR mice (Moy et al., 2007; Pearson et al., 2011; Pobbe et al., 2011). The reduced anxiety-related behavior in BTBR mice observed in this study using the elevated plus maze is different from that observed in studies that reported either increased (Pobbe et al., 2011) or similar anxiety-related behaviors (Silverman et al., 2010a, 2010b) when compared to C57 mice. These behavioral differences in elevated plus maze data for BTBR across these three studies may relate to differences in testing conditions (e.g., testing in light versus in darkness, or apparatus dimensions). Thus, 129Sv, AJ and BTBR mice could be distinguished from C57 mice by a hypo- and hyper-reactive profile, respectively.

Behavioral flexibility: Developmental changes in repetitive behavior, i.e. self-grooming, were observed in C57, BTBR and AJ mice, but not in 129Sv mice for which levels of self-grooming were low throughout development. BTBR and C57 mice, however, showed similar (high) levels of self-grooming during pre-adolescence, but the decrease in repetitive behavior over time as seen in C57 and AJ mice was not found in BTBR mice. Repetitive behavior is a normal and adaptive phenomenon in typically developing children which declines with age (Evans et al., 1997). However, persistent repetitive behavior is an important symptom of many psychiatric disorders and may severely impair daily functioning of patients (Turner, 1999). The atypical trajectory of persistent repetitive behavior in BTBR mice specifically may represent a valuable translational phenotype of psychiatric disease.

The behavioral rigidity profile of BTBR mice was paralleled by cognitive rigidity that was observed in the set-shifting task. During the reversal learning task, adult BTBR mice showed more perseverative errors than C57 and 129Sv mice. These observations indicate that BTBR mice had difficulties in withholding their response to the stimulus that was previously associated with the food reward. This finding is in line with a recent report on cognitive inflexible behavior in BTBR mice in a probabilistic reversal learning task (Amodeo et al., 2012). As BTBR mice are also known for their high levels of repetitive behavior (Blanchard et al., 2012; Pearson et al., 2011), there may be a common corticostriatal pathway involved in both motor and cognitive rigidity (Langen et al., 2011). Supporting this notion, an earlier study in deer mice also reported an association between repetitive behavior, i.e. self-grooming, jumping, and reversal learning deficits (Tanimura et al., 2011). Thus, persistent high levels of behavioral rigidity during adolescence may be predictive of cognitive rigidity later in life.

Cognitive performance: Considering the development of cognitive abilities, 129Sv and BTBR mice showed a clear delay in object discrimination compared to C57 mice, i.e. whereas C57 mice displayed successful discrimination capacity already at adolescence (6 weeks of age), 129Sv and BTBR mice showed this ability no earlier than young adulthood (8 weeks of age). AJ mice showed impaired discrimination capacity throughout development. Interestingly, specific long-term memory deficits were also observed in 129Sv, BTBR and AJ mice in adulthood, suggesting that a transient delay or lack of short-term memory may predict general adult cognitive deficits. Similar observations have been made in human studies and showed that short-term memory problems can indicate later learning or psychiatric problems (Gonzalez-Ortega et al., 2013; Johnson et al., 2011; Poirier et al., 2011). While adult 129Sv mice had general difficulties in the long-term discrimination of both object and conspecifics, AJ mice, showed impaired object discrimination throughout in both development and adulthood, while social discrimination was intact. In contrast, adult BTBR mice specifically showed impairments in long-term memory in a social context. This fits well with the current literature on BTBR mice as this strain is consistently showing social cognitive deficits in the absence of object recognition impairments (Chadman, 2011; Defensor et al., 2011; McFarlane et al., 2008).

The memory deficits observed in adult 129Sv and BTBR did not, however, prevent them from acquiring successful discrimination in the intra- and extra-dimensional set shifting task where they were required to learn the association between either an odor or digging medium with the location of a food reward. An explanation for this finding could be that, for the acquisition of discrimination learning in the set shifting task, multiple consecutive trials were performed that could have facilitated consolidation of memory. Furthermore, during this task mice were food deprived which could have increased the motivation to acquire the task and, therefore, enhanced memory formation.

Our findings support the concept of domain-interplay that was put forward previously by (Kalueff et al., 2008) who suggested that the *association* between domains of behavior rather than the domain or behavior *itself* may relate to genetic variants and neurobiological pathways. Furthermore, additional research is needed in order to understand how some of the observed early life neurological/sensory deficits (as observed in the extended SHIRPA) may influence the adult impairments in behavioral and cognitive performance. Understanding their functional relationship is highly relevant for the identification of early life indicators for these deficits and for unraveling their etiological mechanisms.

Clinical implications

Recent studies have detected typical patterns of behavioral development that drive different outcome in autism (Bolton et al., 2012; Fountain et al., 2012; Thomas et al., 2009) and ADHD (Pingault et al., 2011, 2013). It seems that the expression of repetitive and social behaviors in neurodevelopmental disorders is highly variable and warrants a more complete and developmental explanation of associated cognitive and behavioral deficits (Flores et al., 2011; Moss et al., 2009; Sayers et al., 2011). Other studies have indicated traits in adolescence that indicate trajectories towards schizophrenia (Cullen et al., 2011; Jahshan et al., 2010). Together, these studies indicate a shift from categorical phenotypes towards dimensional and developmental profiles. This movement should be paralleled by developmental screening of animal models. The developmental phenotypes we found in this study are promising in this respect as they indicate how common early cognitive defects may lead to common but heterogeneous adult phenotypes. The prospect of comparing human disorder trajectories to those observed in (genetic) animal models could be very powerful to detect novel etiological subtypes. These will be a valid starting point for early intervention in preclinical studies.

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Conflict of interest

All authors declare no conflict of interest with respect to the research presented in this manuscript.

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SUPPLEMENTARY INFORMATION

Materials and methods

Open field. The apparatus consisted of a circular arena with a diameter of 80 cm. Mice were exposed once during 15 min. Mice were placed near the wall of the arena. After each trial, the apparatus was thoroughly cleaned using paper towels and Trigene solution (0.5%). An overhead camera connected to a PC with video tracking software (EthoVision 7.0, Noldus Information Technology, Wageningen, The Netherlands) recorded the distance travelled for each animal.

Elevated plus maze. Mice were exposed to an elevated maze that was cross-shaped and consisted of a central platform and four arms, two of which are enclosed. Mice were put in on the center platform, facing an open arm, and allowed to freely explore the maze for 5 min. The time spent on, and number of entries into, the open and closed arms as well as the total distance travelled were recorded using an overhead camera and video tracking software (EthoVision 7.0, Noldus Information Technology, Wageningen, The Netherlands).

Home cage screen. Automated home cages consisted of clear, Perspex cages with a PhenoTyper topunit (Noldus Information Technology). The topunit featured a built-in infrared sensitive camera for videotracking, and infrared lighting and filter to allow videotracking independent of lighting conditions. The cages were equipped with bedding material, a shelter, feeding stations and water bottle. The location of the mouse in the cage was recorded continuously and distance travelled was calculated per day. Infrared sensors are placed at the feeding platforms and drinking bottle to allow recordings of food and water intake. As mice needed up to three days to adapt to the novel cage, data of day 4 was taken as a baseline measure.

Table S1. SHIRPA measures and scoresheet

Age	Task	batch	
Jar	Body position	0	inactive
		1	active
		2	excessive activity
	Tremor	0	absent
		1	present
	Palpebral closure	0	eyes open
		1	eyes closed
	Coat appearance	0	tidy and well-groomed
		1	irregularities, e.g. piloerection
	Whiskers	0	present
		1	absent
	Lacrimation	0	absent
		1	present
	Defecation	0	present
		1	absent
In arena	Transfer arousal	0	extended freeze (>5 s)
		1	brief freeze followed by movement
		2	immediate movement
Gait		0	fluid movement
		1	lack of fluidity
Tail elevation		0	dragging
		1	horizontal extension
		2	Straub tail
Startle response		0	none
		1	Preyer reflex
		2	reaction in addition to Preyer
Touch escape		0	no response
		1	response to touch
		2	flees prior to touch
Above arena	Positional passivity	0	struggles when held by tail
		1	struggles when held by neck
		2	struggles when laid supine
Skin color		0	blanched
		1	pink
		2	bright, deep red flush
Trunk curl		0	absent
		1	present
Limb grasping		0	absent
		1	present
Pinna reflex		0	present
		1	absent
Corneal reflex		0	present
		1	absent
Grip strength		0	no grip
		1	proper grip
Evidence of biting		0	none
		1	biting in response to handling
Vocalization		0	none
		1	vocal

Table S2. Order of testing per time point and batch

Age	Task	batch	n
4 weeks	Extended SHIRPA screen	all mice	22-26 per strain
6 weeks	Extended SHIRPA screen	all mice	22-26 per strain
8 weeks	Extended SHIRPA screen	all mice	22-26 per strain
Adult	Extended SHIRPA screen	batch 1	12-14 per strain
	Open field	batch 1	id
	Elevated plus maze	batch 1	id
	Object discrimination	batch 1	id
	Social discrimination	batch 1	id
	Home cage screen	batch 2	8-12 per strain
	Set shifting/reversal task	batch 2	8-10 per strain

Table S3. Scores per strain for modified SHIRPA primary screen

SHIRPA parameter	C57	129Sv	BTBR	AJ
Body position	1[a]	0.5[b]	1[a]	0[a]
Tremor	0	0	0	0
Palpebral closure	0	0	0	0
Coat appearance	0[a]	0[a]	1[b]	0[a]
Whiskers	0	0	0	0
Lacrimation	0	0	0	0
Defecation	0	0	0	0
Transfer arousal	2	1	2	2
Gait	0	0	0	1
Tail elevation	1	1	1	0
Startle response	1	1	1	1
Touch escape	1[a]	0[b]	1[a]	1[a]
Positional passivity	1	1	1	1
Skin color	1	1	1	1
Trunk curl	0	0	0	0
Limb grasping	0	0	0	0
Pinna reflex	0	0	0	0
Corneal reflex	0	0	0	0
Grip strength	1	1	1	1
Evidence of biting	0	0	0	0
Vocalization	1	1	1	0

Table S4. Variables measured in the Elevated Plus Maze (Mean ± SEM)

	C57	129Sv	BTBR	AJ
Distance moved	891.52 ± 50.83	1056.39 ± 105.54	2297.52 ± 120.19	370.43 ± 55.88
Open arm duration	56.62 ± 13.55	79.32 ± 7.68	146.77 ± 16.68	5.91 ± 2.5
Closed arm duration	185.26 ± 11.96	133.51 ± 11.85	87.15 ± 10.69	185.89 ± 19.49
Percentage open (duration)	0.23 ± 0.05	0.38 ± 0.03	0.61 ± 0.05	0.03 ± 0.01
Open arm entries	11.85 ± 1.31	16.58 ± 2.01	32.54 ± 3.6	3.67 ± 1.21
Closed arm entries	18.69 ± 2.3	24.51 ± 1.99	26.02 ± 3.81	20.75 ± 3.33
Percentage open (entries)	0.40 ± 0.04	0.40 ± 0.04	0.57 ± 0.04	0.16 ± 0.06

Chapter 2

Limited impact of *Cntn4* mutation on autism-related traits in developing and adult C57BL/6J mice

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ABSTRACT

Background

Mouse models offer an essential tool to unravel the impact of genetic mutations on autism-related phenotypes. The behavioral impact of some important candidate gene models for autism spectrum disorder (ASD) has not yet been studied, and existing characterizations mostly describe behavioral phenotypes at adult ages, disregarding the developmental nature of the disorder. In this context, the behavioral influence of *CNTN4*, one of the strongest suggested ASD candidate genes, is unknown. Here, we used our recently established developmental test battery to characterize the consequences of disruption of *contactin 4* (*Cntn4*) on neurological, sensory, cognitive, and behavioral phenotypes across different developmental stages.

Methods

C57BL/6J mice with heterozygous and homozygous disruption of *Cntn4* were studied through an extensive, partially longitudinal, test battery at various developmental stages, including various paradigms testing social and restricted repetitive behaviors.

Results

Developmental neurological and cognitive screenings revealed no significant differences between genotypes, and ASD-related behavioral domains were also unchanged in *Cntn4*-deficient versus wild-type mice. The impact of *Cntn4*-deficiency was found to be limited to increased startle responsiveness following auditory stimuli of different high amplitudes in heterozygous and homozygous *Cntn4*-deficient mice and enhanced acquisition in a spatial learning task in homozygous mice.

Conclusions

Disruption of *Cntn4* in the C57BL/6J background does not affect specific autism-related phenotypes in developing or adult mice but causes subtle non-disorder specific changes in sensory behavioral responses and cognitive performance.

INTRODUCTION

Autism spectrum disorder (ASD) is a behaviorally defined developmental disorder with a strong genetic component (Am. Psychiatr. Assoc., 2013; Chen et al., 2015). The identification of genetic risk factors such as common genetic variants, rare inherited and *de novo* mutations have led to the implication of hundreds of different genes (Pinto et al., 2014; De Rubeis et al., 2014). These findings illustrate the complexity and heterogeneity of the genetic architecture of ASD.

Subsequently, mouse models are being used to unravel the functional impact of implicated genes on ASD phenotypes in a controlled genetic and environmental background. However, knowledge of the impact on cognitive and behavioral development of the majority of these genes is missing or incomplete (Abrahams et al., 2013), and most behavioral characterizations of animal models are limited to adult phenotypes disregarding the developmental nature of ASD (Molenhuis et al., 2014). Here, we characterize the impact of *contactin 4* (*Cntn4*) null mutation on behavioral development, using our recently developed longitudinal test battery for mice that tests a wide array of neurological, cognitive, and behavioral parameters across development starting from 3 weeks of age (Molenhuis et al., 2014).

CNTN4 is an axonal glycoprotein belonging to the contactin family, a six-member subgroup of the immunoglobulin superfamily of cell adhesion molecules (Zuko et al., 2013). CNTN4 is known to act as an axon guidance molecule in the establishment of olfactory neural circuitry during neural development (Kaneko-Goto et al., 2008) and promotes target-specific axon arborization of a subset of retinal ganglion cells onto the nucleus of the optic tract (Osterhout et al., 2015). Knowledge of the neurobiological functions of CNTN4 in normal and abnormal development of brain systems are far from complete.

The *CNTN4* gene has been implicated in ASD due to its presence in the genetic locus of the 3p-deletion syndrome, a mental retardation syndrome (Fernandez et al., 2004). Subsequently, evidence for a role of CNTN4 has been accumulated (Glessner et al., 2009; Guo et al., 2012; Pinto et al., 2010; Roohi et al., 2009) but has also been questioned (Cottrell et al., 2011; Murdoch et al., 2015). Three cases carrying a copy number variant (CNV) in the *CNTN4* gene were reported by the Autism Genome Project Consortium (AGP) (Pinto et al., 2010). Deletions and duplications in the *CNTN4* gene or its promoter region were found in 10 families of the Autism Genetic Resource Exchange (AGRE) collection (Glessner et al., 2009). In the Autism Case-Control cohort (ACC), deletion in the promoter region of *CNTN4* was found in three cases but not in controls (Glessner et al., 2009). Association of

CNTN4 with developmental disorders such as ASD further seems supported by the protein's neurobiological functions (Chen et al., 2015; Zuko et al., 2013).

Following these observations, we performed a careful longitudinal functional characterization of homozygous and heterozygous disruptions of *Cntn4* to resolve the impact of this gene on behavioral and cognitive development.

METHODS

Generation and breeding of Cntn4 mice

Cntn4-deficient mice were kindly provided by Dr. Yoshihiro Yoshihara (RIKEN, Japan) (Kaneko-Goto et al., 2008). These mice were generated using a standard gene-targeting method as previously described. A targeting vector was designated to mutate the translation start codon (ATG) in the exon 2 of the *Cntn4* gene into a stop codon (TAG) and introduce a pgk-neo selection marker. Consequently, these mice were backcrossed with C57BL/6 mice more than nine times. Upon arrival in the University Medical Center Utrecht, the mice were re-derived, followed by heterozygous breeding for the use in our experiments. All animals were born and weaned at the University Medical Center Utrecht. Average nest size of Cntn4 litters was 7.2, and the litters larger than 10 animals per litter were culled back to average 7. The minimum litter size used for behavioral experiments was four animals per litter. Detailed information on the genotyping of *Cntn4* mice is provided in Additional file 1.

Given the extensive number of tests, the mice were spread over four different batches. Table 1 provides the order of behavioral testing and the number of animals per genotype per batch. Phenotypic assessments in batches 1, 2, and 3 were performed at the University Medical Center Utrecht. Batch 4 was transported and tested at Sylics (Synaptologics BV, Amsterdam, The Netherlands). All experiments were approved by the ethical committee for animal experimentation of the University Medical Center Utrecht and Free University Amsterdam and performed according to the institutional guidelines that are in full compliance with the European Council Directive (86/609/EEC).

Table 1. Overview of the behavioral tests per batch.

Age	Task	Batch	per genotype
3 weeks	Juvenile social interaction	batch 1 + 2	6-7 genotype matched pairs per genotype
4 weeks	Extended SHIRPA screen	batch 1 + 2	19-26 per genotype
6 weeks	Extended SHIRPA screen	batch 1 + 2	19-26 per genotype
8 weeks	Extended SHIRPA screen	batch 1 + 2	19-26 per genotype
Adult	Extended SHIRPA screen	batch 1	10-15 per genotype
	Open field	batch 1	10-15 per genotype
	Elevated plus maze	batch 1	10-15 per genotype
	Social discrimination	batch 1	10-15 per genotype
	Buried food test	batch 2	9-11 per genotype
	Set shifting - reversal task	batch 2	9-11 per genotype
	Social approach in 3-chamber	batch 3	12 per genotype
	Novel object exploration task	batch 3	12 per genotype
	Barnes maze - reversal task	batch 4	16 per genotype
	Pre-pulse inhibition	batch 4	16 per genotype

Animals in batch 1 + 2 were weaned at post-natal day 21, batch 3 + 4 animals at post-natal day 28.

Developmental neurological and behavioral screening

Cntn4 $^{-/-}$, *Cntn4* $^{+/-}$, and wild-type male littermates were subjected to our previously described longitudinal screening strategy (extended SHIRPA battery) testing an array of neurological, behavioral, and cognitive parameters at 4, 6, 8, and 11 weeks of age (Molenhuis et al., 2014; Rogers et al., 1999). The longitudinal test battery includes the assessment of autism-related traits such as motor stereotypies (e.g., self-grooming) and sensorimotor coordination (e.g., latency to fall from the rotarod). Detailed information on behavioral testing is provided in Additional file 1.

Screening of social behaviors and restricted repetitive behaviors

Abnormalities in social interaction behaviors were assessed in the juvenile social interaction test (3 weeks of age) (Silverman et al., 2010), followed by a three-chamber social approach (Silverman et al., 2010), and a 2-day social discrimination paradigm in adult age animals (Bruining et al., 2015). Stereotypic movements, restricted interests, and repetitive patterns of behavior were analyzed in the novel object investigation task during exposure to four novel toys (Pearson et al., 2011). Cognitive flexibility was assessed by multi-trial associative learning in an extensive set-shifting paradigm (Molenhuis et al., 2014), as well as a Barnes maze spatial learning task including reversal (Seigers et al., 2015). Acoustic startle response and

sensorimotor gating were assessed in the pre-pulse inhibition (PPI) test (Bruining et al., 2015). Anxiety-related behaviors were tested in the elevated plus maze and open field (Molenhuis et al., 2014). Statistical analyses are described in the Supplemental Material section.

RESULTS

eSHIRPA assays

The eSHIRPA (extended SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment) screen did not show differences between the *Cntn4* $^{+/-}$ mice, *Cntn4* $^{-/-}$ mice, and wild-type controls at 4, 6, 8, or 10 weeks of age in general health, body weight, and neurological reflexes nor in the development of various locomotor parameters including total distance moved, movement velocity, and movement duration (Figs. 1A–D, Table 2). Moreover, we found no developmental differences in the amount of self-grooming or sensorimotor coordination on the rotarod (Figs. 1E–F).

Social interaction behavior

The juvenile social interaction test revealed no differences in the amount of social sniffing, anogenital sniffing, or social grooming (Fig. 1G) between the *Cntn4* $^{+/-}$, *Cntn4* $^{-/-}$, and wild-type control mice. In the adult three-chamber test, all genotypes showed a clear preference for exploration of a mouse over an object, and no genotype differences in the amount of social exploration were observed (Fig. 1H). Furthermore, genotypes were equally capable to distinguish between a familiar mouse and a novel mouse in a social recognition paradigm, both at 5 min and 24 h after initial exposure (Fig. 1I).

Restricted and repetitive behaviors

There were no differences in the grooming behavior between the *Cntn4* $^{+/-}$, *Cntn4* $^{-/-}$, and wild-type control mice at adult age, in line with the amount of grooming observed in the longitudinal eSHIRPA screening (Fig. 2A). We also found no genotype differences in restricted interest or in repetitive patterns of behavior in the novel object investigation task during the exploration of the four novel toys (Fig. 2B, C).

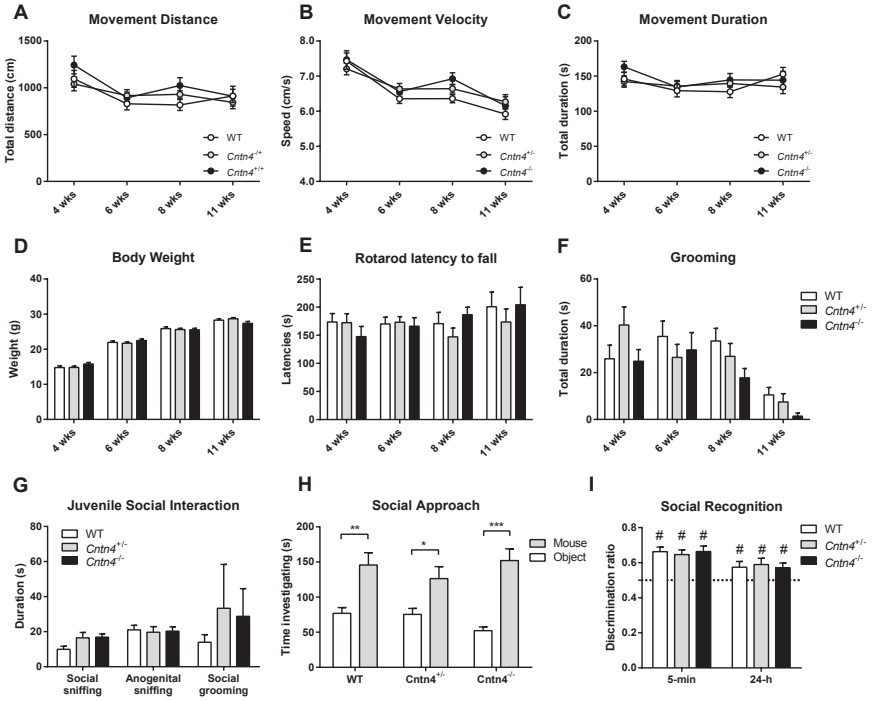

Figure 1. Developmental neurological and behavioral screen and analysis of social behaviors in deficient mice. **A**) Distance moved (rmANOVA genotype, $F(2,63) = 0.760$, $p = .472$), **B**) movement velocity (rmANOVA genotype, $F(2,63) = 1.256$, $p = .292$), **C**) movement duration (rmANOVA genotype, $F(2,63) = 0.342$, $p = .479$), **D**) body weight (rmANOVA genotype, $F(2,63) = 0.588$, $p = .558$), **E**) latency to fall of the accelerating rotarod (rmANOVA genotype, $F(2,64) = 0.110$, $p = .896$), and **F**) time spent self-grooming (rmANOVA genotype, $F(2,64) = 1.038$, $p = .360$) at pre-adolescence (4 weeks), adolescence (6 weeks), early adulthood (8 weeks), and adulthood (10 weeks) (n = 19–26 per genotype) during the eSHIRPA test. **G**) Social sniffing (owANOVA, $F(2,16) = 2.926$, $p = .083$), anogenital sniffing (owANOVA, $F(2,16) = 0.055$, $p = .946$), and social grooming (owANOVA, $F(2,16) = 0.334$, $p = .721$) during the juvenile social interaction test in genotype-matched mice at post-natal day 21 (n = 6–7 pairs of genotype-matched interacting animals per genotype). **H**) Social exploration (owANOVA between genotypes, $F(2,32) = 0.599$, $p = .556$) as a function of exploration of the cage with the novel mouse versus the empty cage during the three-chamber task (n = 12 per genotype). **I**) Social exploration during the social discrimination test following a 5-min inter-trial interval (owANOVA between genotypes $F(2,36) = 0.138$, $p = .872$) and a 24-h inter-trial interval (owANOVA between genotypes $F(2,36) = 0.096$, $p = .909$), with exploration of the novel mouse as fraction of the total duration of social exploration (n = 10–15 per genotype). Data are presented as means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; #$p < 0.05$.

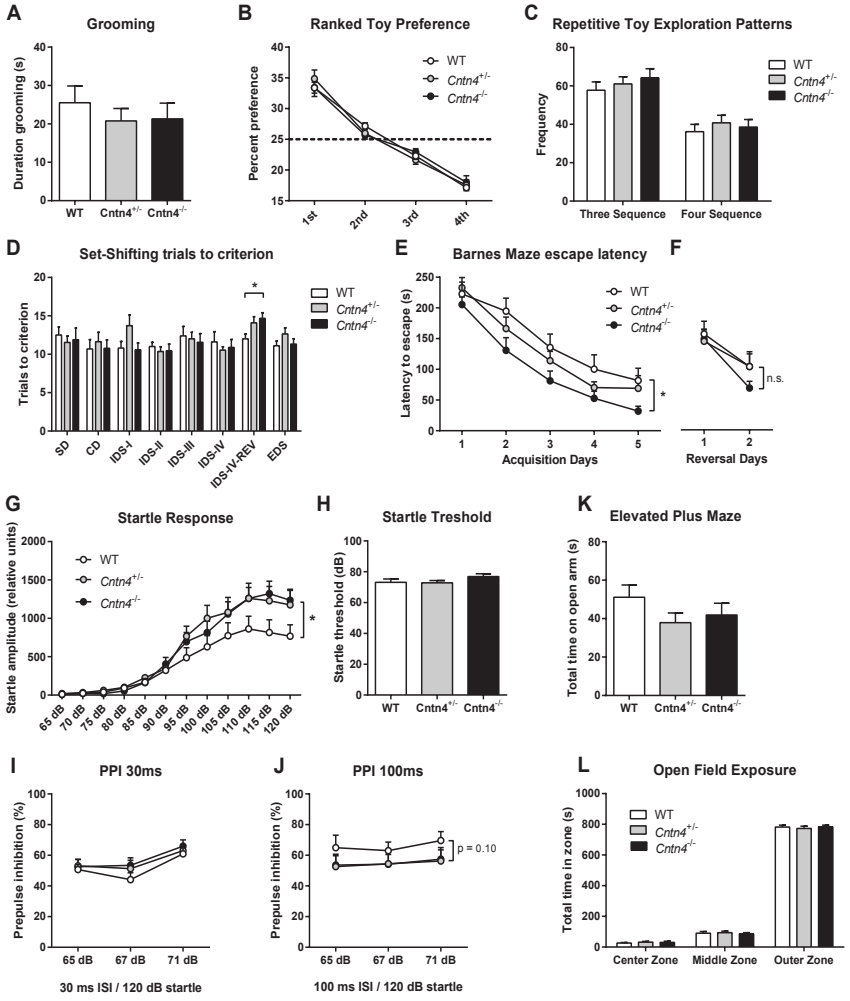

Figure 2. Restricted repetitive behaviors and sensory-sensitivity screening of adult Cntn4 mice. Restricted and repetitive behavior in the novel object investigation task. **A**) Stereotypic movements as total time grooming (owANOVA, F (2,33) = 0.431, p = .653). **B**) Restricted interest as frequency-based percentage preference of exploration of each of the four novel toys (1st preference owANOVA, F (2,33) = 0.446, p = .644; 2nd preference owANOVA, F (2,33) = 1.569, p = .223; 3rd preference owANOVA, F (2,33) = 1.208, p = .312; 4th preference owANOVA, F (2,33) = 0.236, p = .791). **C**) Repetitive toy exploration patterns based on repetitive sequences of three elements (owANOVA, F (2,33) = 0.760, p = .476) and four elements (owANOVA, F (2,33) = 0.227, p = .798) (n = 12 per genotype) Reversal learning during the set-shifting reversal-learning task **D**), where X-axis represents the different sub-tasks. Y-axis represents the total number of trials that were needed

to reach the criterion of 8 correct digs in 10 consecutive trials (n = 9–11 per genotype). **E-F**) Spatial learning and reversal learning during the Barnes maze paradigm. Y-axis represents the daily mean of latency to find the escape hole during **E**) the acquisition phase (rmANOVA genotype, F (2,44) = 4.151, p = .022) and **F**) the reversal-learning phase (rmANOVA genotype, F (2,43) = 0.830, p = .830) after replacing the escape to the other side of the maze (n = 16 per genotype). Startle and PPI results in Cntn4 mice, with **G**) startle magnitude as function of startle stimulus in all genotypes (MANOVA, F (24,70) = 1.984, p = 0.014), **H**) startle threshold (owANOVA, F (2,45) = 1.542, p = 0.225), **I–J**) pre-pulse inhibition tested with different pre-pulse intensities with inter-stimulus interval (ISI) at 30 ms (two-way ANOVA, F (2,135) = 2.376, p = 0.096) and at 100 ms (two-way ANOVA, F (2,135) = 1.1927, p = 0.306; n = 16 per genotype). Anxiety behavior during the elevated plus maze test and open-field test measured as **K**) elevated plus maze anxiety and as total time spent on the open arms (owANOVA, F (2,36) = 1.450, p = 0.248), l) total time spent in the center (owANOVA, F (2,36) = 0.165, p = 0.848), middle (owANOVA, F (2,36) = 0.413, p = 0.665), and outer zones (owANOVA, F (2,36) = 0.125, p = 0.883) of the open field (n = 10–15 per genotype). Data are presented as means ± SEM; *p < 0.05. SD simple discrimination, CD compound discrimintion, IDS I–IV intra-dimensional shift I–IV, IDS-reversal reversal of intra-dimensional shift IV (owANOVA, F (2,27) = 3.487, p = .045; Dunnett's t, WT vs HET p = 0.037, WT vs KO p = 0.092), EDS extra-dimensional shift (owANOVA, F (2,27) = 1.416, p = .260).

We additionally analyzed cognitive flexibility through the assessment of reversal learning in a set-shifting task. Prior to this task, we ascertained intact olfactory capacities, as we found no genotype differences in the latency to find a buried piece of food. In the set-shifting task, all genotypes were equally able to associate a food reward with a specific digging material or odor, as was evident through the performance of simple, compound discrimination and intra- and extra-dimensional shifts (Fig. 2D).

The reversal-learning phase of this test yielded an inconclusive result, as the genotype effect that we observed on reversal learning was only significant (ANOVA $p = 0.04$) in one of the two outcome measures (i.e., the number of trials to reach the criterion but not in errors to reach criterion). Moreover, a shift cost in wild-type mice was observed in errors to reach criterion, although not in the number of trials to reach criterion. Given this inconclusive result, we tested reversal-learning performance in a different paradigm. In this Barnes maze reversal-learning paradigm, we confirmed that *Cntn4* does not affect reversal learning, as all genotypes needed equal amount of time as well as distance before reaching the re-located escape hole (Fig. 2F). In contrast to the set-shifting test, we observed a shift cost that was observed in all genotypes in the reversal phase of the Barnes maze test (Fig. 2E, F).

Responses to sensory stimuli and anxiety-related behaviors

The startle response was consistently increased in the *Cntn4*$^{+/-}$ and *Cntn4*$^{-/-}$ mice at different high amplitudes, although the startle threshold was not significantly different between genotypes (Figs. 2G, H). No significant effects were found on pre-pulse inhibition at both inter-stimulus intervals of 30 and 100 ms (Figs. 2I, J).

The increased startle response to auditory stimuli of the different high amplitudes seemed not to result from increased anxiety levels, as *Cntn4*$^{+/-}$, *Cntn4*$^{-/-}$ mice and wild-type controls did not differ in their elevated plus maze (Fig. 2K) and open-field exploratory behaviors (Figs. 2L).

Table 2. Physical and neurological features during the different phases of the extended SHIRPA primary screen. *Cntn4*$^{+/-}$, *Cntn4*$^{-/-}$, and wild-type (WT) control mice were screened in the Perspex jar for body position (active, inactive, or excessively active), tremor (present or not), palpebral closure (eyes open or not), coat appearance (well-groomed or irregularities like piloerection), whiskers (intact or trimmed), and lacrimation (present or not). In the arena, the mice were screened for transfer arousal (freezing or immediate movement), gait (fluid or not), tail elevation (dragging, horizontal, or straub tail), startle response (preyer reflex, no response, or additional response), and touch escape (response to touch or flight prior to touch while finger approaches). Mice were transferred out of the arena to observe positional passivity (struggling by different types of handling), skin color (color of plantar surface of forelimbs), trunk curl (forward curling with head to abdomen), limb grasping (clasping of rear limbs), pinna reflex (presence of ear retraction), corneal reflex (presence of eyeblink), contact righting reflex, evidence of biting, grip (grasping of grid), vocalization, and puberty (presence of sex organs). Sig. represents statistical significance of between-genotype differences. Data are presented as a percentage of the total number of animals per genotypes, except for defecation (count of the fecal boli).

Age (weeks)	4				6				8				Adult			
Test	**WT**	**+/-**	**-/-**	**Sig.**	**WT**	**+/-**	**-/-**	**Sig.**	**WT**	**+/-**	**-/-**	**Sig.**	**WT**	**+/-**	**-/-**	**Sig.**
Subjects (n)	19	26	22		19	26	22		19	26	22		13	15	10	
Body position (active)	95	96	100	0.22	100	100	100	1.00	100	96	100	0.46	100	100	100	1.00
Body position (inactive)	0	4	0		0	0	0		0	4	0		0	0	0	
Body position (excessive activity)	5	0	0		0	0	0		0	0	0		0	0	0	
Tremor (absent)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Palpebral closure (eyes open)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Coat appearance tidy & groomed	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Whiskers (present)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Lacrimation (absent)	100	96	100	0.45	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Defecation (quantity)	1.9	1.6	2.0	0.47	3.1	2.5	1.9	0.06	2.8	2.3	2.4	0.61	3.8	3.7	3.2	0.70
± SEM	0.3	0.3	0.3		0.3	0.3	0.3		0.4	0.3	0.4		0.7	0.7	0.9	
Transfer arousal (brief freeze)	11	15	14	0.70	11	19	9	0.71	11	8	0	0.16	8	0	10	0.50
Transfer arousal (immediate movement)	89	85	86		89	81	91		89	92	100		92	100	90	
Gait (fluid)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Tail elevation (horizontal extension)	100	100	100	1.00	95	100	100	0.28	100	100	100	1.00	100	100	100	1.00
Tail elevation (Straub tail)	0	0	0		5	0	0		0	0	0		0	0	0	
Startle response (Preyer reflex)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Touch escape (flees prior to touch)	79	73	77	0.88	89	85	73	0.35	63	88	68	0.05	85	87	100	0.45
Touch escape (response to touch)	21	27	23		11	15	27		37	12	32		15	13	0	
Positional passivity (struggles)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Skin color (pink)	95	100	100	0.28	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Skin color (blanched)	5	0	0		0	0	0		0	0	0		0	0	0	
Trunk curl (absent)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Limb grasping (absent)	100	92	100	0.20	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Pinna reflex (present)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Corneal reflex (present)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Contact righting reflex (present)	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00
Evidence of biting (none)	79	77	77	0.99	84	88	100	0.18	100	100	100	1.00	100	100	100	1.00
Grip (OK)	100	96	100	0.36	95	96	100	0.59	100	100	100	1.00	100	100	90	0.24
Full puberty	89	81	100	0.30	100	100	100	1.00	100	100	100	1.00	100	100	100	1.00

DISCUSSION

We present a comprehensive assessment of the impact of the ASD candidate gene *CNTN4* on a variety of neurological, behavioral, and cognitive aspects of development. We found no effect of *Cntn4* deficiency on ASD-related behavioral mouse paradigms such as the juvenile social interaction test, the three-chamber test, grooming behavior, and sensorimotor coordination. We also did not observe developmental neurological, behavioral, or cognitive abnormalities in the extended SHIRPA screen.

The reversal-learning phase of the set-shifting task yielded an inconclusive result, as no shift cost was observed in wild-type animals for the number of trials to reach criterion. Indeed, the genotype difference that was observed in the number of trials to reach the criterion in the reversal-learning phase was rather caused by lack of shift cost in wild-type animals than a reversal-learning deficit in the *Cntn4*$^{-/-}$ mice. In line with this reasoning, when we compared the outcome of *Cntn4*$^{-/-}$ mice in the reversal-learning phase with the results obtained for C57BL/6J mice obtained in a previous study, we find no genotype difference in this phase of the set-shifting task (Molenhuis et al., 2014). In the Barnes maze reversal-learning paradigm, we also did not find a reversal-learning deficit. Based on the summary of these data, we conclude that *Cntn4* disruption has no effect on cognitive flexibility.

Non-ASD-specific effects at adult ages were indicated by increased startle response to auditory stimuli of different high amplitudes and by faster escape hole finding during subsequent days of acquisition in the Barnes maze.

Hyper-responsivity to acoustic stimuli is related to many neurodevelopmental disorders and has also been reported in ASD (Green et al., 2015). In addition, acoustic hyper-responsivity in patients with fragile X syndrome is known to be consistent with animal model data (Chen and Toth, 2001). Similar to our findings, an increased startle response to acoustic stimuli was recently described in children with ASD (Takahashi et al., 2014), although these were in response to weak stimuli in contrast to the high amplitudes we found. The observed hyper-responsivity in *Cntn4*-deficient mice was unrelated to anxiety levels, as mice showed similar exploratory behaviors in classical anxiety behavioral tests, such as the elevated plus maze and open field. Although speculative, the increased startle response together with the enhanced acquisition in the Barnes maze could indicate that *Cntn4* deficiency in the C57BL/6J background leads to a state of increased behavioral responsiveness without overt anxiety or avoidance behavior in mice (Blanchard et al., 2003).

Together, the findings show that in the C57BL/6J background, disruption of *Cntn4* does not lead to substantial behavioral defects related to autistic development. A limited behavioral penetrance of *CNTN4*mutations on autistic development may be

consistent with a recent study that revisited the association of contactins, including *CNTN4*, with ASD (Murdoch et al., 2015). The behavioral phenotype of *Cntn4* mice contrasts other previously studied genetic ASD models (Silverman et al., 2010). For instance, *Shank3* or *Pten* mice show extensive impairments in social interaction and sensorimotor phenotypes and are therefore regarded as translational models for ASD (Ey et al., 2011; Jiang and Ehlers, 2013; Peca et al., 2011).

Although ASD-related phenotypes in *Cntn4*-deficient mice were observed, our findings do have relevance for neurodevelopmental disorder research. The specific phenotypes observed in *Cntn4*-deficient mice may be used to study the mechanisms underlying increased responsiveness or vigilance, a trait observed across many different human disorders such as attention-deficit hyperactivity disorder, post-traumatic stress disorder, and schizophrenia (Dalgleish et al., 2001; Freedman et al., 1991; Parker et al., 2004). *Cntn4*-deficient mice may serve as a model to study the mechanistic underpinnings of behavioral states in which vigilance is altered. Indeed, common single-nucleotide variants in the *CNTN4* locus have recently been associated with other neuropsychiatric disorders, such as schizophrenia (Ripke et al., 2014), perhaps pointing to a non-disorder specific contribution of this cell adhesion gene in neuropsychiatric pathogenesis. In addition, a role for *Cntn4*, was recently shown in target-specific arborization during development of the accessory optic system (Osterhout et al., 2015). Our study shows the importance of detailed developmental neurological, behavioral, and cognitive characterization of genetic animal models to complement human genetic studies in ASD and related disorders.

Conclusions

In our test battery, disruption of *Cntn4*, a prominent ASD candidate gene, had no effect on cognitive and behavioral development or ASD-specific phenotypes. At adult age, we could detect an effect of *Cntn4*-disruption on an adult sensory behavioral and a spatial cognitive feature.

Abbreviations

ASD: autism spectrum disorder; eSHIRPA: extended SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment; PPI: pre-pulse inhibition.

Competing

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RTM participated in the design of the study, carried out the experiments and genotyping, analyzed the data, interpreted the results, and wrote the manuscript. HB provided input into the study design, interpreted the results, and wrote the manuscript. ER and LV participated in the design of the study and coordination of the experiments. ML participated in the design of the study, data analysis, and interpretation of the results. JPHB aid in the genotyping and participated in the interpretation of the results. MJHK contributed to the design of the study, development of the methods, coordination of the statistical analyses, and interpretation of the data. All authors contributed to the reviewing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTAL MATERIAL

Generation and breeding of Cntn4 knockout mice. Upon arrival in the University Medical Center Utrecht, mice were re-derived, followed by heterozygous breeding for the use in our experiments. The following primers were used for genotyping Cntn4-progeny: 5'-tggtagatggatcgatggcaaacatgtccc-3' (mutant allele forward); 5'-agccccagtttttgcctaagcatt-3' (wild-type allele forward); 5'-ttcatcactcctgaatcacacatgtcagg-3' (mutant and wild-type allele reverse). Absence of protein was confirmed by western blot.

Developmental neurological and behavioral screening. The original SHIRPA primary screen [2] of general health and neurological reflexes was modified and extended with additional tests to increase the number of behavioral domains measured as we have performed previously [3]. Mice were placed in a Perspex jar and recorded for 5 min. The following phenotypes were observed in the jar: body position (active, inactive or excessively active), tremor (present or not), palpebral closure (eyes open or not), coat appearance (well-groomed or irregularities like piloerection), whiskers (intact or trimmed), lacrimation (present or not), defecation (count of the fecal boli). Subsequently mice were transferred to an arena made of Perspex (Macrolon type III cage). During the time the mice spent in the arena an automated recording of locomotor activity was made for 5 min using video-tracking software Ethovision (Noldus Information Technology). In the arena the following phenotypes were observed: transfer arousal (freezing or immediate movement), gait (fluid or not), tail elevation (dragging, horizontal or straub tail), startle response (preyer reflex, no response or additional response) and touch escape (response to touch or flight prior to touch while finger approaches). Mice were transferred out of the arena and the following phenotypes were observed: positional passivity (struggling by different types of handling), skin color (color of plantar surface of forelimbs), trunk curl (forward curling with head to abdomen), limb grasping (clasping of rear limbs), pinna reflex (presence of ear retraction), corneal reflex (presence of eye-blink), contact righting reflex, evidence of biting, grip (grasping of grid), vocalization and puberty (presence of sex organs). Self-grooming was manually quantified based on 5-min video recordings of the time the mice spent in the arena, using video software The Observer (Noldus Information Technology). As part of the longitudinal screening, all mice were also tested the RotaRod (Ugo-Basile, Varese, Italy) to test the sensorimotor competence [4]. The latency for the time to fall off the rotating beam is a measure for the motor coordination and balance. The beam starts rotating at a speed of 4 rpm. During a 5-min trial the beam is

accelerating until 40 rpm. Two passive rotations during two complete turns were also counted as a fall.

Juvenile social interaction test. Juvenile social interactions were assessed between two genotype matched, non-cage mate animals at postnatal day 21 as was shown previously [5]. Habituation and testing took place in clean polycarbonate type II cages (mice: 22 × 16 × 14 cm) with fresh bedding. Social interactions, digging and grooming were scored manually based on video recordings using The Observer software (Noldus Information Technology).

Three chamber social approach test. Preference for social approach was measured using the three-chambered apparatus [5]. The apparatus was a rectangular box made of polycarbonate and was divided into three compartments by partitions with openings, which allowed free access to all compartments. The paradigm consisted of three trials each lasting for ten minutes. Initially, mice were habituated to the empty arena for 5 min after which empty wire cages were introduced and the mice were allowed to inspect these cages for another 10 min. The second phase of the test measured social preference involving a 10-min session where the experimental mouse was exposed to an inanimate object, one of the empty wired cages and a wired cage covering a stimulus mouse. The placement of both social and non-social targets was counterbalanced between experimental subjects. Parameters measured were a) time spent in each chamber, b) time spent sniffing each target. Behavioral scoring occurred manually using Observer software (Noldus Information Technology). Both the time the mice spent in the three chambers as well as the time the mice spent sniffing the cages was scored manually using Observer software (Noldus Information Technology).

Social recognition test. Social recognition capacity was tested using out two-day social discrimination paradigm, see our previous studies [2], [4]. Before testing all mice were group-housed. To avoid exposure to odor prior to testing, intruder animals were housed in separate rooms under identical conditions, e.g., non-gender matched. Edding 30 permanent marker (tip 1.5-3 mm, filled with neutral smelling water- based ink, green; Edding International) was used for labeling the intruder animals. Habituation and testing took place in clean polycarbonate type II cages (mice: 22 × 16 × 14 cm) with fresh bedding. Disposable gloves were used for handling the animals and changed after each trial to avoid odor contamination. Test animals were habituated in the test cage for 5 min and initially exposed to an age- and gender-matched A/J conspecific for 2 min and then, after inter-trial intervals (ITI) of 5 min exposed to the familiar conspecific and a first novel A/J conspecific for 2 min. After testing, the test animals were returned to their home cage. On day 2 for the 24 h ITI testing, again a clean cage with fresh bedding was used and the

test animal was habituated for 5 min and re-exposed to the same familiar intruder of day 1 and to a different novel intruder animal from a different cage and housing room than the intruder of day 1 for 2 min. Social investigation was defined as the total time mice engaged in social sniffing, anogenital sniffing and allogrooming. These behaviors were manually scored from video recordings using the Observer software (Noldus Information Technology, Wageningen, The Netherlands). The time spent by the test animal in investigating each intruder animal was measured by a trained observer blind to the animal's genotype.

Restricted interest and repetitive behavioral patterns. Restricted interest and repetitive patterns of novel object exploration were assessed in the novel object investigation task, during ten-minute exposure to four novel toys, a paradigm described previously [6]. In this test, a blue lego, miniature bowling pin, dice and green marble. Object-exploration behaviors and self-grooming were manually scored from video recordings using the Observer software (Noldus Information Technology, Wageningen, The Netherlands). Analyses of restricted interest and repetitive sequences of object-exploration were performed as reported previously[6].

Buried food test. Olfactory capabilities were assessed by the latency to locate buried food[7]. Testing took place in bedding-filled transparent plastic cages (42.5 cm x 26.5 cm x 18 cm, length x width x height).

Set shifting and reversal learning task. Mice were tested on a variant of the reversal/set-shifting task, as we previously reported [2], [4]. Mice were required to learn the location of a hidden food reward in one of two food cups that were placed in a testing cage. Three days prior to this task, mice were housed solitary and food restricted up to 85% of their ad libitum body weight to ensure appropriate motivation. The reward was hidden under a scented digging medium. Odor and digging medium represented two different dimensions, mice had to learn to focus on the relevant dimension and ignore the other dimension during the different learning phases. Different combinations of odors and digging media were used. The task consisted of 8 sub-tasks: simple discrimination, compound discrimination, intra-dimensional shifts I-IV and intra-dimensional shift IV reversal. During simple discrimination, mice had to associate either a medium or an odor with the reward, the second dimension was kept constant for both cups. During compound discrimination, both dimensions were different, mice had to learn to ignore the non-relevant dimension and focus on the previously learned association between a certain medium or odor and the reward. During the four intra-dimensional shifts, the relevant dimension did not change, but the specific pairings within the dimension were changed in such a way that the animal had to learn a new association in every task.

During the reversal task, the cues stayed the same, except that the previously rewarded medium or odor was no longer reinforced. Thus, mice had to switch to choose the previously not rewarded cue. During the extra-dimensional shift, the relevant dimensions were changed (i.e. mice that previously had to associate a medium with the reward now had to associate an odor, and vice versa). Each phase lasted about 8-30 trials, depending on the learning rate. Criterion for acquisition of each sub-task was set at 8 out of 10 correct consecutive trials. The total task duration was 4 days. The latency to find the reward, the number of trials to reach criterion and the number of errors were recorded for each of the individual tasks.

Barnes maze and reversal learning. The Barnes Maze is a test for spatial learning and memory, performed as previously reported [8]. The test is similar to the Morris Water Maze, with the important difference that this test does not require swimming. As such, the emotional distress of the Barnes Maze is suggested to be lower than the emotional load of the Morris Water Maze. The Barnes maze contains 24 holes situated at the edge of the maze. One of these holes is connected to an escape box. The testing room is equipped with several visual cues mounted on the wall. After multiple training trials, mice learn to locate the escape hole using the visual cues around the maze.

The Barnes maze consisted of a circular grey platform (diameter 120 cm) elevated 100 cm above the floor with 24 holes (4.5 cm diameter) spaced at equal distance 5 cm away from the edge of the platform. One hole was designated as escape hole, and equipped with a cylindrical entrance (4.5 cm diameter x 5 cm depth) mounted underneath the maze providing access to an escape box (15.3 x 6.4 x 6.1 cm) containing a metal stairway for easy access that was not visible unless mice approached the hole closely. Other holes were equipped identical cylindrical entrances, but without escape box. Visual extra-maze cues (50 x 50 cm) composed of black and white patterns were mounted on the walls ~70 cm away from the maze. Three fans surrounding the maze (60 cm away from the maze spaced ~120° apart) produced a variable airflow across the entire maze by a slow 90° horizontal movement, proving both an aversive environment as well as dispersion of any odor cues. Several fluorescent tube lights mounted at the ceiling provided bright illumination (1000 lx). A speaker mounted to the ceiling provided background sound.

Mice received training sessions twice a day, in the morning and afternoon. Mice were introduced in an opaque cylinder placed in the center of the maze, after which the experimenter left the room and closed the door. The cylinder was pulled upwards 30 s later, and mice could explore the maze to locate the escape hole. If the latency to enter the escape hole exceeded 300 s, mice were gently guided toward the escape hole. During the first 2 habituation sessions, the escape box contained

cage enrichment of a mouse's own home cage, and once in the cylinder, mice were left in there for 60 s. After each mouse, the platform and escape box were thoroughly cleaned with 70 % ethanol. The platform was rotated 90° after each trial to avoid the use of any odors cue. During a 300 s probe trial the escape hole was identical to all 23 other holes.

The path travelled by a mouse was video tracked by an overhead camera and analyzed using Viewer 2 software with Barnes maze plugin (BIOBSERVE GmbH, Bonn, Germany). The distance and latency to reach the target location were recorded. Barnes maze training consisted of two training sessions per day, for five days. On the eighth day, two additional training sessions were given. Reversal learning was forced by replacing the escape to the other side of the board on day nine and ten.

Acoustic startle and pre-pulse inhibition. Acoustic startle and PPI were measured during one 45-min session in four Plexiglas cylinders in ventilated sound-attenuating chambers (Med Associates, St. Albans, VT), placed on separate heavy passive vibration-free tables (Newport Corporation, Irvine, CA) as reported before [4]. During testing, a separate speaker provided white noise background of 65 dB. The intensity of the startle stimuli was calibrated with a microphone placed inside the Plexiglas cylinders in a closed chamber, while white background noise was switched off. Hence, the reported prepulse and pulse intensities are lower than the actual cumulative sound pressure level during testing (e.g., a 65 dB pulse in addition to a 65 dB background noise add up to 68 dB intensity). The session started with a habituation period of 5 min, followed by a total of 260 trials with pseudo-randomized interval periods (5-15 s) consisting of acoustic startle trials with white noise bursts at various intensities (65, 70, 75, 80, 85, 90, 95, 100, 105, 110, and 115 dB; 10 trials per intensity) and prepulse inhibition trials with white noise bursts at various prepulse intensities (0, 65, 67 and 71 dB; 30 trials per prepulse intensity; startle intensity always 120 dB) in pseudo randomized order such that all 4 boxes produced startle stimuli at exactly the same time. In half of the prepulse trials, onset of white noise prepulse stimuli (20 ms; 1 ms programmed rise/fall time) and startle stimuli (40 ms; no programmed rise/fall time) was separated by a 30-ms, in the other half by 100-ms interval. In each trial, the highest startle intensity peak (in relative machine units) was collected during the 100-ms interval after the startle stimulus, from which the individual mean highest startle intensity peak during the 100-ms null-period prior to prepulse stimuli was subtracted. The ISI of 30 ms was used as the PPI in mice is at its maximum around 30-ms ISI [9] whereas for humans the maximum PPI is found at 100 ms [10]. The equipment was calibrated to allow for a wide range of startle intensities, however, the force generated by some mice at the highest pulse intensities could exceed the dynamic range of the equipment (maximum of 2047 units) artificially reducing the percentage of PPI in subsequent

analyses. Therefore, when the number of such censored 120-dB pulse trials was more than 33% (e.g., more than 5 out of 15) no PPI was calculated. The percentage of PPI was calculated as follows: PPI = 100 * [(mean startle intensity pulse) - (mean startle intensity pulse with prepulse) / (mean startle intensity pulse)].

Anxiety-like behavior. As we reported previously, anxiety behavior was measured in an open field test and in the elevated plus maze test, making use of the natural tendency of rodents to avoid open spaces in a novel environment [3]. Mice were exposed to an elevated maze that was cross-shaped and consisted of a central platform and four arms, two of which are enclosed. Mice were put in on the center platform, facing an open arm, and allowed to freely explore the maze for 5 min. The time spent on, and number of entries into the open and closed arms as well as the total distance travelled were recorded using an overhead camera and video tracking software (EthoVision 7.0, Noldus Information Technology, Wageningen, The Netherlands).

The open field consisted of a circular arena with a diameter of 80 cm. Mice were exposed once for a 15 min session. Mice were placed near the wall of the arena. After each trial, the apparatus was thoroughly cleaned using paper towels and Trigene solution (0.5%). An overhead camera connected to a PC with video tracking software (EthoVision 7.0, Noldus Information Technology, Wageningen, The Netherlands) recorded the movement of each animal to measure the total amount of time the mouse spent in the arena center.

Statistical analyses. Genotype differences in each single trial were determined and analyzed using one-way ANOVA (owANOVA) [3]. Social approach in the 3-chamber social interaction task was additionally tested using a paired t-test, comparing the time investigating the object versus investigating the mouse. Social discrimination ratios in the social recognition paradigm were additionally tested against chance level (a ratio of 0.5) within strain for each interval (T1 and T24) using a one-sample T-test [3]. For repeated measurements, a repeated measures ANOVA (rmANOVA) was performed with 'time' as within-subjects factor and 'genotype' as between-subjects factor [3]. In case of a significant F-value, post-hoc comparisons were performed using Dunnet's test with wild type as control group. Genotype differences in startle response were compared using the MANOVA Pillai's trace statistic as the Mauchly's indicated violation of sphericity. PPI data were compared using a 2-way ANOVA. Non-normally distributed SHIRPA scores were compared using a non-parametrical Kruskal–Wallis test with Mann–Whitney U tests for post-hoc comparisons. Significance was set at $p<0.05$. SPSS 20.0 for Windows was used for analyses.

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Chapter 3

Mouse models for ASD respond differently to novelty

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ABSTRACT

Background

Behavioral testing in mouse models is often performed after the mouse has been transferred from its home-cage environment into a novel behavioral testing arena. Since resistance to change is a common feature in Autism Spectrum Disorders (ASD), we hypothesized that exposure to environmental change may induce behavioral abnormalities in genetic animal models for ASD. Here, we tested this hypothesis and aimed to identify optimal environmental conditions that can be used to maximize behavioral differences in animal models for ASD.

Methods

To investigate the impact of environmental change on mouse models for ASD, we first performed a systematic literature review and compared behavioral activity during baseline conditions versus during exposure to a novel behavioral testing environment. To identify optimal conditions for future phenotype-driven studies related to ASD, we then tested behavioral activity in the BTBR inbred strain, a model for ASD, across a variety of assays and environmental exposures. These included longitudinal home-cage observations and exposure to a novel environment with four novel objects.

Results

Based on the literature review, abnormalities in behavioral activity in mouse models for ASD are more prominent after environmental change than during baseline conditions. BTBR mice pervasively showed novelty-induced hyperactivity, which was most prominent during combined exposure to a novel environment and novel objects. In contrast, BTBR mice displayed *hypo*-activity relative to C57 mice *after* habituation to a novel home-cage.

Conclusion

Exposure to a behavioral testing environment induces the expression of behavioral abnormalities in mouse models for ASD, and behavioral activity in novel environments is affected in most of these models. These findings suggest that exposure to a novel environment may act as a potential modifier in mouse models for ASD, and also indicate that short behavioral assays with a high degree of novelty can help to potentiate the expression of behavioral phenotypes in animal models for ASD.

INTRODUCTION

Repetitive and restricted interests and behaviors are a core feature of Autism Spectrum Disorders (ASD), and include distress when confronted with relative small changes or transitions (Am. Psychiatr. Assoc. 2013). Caregivers commonly report that these behaviors are among the most challenging aspects of ASD (Boyd et al. 2012), and the severity of repetitive behavior symptoms at preschool age has been linked with poor outcome later in life (Troyb et al. 2015). Despite the impact of repetitive behaviors on daily life functioning in people with ASD, there are currently no pharmacological interventions to treat symptoms in this domain.

Repetitive behaviors have been subdivided into "lower-level" motor actions such as stereotyped movements and tics, and more complex or "higher-level" behavioral inflexibility related to insistence on sameness and resistance to change (Turner 1999; Lewis and Kim 2009; Geurts et al. 2009). Emerging evidence suggests that both low-level motor stereotypies as well as high-level resistance to change are regulated by common cortico-striatal circuits (Lewis and Kim 2009; Langen et al. 2011b, 2011a). The assessment of autism-like behavioral domains in animal models is typically performed using standardized behavioral testing paradigms (Kas et al. 2014). Although a wide range of tests have been developed, most tests have in common that they involve human handling and transfer to a novel behavioral testing environment. Since resistance to change is regarded as an element of repetitive and restricted interests and behaviors in ASD (Hutt 1969), we hypothesized that the exposure to a novel environment contributes to the expression of behavioral abnormalities observed in genetic animal models for ASD, and that exposure to novelty could be a useful environmental challenge to potentiate behavioral mouse phenotypes relevant for ASD studies.

In order to test this hypothesis, we first reviewed existing literature to investigate the response of ASD animal models to unexpected and novel situations by comparing behavioral phenotype expression during short behavioral tests with behavior during baseline conditions. Next, we set out to identify potentiating novelty conditions by comparison of C57BL/6J (C57) and BTBR T+tf/J (BTBR) mouse inbred strains across a variety of exposures. Based on our findings, we conclude that short assays that incorporate novelty are well suited to study behavioral trait variation in animal studies in the context of ASD.

METHODS

Systematic literature review of novelty behaviors in genetic animal models of ASD

First, we performed a systematic literature study to compare behavioral abnormalities in ASD mouse models during baseline conditions versus during exposure to a novel behavioral test environment. For this comparison, we focused on behavioral activity as our main readout, because long-term behavioral observations are often limited to measurements of locomotor activity. We first collected published locomotor activity data from individual mouse model studies based on prominent ASD risk-genes, and then combined these data per ASD risk-gene.

For this search, we focused on genetic animal models for ASD that we selected based on prominent ASD-risk genes defined as genes in category 1 and 2 of the autism gene scoring module in the SFARI knowledgebase (Abrahams et al. 2013) (https://gene.sfari.org/autdb/GS_Home.do accessed 2017-11-12). To identify relevant mouse behavioral studies relating to these ASD-risk genes, we performed a Boolean PubMed search after retrieval of orthologous mouse gene names from BioMart (Smedley et al. 2015) (https://www.ensembl.org/biomart accessed 2017-11-12):

> Adnp OR Ank2 OR Ankrd11 OR Arid1b OR Ash1l OR Asxl3 OR Bckdk OR Bcl11a OR Cacna1d OR Cacna1h OR Cacna2d3 OR Chd2 OR Chd8 OR Cic OR Cntn4 OR Cntnap2 OR Ctnnd2 OR Cul3 OR Ddx3x OR Deaf1 OR Dip2c OR Dscam OR Dyrk1a OR Erbin OR Foxp1 OR Gabrb3 OR Gigyf2 OR Gm42715 OR Gria1 OR Grin2b OR Grip1 OR Ilf2 OR Ints6 OR Irf2bpl OR Kat2b OR Katnal2 OR Kdm5b OR Kdm6a OR Kmt2a OR Kmt2c OR Kmt5b OR Magel2 OR Mboat7 OR Mecp2 OR Med13 OR Med13l OR Met OR Myt1l OR Naa15 OR Nckap1 OR Nlgn3 OR Nrxn1 OR Phf3 OR Pogz OR Ptchd1 OR Pten OR Ranbp17 OR Reln OR Rims1 OR Scn2a OR Scn9a OR Setd5 OR Shank2 OR Shank3 OR Slc6a1 OR Smarcc2 OR Spast OR Srcap OR Srsf11 OR Syngap1 OR Tbl1xr1 OR Tbr1 OR Tcf20 OR Tnrc6b OR Trip12 OR Ubn2 OR Upf3b OR Usp7 OR Wac OR Wdfy3 OR Zfp957) AND autism AND mouse AND (behavior OR behavioral) AND (locomotion OR locomotor OR hyperactive OR hyperactivity OR hypoactive OR hypoactivity OR responsiveness)

This literature search yielded 47 studies, of which 25 could be used to investigate locomotor activity after transfer into a novel behavioral test environment (i.e. within 30 minutes after transfer) versus at baseline levels derived from relative long-term observations. We confined our analysis to data obtained from male mice, since only few studies reported data from both male and female mice. We first collected the

locomotor activity data from individual mouse models, and then combined these data per ASD risk-gene to derive gene-based scores for abnormal locomotor activity (i.e. in comparison to wild-type mice) after transfer and at baseline (Table 1). Genes lacking either one of these scores were excluded from further analysis, which resulted in a total of 11 gene-based scores used for final analysis.

Table 1. Scoring of abnormalities at baseline and after environmental change

Gene-based score	Definition
↑↑	Consistent higher activity, or highly significant (p-value < 0.001)
↑	Higher activity (p-value < 0.05)
↔	No difference in activity
↓	Lower activity (p-value < 0.05)
↓↓	Consistent lower activity, or highly significant (p-value < 0.001)

Novelty-related experiments in BTBR mice

For the study of novelty-related behavioral differences in BTBR mice, breeding pairs of C57BL/6J (C57) and BTBR T+tf/J (BTBR) strains were originally purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Strain colonies were subsequently bred in-house. Phenotypic assessments were performed in two different cohorts of adult male mice to limited repeated exposure to multiple behavioral assays (Table 2). All experiments were approved by the ethical committee for animal experimentation of the University Medical Center Utrecht and Free University Amsterdam and performed according to the institutional guidelines that are in full compliance with the European Council Directive (86/609/EEC).

Table 2. Phenotypic assessments in two different cohorts

Cohort	Assay	Animals (n)	Laboratory
Cohort 1	Home-cage observation	14 BTBR; 30 C57	VU Amsterdam (Sylics)
Cohort 2	Novel object exposure	10 BTBR; 11 C57	UMC Utrecht

Automated observation of novelty and baseline activity

Eight to 12-week-old male mice (cohort 1) were singly housed on sawdust in standard Makrolon type II cages enriched with cardboard nesting material for at least 1 week prior to experiments, with water and food ad libitum (7:00/19:00 lights on/off; providing an abrupt phase transition). We only used male mice to avoid possible impact of estrous cycle on longitudinal behavioral assessments. Observation of was performed over 2.5 days in a home-cage environment (PhenoTyper model 3000, Noldus Information Technology, Wageningen, The Netherlands), described in detail previously (Maroteaux et al. 2012). Mice were introduced in the cage in the second half of the subjective light phase (14:00–16:00 h), and video tracking started at the onset of the first subjective dark phase (19:00 h). The X–Y coordinates of the center of gravity of mice sampled at a resolution of 15 coordinates per second were acquired and smoothed using EthoVision software (EthoVision HTP 2.1.2.0, based on EthoVision XT 4.1, Noldus Information Technology, Wageningen, The Netherlands), as previously reported (Loos et al. 2014). We here report longitudinal PhenoTyper data per one-hour time bins.

short exposure to novel objects

Fifteen- to 20-week-old male mice (cohort 2) were socially housed in Makrolon type II cages, with water and food ad libitum (7:00/19:00 lights on/off; providing an abrupt phase transition). Observation was performed in the middle of the subjective light phase (11:00 – 15:00 h) during a 10-miute exposure to a novel testing cage (26.5 x 17 x 11.4 cm) with wood chip bedding and four novel objects placed ± 1 cm from the walls: a dice (19x19x19 mm), a marble (Ø 35 mm), a miniature bowling pin (39x14x14 mm) and a blue piece of Lego (36x15x15 mm). The four novel objects were made of dense material, in order to prevent chewing. Before each trial, objects were thoroughly cleaned with Trigene to avoid object preference based on odor. All trials were recorded with a high definition video recorder and X–Y coordinates of the center of gravity analyzed using EthoVision XT9 (Noldus Information Technology, Wageningen, The Netherlands).

Statistics

For our behavioral experiments, phenotype distributions were inspected for normality, and in the case of non-normality – which was observed only for baseline locomotor-activity – a log-transformation was applied to improve normality for statistical testing. Group differences were determined using a Welch Two Sample t-test.

Cohen's *d* was determined as described by Coe (Coe 2002), by calculating the mean difference between BTBR and C57 groups, and then dividing the result by the pooled standard deviation: $SD_{pooled} = \sqrt{\frac{(N_{BTBR}-1)\cdot SD_{BTBR}{}^2+(N_{C57}-1)\cdot SD_{C57}{}^2}{N_{BTBR}+N_{C57}-2}}$. Confidence intervals for effect sizes were calculated according to Hedges and Olkin (Hedges and Olkin 1985) p86, based on standard deviation $\sigma[d] = \sqrt{\frac{N_{BTBR}+N_{C57}}{N_{BTBR}\times N_{C57}} + \frac{d^2}{2(N_{BTBR}+N_{C57})}}$. The 95% confidence intervals for *d* were subsequently calculated from $\mathrm{d} - 1.96\,\sigma[\mathrm{d}]$ to $+1.96\,\sigma[\mathrm{d}]$. All statistical analyses were performed withthe statistical software R (R core team 2016).

RESULTS

Behavioral abnormalities in ASD mouse models after change

We performed a systematic literature study to compare behavioral activity in genetic mouse models for ASD during baseline conditions versus during exposure to a novel behavioral test environment. We focused on behavioral activity as our main readout, because long-term behavioral observations are often limited to locomotor activity. We first collected locomotor activity data from individual mouse model studies based on prominent ASD risk-genes, and then combined these data per ASD risk-gene. Following this approach, we were able to calculate gene-based scores for both abnormalities during baseline conditions and during exposure to a novel test environment for a total of 11 prominent ASD risk-genes (Table 3a and 3b). Based on these data, we found that mouse models related to 10 out of 11 different ASD risk-genes displayed abnormal behavioral activity after environmental change, while abnormalities during baseline conditions were observed in models related to 7 out of 11 genes. In addition, we found that abnormalities during exposure to a novel behavioral test environment were more severe relative to baseline measures for models related to *Cntnap2, Met, Nrxn1, Ptchd1* and *Pten* (Figure 1). These findings indicate that behavioral activity abnormalities in genetic mouse models for ASD are more pronounced after environmental change than during baseline conditions.

Table 3a. Systematic literature review and assays for locomotor activity

Human gene	Publication	Mouse model	Behavioral assays
CNTN4	(Molenhuis et al., 2016)	*Cntn4* HET + KO	Open field (15 min) and home-cage locomotor activity
CNTNAP2	(Peñagarikano et al., 2011)	*Cntnap2* KO	Open field (20 min)
	(Thomas et al., 2017)	*Cntnap2* KO	Open field (20 min, 5-min bins)
	idem	*Cntnap2* KO	Activity monitoring (24 hours)
FOXP1	(Araujo et al., 2017)	*Foxp1C* KO *(neuron-specific)*	Activity monitoring in novel cage (120 min, 5-min bins)
PTCHD1	(Wells et al., 2016)	*Ptchd1* KO	Open field (60 min, 5-min bins)
	(Ung et al., 2017)	*Ptchd1* KO	Home-cage locomotor activity
MECP2	(Santos et al., 2007)	*MeCP2* KO	Open field (5 min)
	(Nag et al., 2009)	*MeCP2* KO	Activity monitoring (8 hours)
	(Lioy et al., 2011)	*MeCP2* KO	Open field (20 min) and home-cage-like cage (10 min)
	(Johnson et al., 2012)	*MeCP2* KO	Running wheel assay (24 hours)
	(Zhou et al., 2017)	*MeCP2* KO	Open field (15 min)
	(Chang et al., 2016)	*MeCP2* KO *(GABA-specific)*	Locomotor activity in empty cage (10 min)
	(Xu et al., 2018)	*MeCP2* mutant	Open field (30 min)
MET	(Thompson and Levitt, 2015)	*Met* KO *(neuron-specific)*	Activity monitoring (30 min)
NLGN3	(Jaramillo et al., 2017)	NL3R451C mutant	Novel cage activity test (120 min, 5-min bins)
NRXN1	(Laarakker et al., 2012)	*Nrxn1-alfa* KO *(mixed background)*	Open field (5-min, repeated exposures)
	(Grayton et al., 2013)	*Nrxn1-alfa* HET + KO *(fixed background)*	Home-cage locomotor activity
PTEN	(Ogawa et al., 2007)	*Pten* KO *(neuron-specific)*	Activity monitoring in open field (72 hours)
SHANK2	(Schmeisser et al., 2012)	Shank2 HET + KO	Open field (30 min, 5-min bins)
	(Ha et al., 2016)	Shank2 KO *(Purkinje cell-specific)*	Open field (60 min) and activity monitoring (96 hours)
SHANK3	(Lee et al., 2015)	*Shank3(Δ9)* KO	Open field (60 min) and 72-hour activity monitoring
	(Speed et al., 2015)	*Shank3* mutant	Open field (10 min) and activity test (120 min, 5-min bins)
	(Wang et al., 2016)	*Shank3* KO	Open field (60 min) and home-cage observation
	(Chang et al., 2016)	*Shank3B* KO	Locomotor activity in empty cage (10 min)
	(Copping et al., 2017)	*Shank3B* HET	Open field (30 min)

Table 3b. Literature findings and gene-based scores for locomotor activity at baseline and after transfer into a novel environment

Human gene	Publication	Behavioral data	Gene-based scores		
			After transfer	Baseline	delta
CNTN4	(Molenhuis et al., 2016)	No difference in open field or long-term measurements	↔	↔	↔
CNTNAP2	(Peñagarikano et al., 2011)	Locomotor activity ↑ ($p<0.001$)	↑↑	↓	↑↑↑
	(Thomas et al., 2017)	Locomotor activity during first 15 min ↑ ($p<0.05$), after 15 min ↔			
	idem	Activity during 24-hour monitoring ↓ ($p<0.01$)			
FOXP1	(Araujo et al., 2017)	Activity ↑ throughout 120 min the trial ($p<0.001$)	↑↑	↑↑	↔
PTCHD1	(Wells et al., 2016)	Locomotor activity in first 15 min ↑ ($p<0.001$), but in last 15 mins ↔	↑↑	↑	↑
	(Ung et al., 2017)	Locomotor activity during habituation ↑ ($p<0.001$), in dark phase ↑ ($p<0.01$)			
MECP2	(Santos et al., 2007)	Distance moved ↓ ($p<0.05$), speed ↓ ($p<0.001$), neurological dysfunction	↓↓	↓↓	↔
	(Nag et al., 2009)	Locomotor activity from hours 2 to 8 ↓ ($p<0.001$)			
	(Lioy et al., 2011)	Locomotor activity in open field ↓ and new cage ↓ ($p<0.01$; $p<0.01$)			
	(Johnson et al., 2012)	Running wheel activity ↓ ($p<0.05$)			
	(Zhou et al., 2017)	Locomotor activity ↓ ($p<0.05$), duration active ↓ ($p<0.001$), anxiety ↑			
	(Chang et al., 2016)	Locomotor activity after 10 min habituation ($p<0.05$) ↓			
	(Xu et al., 2018)	Locomotor activity ($p<0.05$) ↓			
MET	(Thompson and Levitt, 2015)	Distance moved in activity chamber ↓ ($p<0.005$), at end of trial ↔	↓	↔	↓
NLGN3	(Jaramillo et al., 2017)	Activity ↑ throughout the trial ($p<0.05$)	↑	↑	↔
NRXN1	(Laarakker et al., 2012)	Responsivity to novelty ↑ ($p<0.05$)	↑↑	↔	↑↑
	(Grayton et al., 2013)	Baseline activity in KO ↓(trend), in HET 10 min after transition ↑(trend)			
PTEN	(Ogawa et al., 2007)	Locomotor activity on day 1 and 2 ↑ ($p<0.05$), on day 3 ↔	↑	↔	↑
SHANK2	(Schmeisser et al., 2012)	Locomotor activity in HET ↑ and KO ↑ throughout the trial ($p<0.01$; $p<0.001$)	↑	↑	↔
	(Ha et al., 2016)	Open field and long-term measurements ↔			
SHANK3	(Lee et al., 2015)	Open field and long-term measurements ↔	↓	↓	↔
	(Speed et al., 2015)	Activity in first 5 min and open field ↓ ($p<0.001$; $p<0.05$), full 120 min ↔			
	(Wang et al., 2016)	Locomotor activity in open field ↓ ($p<0.05$), escape from novel arena ↑			
	(Chang et al., 2016)	Locomotor activity after 10 min habituation ↑ ($p<0.05$)			
	(Copping et al., 2017)	Open field locomotor activity in HET ↔, activity in KO ↓ ($p<0.05$)			

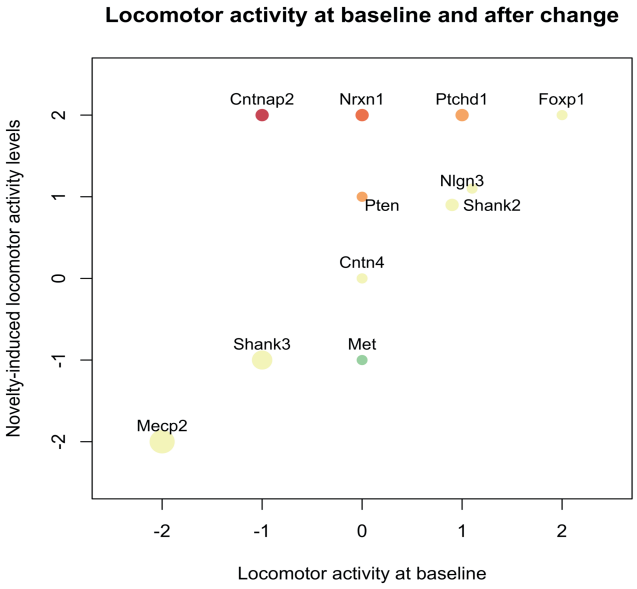

Figure 1. Novelty-induced effects on locomotor activity in genetic mouse models for ASD. Gene-based scores for abnormal locomotor activity at baseline and during exposure to a novel behavioral testing environment (2 = highly significant increase (p-value < 0.001) or consistent higher activity; 1 = higher activity (p-value < 0.05); 0 = no difference; -1 = lower activity (p-value < 0.05); -2 = highly significant decrease (p-value < 0.001) or consistent lower activity). Dot colors represent the difference between novelty-induced locomotor activity scores and locomotor activity scores at baseline (yellow dots indicate similar scores, while red and green dots indicate that novelty-induced locomotor activity scores are higher or lower, respectively). Dot size reflects the number of studies per gene-based score.

Novelty-exposure reveals maximal behavioral differences in BTBR mice

These literature data suggested that exposure to novelty could be a useful modifier to maximize behavioral differences in animal models for ASD. To identify behavioral assays with the maximum capacity to reveal behavioral differences in genetic mouse models for ASD, we then investigated the behavioral activity profile of the BTBR mouse inbred strain model for ASD during novelty and baseline conditions. We performed a longitudinal home-cage observation and found that BTBR mice displayed *hyper*-activity in the first dark-hour of the observation (Figure 2a + 2b), followed by *hypo*-activity later in the experiment (Figure 2a + 2c).

To identify behavioral assays with the maximum capacity to differentiate between C57 and BTBR mice based on novelty-induced hyperactivity, we tested a second batch of BTBR and C57 mice by exposing them to a novel environment with four novel

objects for 10 minutes. In this assay, we observed highly that BTBR mice showed highly increased locomotor activity compared to C57 control mice (Figure 3).

To enable direct comparison of behavioral assays and their capacity to differentiate between C57 and BTBR mice, we then calculated effect sizes for strain differences for different behavioral assays. Combined with the data from (Molenhuis et al. 2014), we found that effect sizes increased with relatively short durations and with exposure to a novel environment and novel objects (Figure 4).

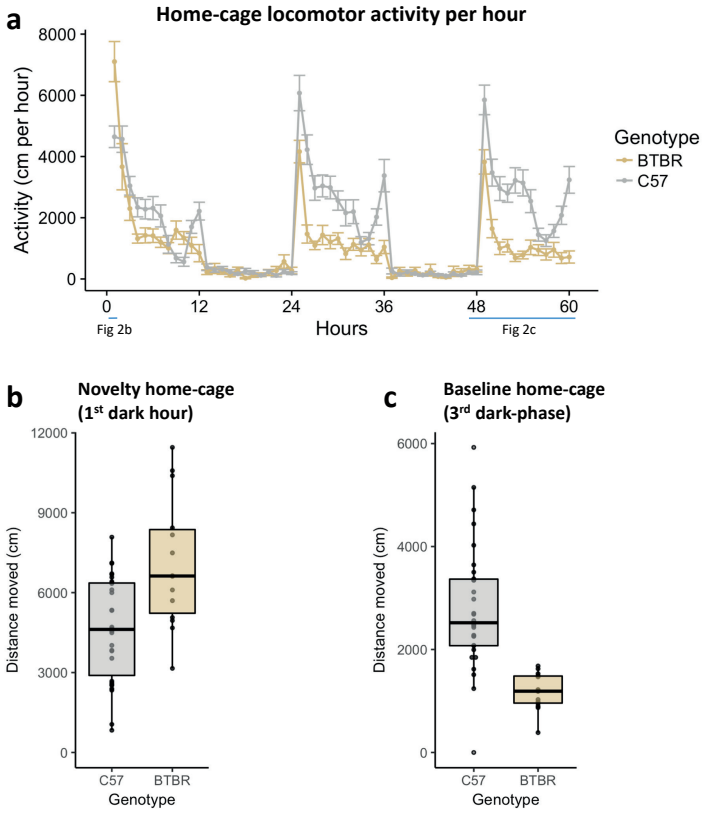

Figure 2. Longitudinal home-cage observations and novelty-exposure in BTBR and C57 mice. **a**) Locomotor activity in BTBR and C57 mice in one-hour time bins. **b**) Locomotor activity differences between BTBR and C57 in the first dark-phase hour; Welch Two Sample t-test: t(20.77)=3.296, p = 0.0035). **c**) Locomotor activity differences between BTBR and C57 mice in their 3rd subjective dark-phase; Welch Two Sample t-test for log-transformed data: t(25.50)=-7.201, p = 1.34x10-7 (Welch Two Sample t-test before log- transformation: t(37.78)=-6.642, p = 7.71x10-8).

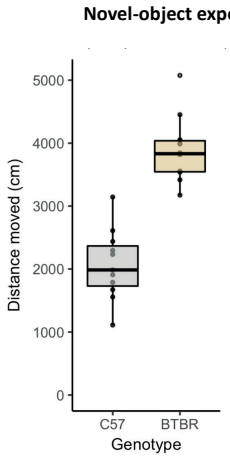

Figure 3. Novel objects exposure and hyperactivity in BTBR mice. Locomotor activity differences between BTBR and C57 mice during 10-miute exposure to four novel objects. Welch Two Sample t-test: t(18.85)= 7.529, p = 4.28x10-7.

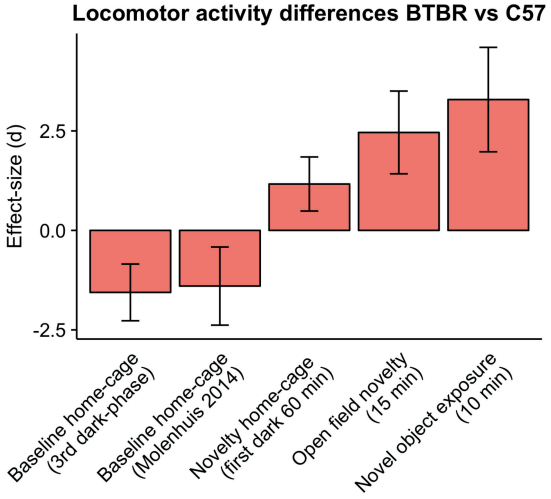

Figure 4. Comparison of BTBR and C57 mice across novelty-exposures and baseline levels. Effect-sizes and 95% confidence intervals for differences between BTBR and C57 mice for baseline home-cage activity (cohort 1); baseline home-cage activity (data from Molenhuis et al. 2014); locomotor (hyper-)activity during first dark hour of novel home-cage (cohort 1); 15 min exposure to open field (data from Molenhuis et al. 2014); and 10 min exposure to a novel cage with four novel objects (cohort 2).

DISCUSSION

We investigated the impact of unexpected or novel situations – inherent to many classical behavioral testing paradigms – on existing gene knockout models for ASD. Since resistance to change is a common ASD feature (Geurts et al. 2009), we hypothesized that behavioral abnormalities observed in these models are more profound after the mouse is transferred from its home-cage into a novel behavioral testing arena. Our findings suggest that transfer to another environment may act as a potential modifier in mouse in mouse models for ASD.

Our results from a systematic literature study indicated that behavioral activity in novel environments is affected in many gene knockout models for most ASD risk-genes, and that exposure to a novel behavioral testing environment contributes to behavioral abnormalities observed in these models. One limitation of this literature study is that the behavioral measurements during baseline conditions as well as exposure to novelty were variable across animal model studies. Over the past decade, various home-cage systems have been developed that allow longitudinal behavioral monitoring (De Visser et al. 2006; Loos et al. 2014). Our findings underscore the added value of such phenotyping strategies, as they allow to assess the precise difference between novelty-induced behaviors versus baseline expression within a single animal and experiment. Another limitation of our study is that we only focused on locomotor activity because long-term behavioral observations are often limited to measurements of locomotor activity. An exciting future direction is to expand longitudinal behavioral profiling beyond the assessment of locomotor activity, for example by the use of automated behavior recognition systems to detect patterns of stereotyped behaviors.

We further aimed to identify optimal environmental conditions to maximize behavioral differences in phenotype-driven future studies. Therefore, we characterized differences in behavioral activity between C57 and BTBR mice when exposed to a variety of environmental conditions. The experiments in BTBR mice showed consistent novelty-induced hyperactivity, combined with *hypo*-activity relative to C57 mice *after* habituation to a novel environment. These findings are consistent with previous observations (Molenhuis et al. 2014), and indicate that novelty-induced hyperactivity increases with the extent of novelty (e.g. exposure to a novel environment and novel objects) and short observation periods (e.g. 10 minutes). The results from previous published data combined with the present behavioral experiments in BTBR mice show that exposure to a novel environment potentiates behavioral phenotype expression in ASD mouse models, and suggest that short lasting assays that incorporate novelty may be best suited to detect genotype-phenotype relationships in the context of ASD.

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Chapter 4

Modeling the quantitative nature of neurodevelopmental disorders using Collaborative Cross mice

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ABSTRACT

Background

Animal models for neurodevelopmental disorders (NDD) generally rely on a single perturbation in one fixed genetic background. Their translational value would be greatly enhanced if genetic insults could be studied in a more quantitative framework across genetic backgrounds.

Methods

To meet this demand, we used the Collaborative Cross (CC), a novel mouse genetic reference population, to investigate the quantitative genetic architecture of mouse behavioral phenotypes commonly used in animal models for NDD.

Results

Classical tests of social recognition and grooming phenotypes appeared insufficient for quantitative studies due to genetic dilution, limited heritability or non-continuous distribution. In contrast, NDD relevant traits such as stereotyped behaviors and locomotor activity were characterized by continuous distribution across our CC sample, and also mapped to quantitative trait loci containing genes associated with corresponding phenotypes in human populations.

Conclusion

These findings show that the CC can move animal model studies beyond comparative single gene-single background designs, and point out which type of behavioral phenotypes are suitable to quantify the effect of developmental perturbations across multiple backgrounds.

INTRODUCTION

Recent studies show that genetic risk factors for neurodevelopmental disorders (NDD) such as autism spectrum disorder (ASD) and attention deficit hyper-activity disorder (ADHD) segregate in the general population and influence continua of behavioral and developmental traits (Robinson et al. 2016; Weiner et al. 2017; Demontis et al. 2017; Bralten et al. 2017). These findings confirm the clinical notion that the extreme tails of trait distributions are most likely to be associated with a clinical diagnosis, and that genetic risk factors for ASD and ADHD confer quantitative rather than categorical effects (Coghill and Sonuga-Barke 2012; Plomin et al. 2009; Constantino 2011). The important implication of these studies is that continuous behavioral dimensions instead of dichotomous outcomes should be studied to acknowledge the quantitative effects of developmental perturbations.

This notion also has implications for animal studies into the biology of NDD. So far, animal models for NDD are generally designed to study the impact of single genetic mutations in a fixed genetic background. Indeed, they have provided crucial insights into neurodevelopmental disease biology and have transformed the landscape of treatment development (de la Torre-Ubieta et al. 2016). A caveat is that their translational value can be limited, as genotype-phenotype relationships observed in a single mouse genetic background are often not generalizable to other backgrounds (Sittig et al. 2016). To move forward, animal model strategies are required that can evaluate the clinical impact of developmental insults on continuous behavioral traits across genetic backgrounds.

To meet this demand, we tested the potential of the Collaborative Cross (CC), a mouse genetic reference population, and establish a quantitative framework to study the impact of NDD etiological factors on neurobehavioral trait distributions. The CC is a panel of mouse recombinant inbred lines (RI lines) with a unique level of genetic diversity derived from 5 classical laboratory strains and 3 wild-derived mouse inbred lines (Figure 1A) (Churchill et al. 2004). The resulting level of genetic diversity is considerably higher compared to existing RI panels, which is a crucial asset to match the human population studies into behavioral variation (Yang et al. 2011; Hall et al. 2012; Churchill et al. 2004; Beck et al. 2000). Using 53 CC lines, we find that general physical and basic behavioral traits such as digging, locomotor activity and stereotyped exploratory behavior show substantial continuous variation and heritability in CC lines, in contrast to mouse phenotypes commonly studied to establish face validity for specific NDDs such as ASD. To confirm translational relevance of neurobehavioral trait variations in the CC population, we find that several of the loci mapped for neurobehavioral traits were enriched for homologous genes implicated in NDD and brain developmental processes.

RESULTS

Structure of the genomes of CC lines in this study

We analyzed quantitative behavioral and physical variation in 53 CC lines available at Tel Aviv University (TAU), Israel (Figure 1a) (Hall et al. 2012). We first reconstructed the genome of each CC line as a mosaic of the eight CC founder strains, by calculating the probabilities of descent at about 11,000 genomic intervals using available genome-wide SNP array data from the MUGA and MegaMUGA platforms (Figure 1b) (Hall et al. 2012). In this set of CC lines, the overall genetic contribution of CC founder strains was within the expected range of about 1/8 = 12.5%, except for CAST/EiJ and PWK/PhJ, contributing 8.4% and 5.9%, respectively (Figure 1c). Despite the lower overall abundance of these founder strains, complete absence of founder haplotypes was only observed at three genomic intervals, on chromosome 2 and 8 (Figure 1d). These results indicate that this set of CC lines represents a substantial mixture of the genetic diversity present in the eight CC founder strains.

Quantitative variation in behavioral and physical traits

We first tested the influence of the genetic variation in these 53 CC lines on basic mouse behavioral traits and quantified digging, locomotor activity and stereotyped exploratory patterns (for quantification of exploratory patterns refer to Supplementary Figure S1). The total population consisted of 285 male CC mice with 5-6 mice on average per CC line. We observed substantial continuous variation for these traits across the tested population (Figure 2a), with significant differences between CC lines (Figure 2b). We then estimated heritability and found that estimates of narrow-sense heritability (estimated from genome-wide genetic differences between lines) were substantial for all 3 basic behavioral traits (Figure 3a and b, top). Similar observations were made for broad-sense heritability (estimated from differences between CC lines) (Supplementary Figure S2). To identify genetic loci contributing to this variation, we performed quantitative trait locus (QTL) analysis and identified significant QTLs for digging behavior on the X chromosome, locomotor activity on chromosomes 1 and 12, and stereotyped exploratory patterns on chromosomes 2 and 12 (Figure 2c & Table S1).

To compare mouse behavioral traits with general physical traits, we quantified brain-weight and body-weight and observed strong variation across CC lines (Figure 2d & e). Moreover, heritability-estimates for corrected brain-weight and total body weight were high relative to basic mouse behavioral traits (Figure 3a and b, middle). We performed QTL-analysis and identified loci for corrected brain-weight on chromosomes 4 and 13. Perhaps surprisingly, we did not detect any locus

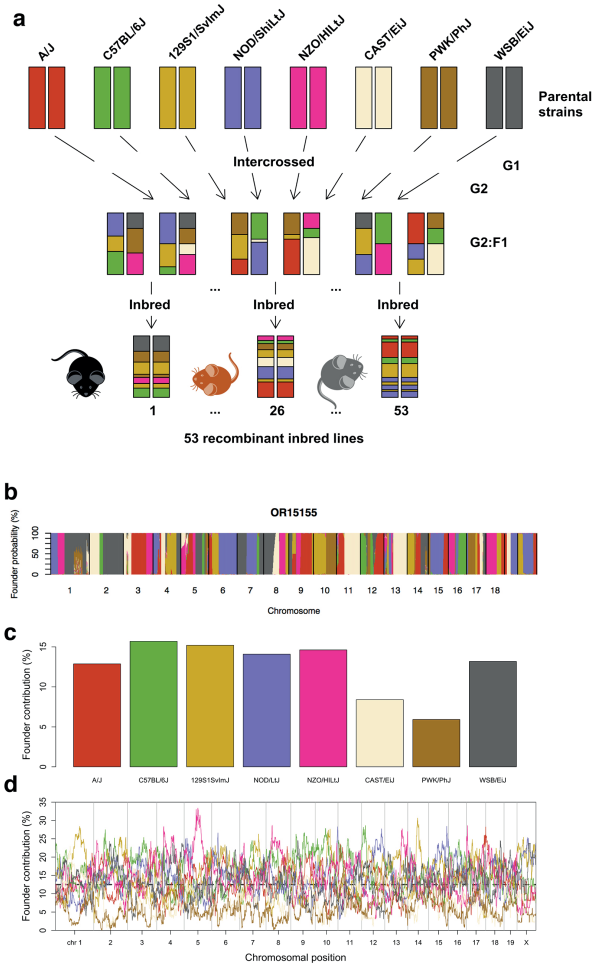
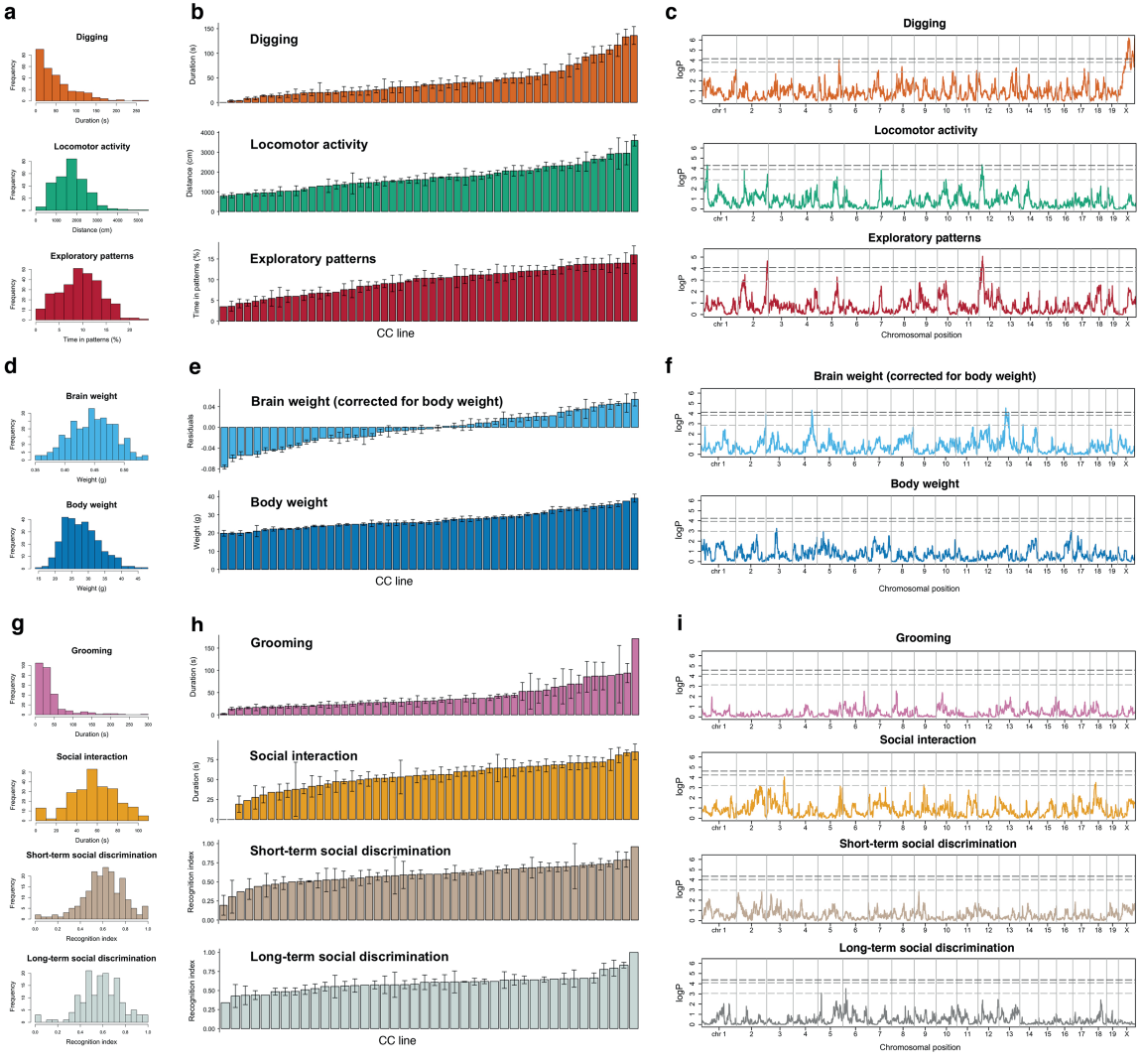


Figure 1. Genome structures across CC lines in this study. (**a**) CC lines used in this study were created from independent three-round intercrosses of eight parental founder strains, five of which are classical laboratory strains (C57BL/6J, A/J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HlLtJ) and three of which are wild-derived inbred strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ). The resulting F1 mice were iteratively inbred and constitute a population of RI lines that is considered fully inbred at generation F20+. (**b**) Example of a genome reconstruction of a CC line, as a mosaic of the eight CC founder strains. Intervals of ambiguity are, either caused by remaining heterozygosity, or are regions where the founder strains have identical haplotypes. (**c**) Overall founder strain contributions in CC lines used for genetic analysis in this study, indicating lower abundance of CAST/EiJ, PWK/PhJ founder haplotypes. (**d**) Genome-wide founder strain contributions indicate absence of PWK/PhJ founder haplotypes at three genomic intervals, on chromosome 2 and 8.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Digging
Frequency
Duration (s)
Locomotor activity
Distance (cm)
Exploratory patterns
Time in patterns (%)
b
Digging
Duration (s)
Locomotor activity
Distance (cm)
Exploratory patterns
Time in patterns (%)
CC line
c
Digging
logP
chr 1
Locomotor activity
Exploratory patterns
Chromosomal position
d
Brain weight
Weight (g)
Body weight
e
Brain weight (corrected for body weight)
Residuals
Body weight
Weight (g)
CC line
f
Brain weight (corrected for body weight)
Body weight
Chromosomal position
g
Grooming
Duration (s)
Social interaction
Short-term social discrimination
Recognition index
Long-term social discrimination
h
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Duration (s)
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CC line
i
Grooming
Social interaction
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Long-term social discrimination
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Figure 2. Quantitative phenotypic variation across CC lines and genetic mapping.(**a**) Histograms of digging, locomotor activity and stereotyped exploratory patterns in 285 CC mice from 53 CC lines. (**b**) Digging, locomotor activity and stereotyped exploratory patterns obtained from CC lines. Plots are expressed as mean ± SEM, with CC lines ordered along the x-axis by mean per phenotype (one-way ANOVA P=2.1x10-25, P=1.1x10-22, P=1.6x10-14; one-way ANOVA after correction for age, batch and origin P=1.3x10-17, P=3.3x10-15, P=3.3x10-7). (**c**) Genome scans for digging, locomotor activity and stereotyped exploratory patterns. Significance thresholds were estimated by 1000 permutations. QTLs were detected for digging on the X chromosome (permuted P<0.001), locomotor activity on chromosomes 1 and 12 (permuted P=0.042 and P=0.042), and exploratory patterns on chromosomes 2 and 12 (permuted P=0.012 and P=0.003). Dashed lines represent genome-wide permuted P<.05, 0.1, and 0.5. (**d**) Histograms of brain-weight and body-weight. (**e**) Brain-weight (residuals after correction for body-weight) and body-weight obtained from 53 CC lines. Plots are expressed as mean ± SEM, with CC lines ordered along the x-axis by mean per phenotype (one-way ANOVA P=1.1x10-44, P=2.0x10-48; one-way ANOVA after correction for age, batch and origin P=1.1x10-32, P=6.0x10-30). (**f**) Genome scans for brain-weight (residuals after correction for body-weight) and body-weight. QTLs were detected for corrected brain-weight on chromosomes 4 and 13 (permuted P=0.034 and P=0.021), but not for body-weight. (**g**) Histograms of grooming, social interaction, short-term and long-term social discrimination. (**h**) Grooming, social interaction, and short-term and long-term social discrimination from up to 53 CC lines. Plots are expressed as mean ± SEM, with CC lines ordered along the x-axis by mean (one-way ANOVA P=2.8x10-5, P=2.8x10-8, P=0.0035 and P=0.33; one-way ANOVA after correction for age, batch and origin P=0.0027, P=2.6x10-5, P=0.18 and P=0.31). (**i**) Genome scans for grooming, social interaction, short-term and long-term social discrimination. No QTLs were detected.

for total body-weight, despite high estimates of narrow-sense heritability (Figure 2f). This is likely because many loci, each contributing a relatively small fraction of variance, contribute to bodyweight.

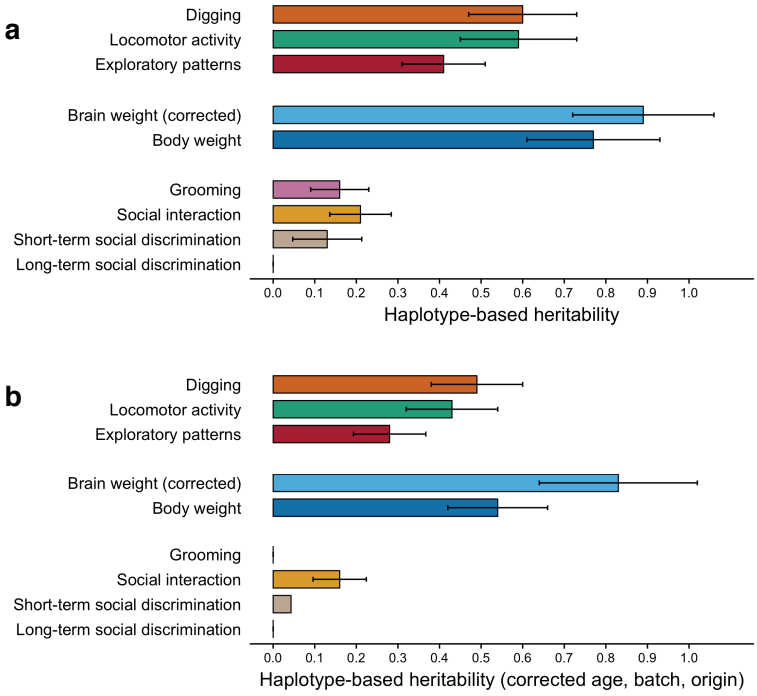

Figure 3. Haplotype-based heritability estimates. (**a**) Genome-wide haplotype-based (narrow-sense) heritability estimates for activity mouse behavioral traits (top), physical traits (middle), and social behavior and grooming-related readouts (bottom). (**b**) Genome-wide haplotype-based (narrow-sense) heritability estimates after correction for effects of age, batch and origin, and quantile normalization.

Quantitative variation in phenotypes commonly used in animal models for ASD

Next, we analyzed behavioral traits commonly studied to establish face validity with human ASD and found that continuous variation in social interaction, short-term and long-term social discrimination (Figure 2g) across CC lines. In contrast, for grooming behavior, we observed a more discrete distribution, indicated by a small fraction (6.0%) of animals with excessive levels (i.e. duration > 120 seconds) that were phenotypically separate from the majority (Figure 2g). For all these ASD-related variables, differences within CC lines were relatively large, with modest differences between lines (Figure 2h). We estimated heritability and found that estimates of narrow-sense heritability of ASD-related variables were low relative to the three basic behavioral and general physical traits (Figure 3a and b, bottom). In contrast to the high heritable phenotypes, no significant loci were identified in the QTL-analysis for these low heritable phenotypes (social cognitive and grooming behavior variables) (Figure 2i).

Intersection of genes in QTLs with human neurodevelopmental risk genes

We then investigated whether behavioral QTLs were enriched for genes relevant to NDD in humans. For that purpose, we identified the human orthologues of genes within each mouse behavioral QTL and ranked these genes on the basis of a recently developed genome-wide prediction algorithm to prioritize genes for ASD based on prior genetic evidence and functional interaction networks.(Krishnan et al. 2016). Following this strategy, we found that homologous genes in the QTL for locomotor activity on chromosome 1 showed enrichment of genes with high ASD-ranking (Supplementary Figure S3, $P = 0.02$). High ASD-ranked genes within this QTL included brain-specific angiogenesis inhibitor 3 (*Bai3*), a member of the BAI subfamily of adhesion G-protein-coupled receptors (GPCRs). These GPCRs regulate synapse development and plasticity and are implicated in various neurological and psychiatric disorders. Moreover they are potential targets for pharmacological intervention (Duman et al. 2016).

Furthermore, the QTL for repetition in exploratory patterns on chromosome 2 contained *Hrh3*, a histamine signaling gene implicated in ASD by expression-analysis in post-mortem brains (Wright et al. 2017). Interestingly, histamine H3R receptor activation in the dorsal striatum was recently shown to trigger stereotypies in mice, and antagonism of H3R was found to attenuate elevated repetitive behavior in an animal model of autism induced by prenatal exposure to valproic acid (Rapanelli et al. 2017; Baronio et al. 2015).

In addition, the QTL for brain-weight on chromosome 13 contained *Atxn1*, a gene whose disruption is known to result in reduced cortical thickness in mice, and causes a spectrum of neurobehavioral phenotypes including hyperactivity in both mice and humans (Lu et al. 2017). The overlapping region of loci for exploratory patterns and locomotor activity on chromosome 12 included a mouse homolog of the human *YWHAE* gene, implicated in the Miller-Dieker syndrome that is located on chromosomal position 17p13.3. Finally, although the QTL for digging behavior on the X-chromosome was very broad, notable genes in this locus included *Dcx*, coding for neuronal migration protein doublecortin, and *Htr2C* gene, coding for a serotonin receptor implicated in mental illnesses including OCD (Sinopoli et al. 2017).

DISCUSSION

We show that CC recombinant inbred lines can strongly complement existing genetic or inbred animal models for neurodevelopmental disorders. The current sample of CC inbred lines show a population distribution of phenotypic variability for multiple NDD traits, and we identify QTLs that harbor genes strongly associated with corresponding NDD phenotypes in humans. Overall, the results show that CC lines can be used to study the quantitative impact of genetic manipulations and environmental perturbations on the basis of controlled genetic background variability. This is a crucial advancement as human genetic association studies have established a genetic architecture of NDD in which genetic background and risk cause a variable continuous distribution of core traits across the entire population.

The obtained results in this CC population show that the choice of behavioral phenotypes is crucial to mimic trait dynamics and genetic associations observed in population studies (Robinson et al. 2016). First, phenotypes should be continuously distributed traits in the study population. We found that grooming behavior was non-continuously distributed in the CC population, indicating that this phenotype may only capture genetic effects in a smaller proportion of the population. Secondly, phenotype expression should be heritable in the study population, similar to ASD and ADHD-like traits in the general human population. In this respect, locomotor activity, a measure of stereotyped exploratory patterns and social interaction seem more suitable compared to low heritability traits such as short-term and long-term social discrimination.

Once these criteria have been met, translational value may be indicated by the intersection of human neurodevelopmental risk genes with genetic associations of homologous genes in mouse populations. As proof of principle, we found several

genes in the QTLs that are implicated in normal and abnormal brain development and genes that are associated with NDD. Interestingly, we identified a gene coding for H3R receptor in the QTL for repetitive behavior patterns. Manipulation of this receptor has been shown to alter repetitive behavior expression both in an anatomical model as in a pharmacological model (Wright et al. 2017; Rapanelli et al. 2017; Baronio et al. 2015). For example, the H3 receptor is highly expressed in striatal nuclei, and histamine H3 receptor activation in the dorsal striatum was recently shown to trigger stereotypies in mice (Rapanelli et al. 2017). This finding highlights the potential to use the CC population to assess the quantitative impact of pharmacological compounds on behavioral dimensions across different genetic backgrounds, e.g. as a genetic reference population. The use of CC for NDD studies can be expanded into more targeted experiments, for example by heterozygous crosses between CC lines and gene knockout lines(Dorman et al. 2016), or CRISPR/Cas9-mediated genome editing to introduce genetic mutations observed in human populations (Hirose et al. 2017).

Quantitative, population-based approaches in animal modelling are needed to complement models based on single genetic mutations in a single background, to sustain the translational value of animal studies into NDD. Our results provide a starting point for modeling the quantitative nature of NDD using the CC population, and demonstrate its unique potential for future studies into mouse models of neurodevelopmental disorders.

MATERIALS AND METHODS

Collaborative Cross mice

We investigated neurobehavioral phenotypes in 53 Collaborative Cross RI lines available at Tel Aviv University (TAU), Israel. The CC lines were initially bred at three different locations from eight common founder strains (Churchill et al. 2004). These included 37 CC lines originating from the International Livestock Research Institute (ILRI) in Kenya and relocated to Tel Aviv University (Israel) in 2006; 10 CC lines originating from Geniad Ltd. In Western Australia (CC-GND); and six CC lines originating from the Oak Ridge National Laboratory (ORNL) in Tennessee that were subsequently relocated to the University of North Carolina in 2009 (CC-UNC). All 285 mice tested in this study were male and born, raised and tested in the small animal facility at TAU. All experimental mice and protocols were approved by the Institutional Animal Care and Use Committee of TAU. Animals were socially housed in type II Makrolon cages (26.5 x 17 x 11.4 cm) with a wood chip bedding, and given tap water and rodent chow *ad libitum*.

Behavioral and morphological mouse phenotyping

Social interaction behaviors were assessed in a 2-day social discrimination paradigm in adult age animals as we performed previously (Molenhuis et al. 2014; Bruining et al. 2015). In brief, test animals were habituated in the test cage for 5 minutes and initially exposed to an age- and gender-matched A/J conspecific for 2 minutes and then, after inter-trial intervals (ITI) of 5 minutes, exposed to the familiar conspecific and a first novel A/J conspecific for 2 minutes. On day 2, after the 24-hour ITI, the test animal was habituated for 5 minutes and re-exposed to the same familiar intruder of day 1 and to a different novel intruder animal from a different cage and housing room than the intruder of day 1 for 2 minutes. Trials that needed to be ceased because of aggression were excluded from further analysis. Basic mouse behaviors including locomotor activity, exploratory patterns, digging and grooming were assessed on day 3. Animals were exposed to four novel objects in a 26.5 x 17 x 11.4 cm arena with wood chip bedding. Quantification of exploratory patterns was performed as previously reported by Bonasera *et al.* (Bonasera et al. 2008) and indicated in Figure S1. Theme software version 5.0 (Noldus, Wageningen) was used, and analysis was repeated using equal locomotor distance per animal (i.e. repeating the analysis and averaging the results for the first and last 800 cm in CC mice). On day 4, animals were euthanized and brain tissues were isolated.

Reconstruction of the genomes of CC lines

Genome structures of CC lines were derived from SNPs array data from the Mouse Universal Genotyping Array (MUGA, 7,500 markers) (Hall et al. 2012) and MegaMUGA (77,800 markers). For each CC line, we reconstructed the founder-based mosaic using the hidden Markov model (HMM) HAPPY package (Mott et al. 2000; Durrant et al. 2011) resulting in a probability matrix of descent from each CC founder that was subsequently pruned to about 11,000 SNPs.

Calculation of narrow-sense heritability

Narrow-sense heritability was calculated using kinship matrix K, based on reconstructed genomes (i.e. probability of descent at about 11,000 genomic intervals). Narrow-sense heritability was estimated using a mixed model applied to the phenotype and kinship matrix K in which the phenotypic Variance-Covariance matrix V is modeled as $V = K\sigma_g^2 + I\sigma_e^2$, where I is the identity matrix and σ_g^2, are the genetic and environmental variance components that are estimated by the mixed model by maximum likelihood. The narrow sense heritability is $h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$. Narrow-sense heritability estimates were calculated before and after correction for age, batch and origin effects and quantile normalization.

Calculation of broad-sense heritability

Broad-sense heritability (H^2) including epistatic but not dominance effects was estimated using the proportion of phenotypic variance explained by differences in CC lines in a one-way ANOVA.(Iraqi et al. 2014) Broad-sense heritability was estimated by $H^2 = V_g / (V_g + V_e)$, in which the environmental component of variance within lines is $V_e = MS_{within}$ and the genetic component of variance among CC lines is $V_g = (MS_{between} - MS_{within})/n$, where n represents the average number of animals per line.

Quantitative trait loci (QTL) analysis

Quantitative Trait Loci (QTL) mapping was performed using the HAPPY package. (Mott et al. 2000) Phenotype data were first corrected for age, batch and origin effects and then quantile normalized, and CC line means were weighted by the number of animals per line. Analysis was performed at 11,000 genomic intervals and significance thresholds were estimated by 1000 permutations. QTLs were considered significant at genome-wide permuted $P<0.05$ and intervals were defined using a cut-off score of max logP-1.

Characterization of QTLs by homologous genes in humans

Human orthologous genes within each QTL interval were identified using Biomart build NCBIM37. Per QTL, these orthologous genes were characterized by their rank in a recently established ranking of all human genes by ASD-implication by Krishnan *et al.* (Krishnan et al. 2016), adjusting for human genes without mouse orthologue.

Statistical analyses

All statistical analyses were performed with the statistical software R (R core team 2016).

Acknowledgements

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Conflict of Interest

The authors declare no competing interests.

Author contributions

Conceptualization, M.J.H.K., H.B., R.T.M.; Methodology, F.A.I., R.F.M., R.T.M.; Investigation, R.T.M., M.J.V.B., P.E.S., H.J.A.; Writing – Original Draft, R.T.M., H.B. and M.J.H.K.; Writing – Review & Editing, R.T.M., H.B. and M.J.H.K., R.F.M., F.A.I., J.P.H.B.; Visualization, R.T.M.; Funding Acquisition, M.J.H.K., R.T.M.; Resources, M.J.H.K., F.A.I.; Supervision, M.J.H.K., H.B., J.P.H.B.

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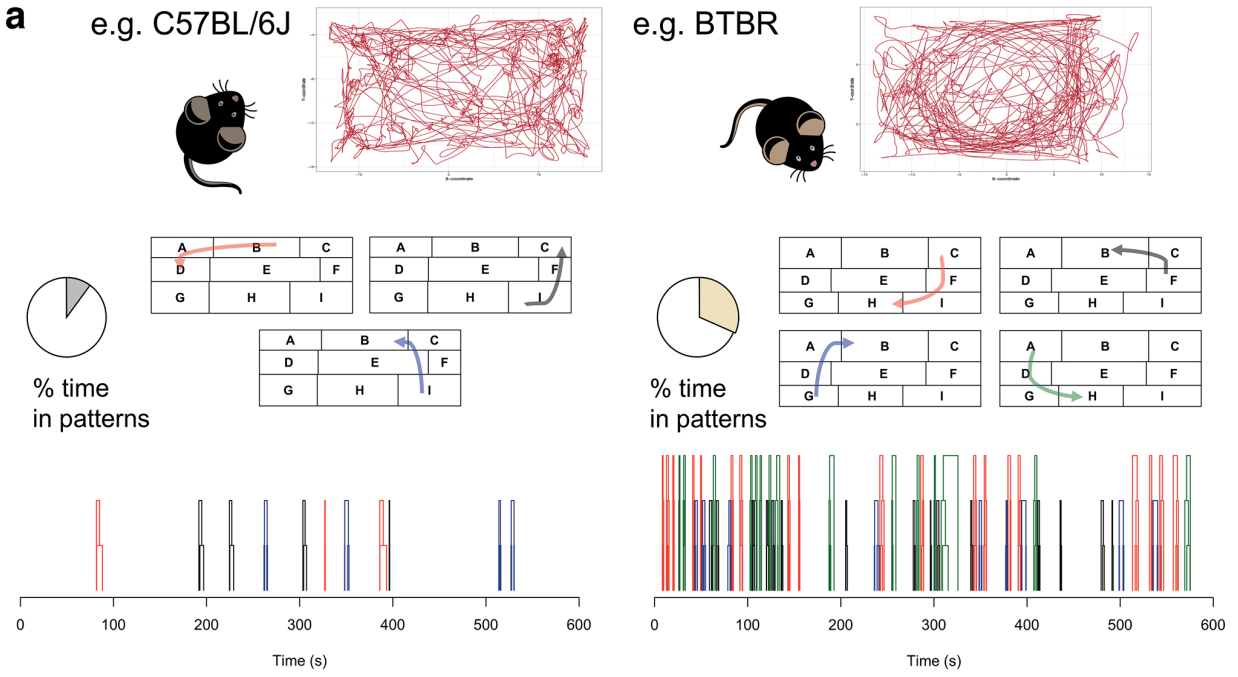
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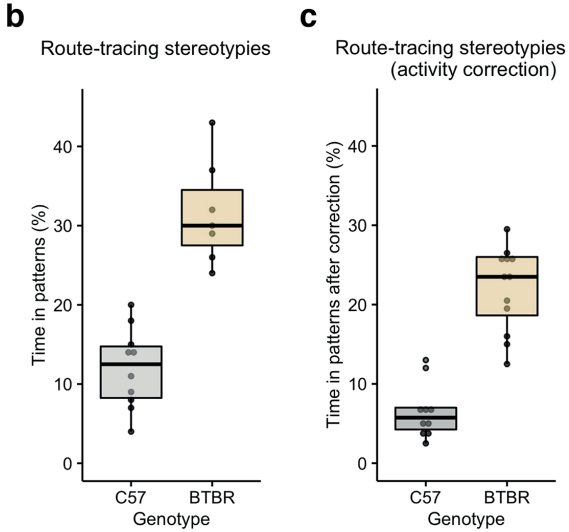

Figure S1. Quantification of stereotyped exploratory patterns. (**a**) Example of exploratory patterns during novelty exposure in C57BL/6J mice and the BTBR inbred strain models for ASD. Exploratory patterns were quantified following Bonasera et al. 2008, by testing whether spatial bins of locomotor activity occur in particular sequences ('patterns') over time at a probability greater than chance. Following the identification of patterns (e.g. red, green, black, blue) with different levels of complexity, scores for exploratory patterns are defined as the total percentage of time the animal spent in patterns (e.g. 7% in C57BL/6J vs. 22% in BTBR). (**b**) Box-plot comparison of exploratory patterns in C57BL/6J and BTBR mice (Welch's t-test t(10.83) = 6.63, P=4.0x10-5). (c) Exploratory patterns after activity-correction (C57BL/6J (n=10) and BTBR mice (n=12), Welch's t-test t(19.07) = 8.20, P=1.1x10-7). Activity correction was applied by considering equal distances across all animals, i.e. first and last 1500cm in C57BL/6J and BTBR mice.

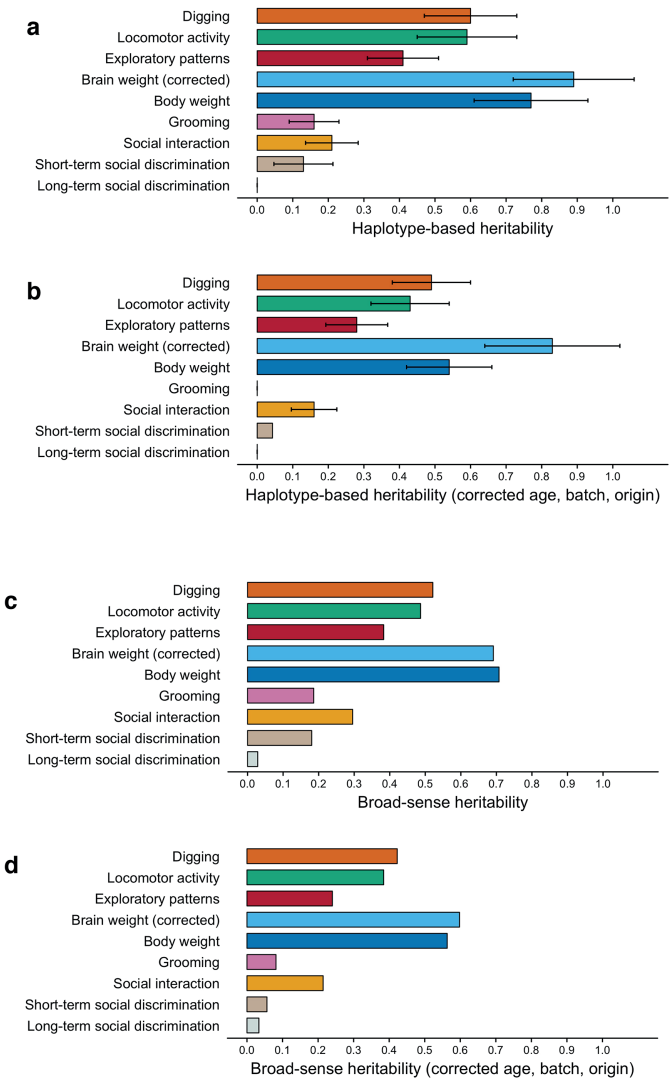

Figure S2. Haplotype-based and broad-sense heritability estimates. (**a**) Genome-wide haplotype-based heritability estimates for basic mouse behavioral traits (top), physical traits (middle), and ASD-related readouts (bottom). (**b**) Genome-wide haplotype-based heritability estimates after correction for effects of age, batch and origin, and quantile normalization. (**c**) Broad-sense heritability estimates for basic mouse behavioral traits (top), physical traits (middle), and ASD-related readouts (bottom). (**d**) Broad-sense heritability estimates after correction for effects of age, batch and origin, and quantile normalization.

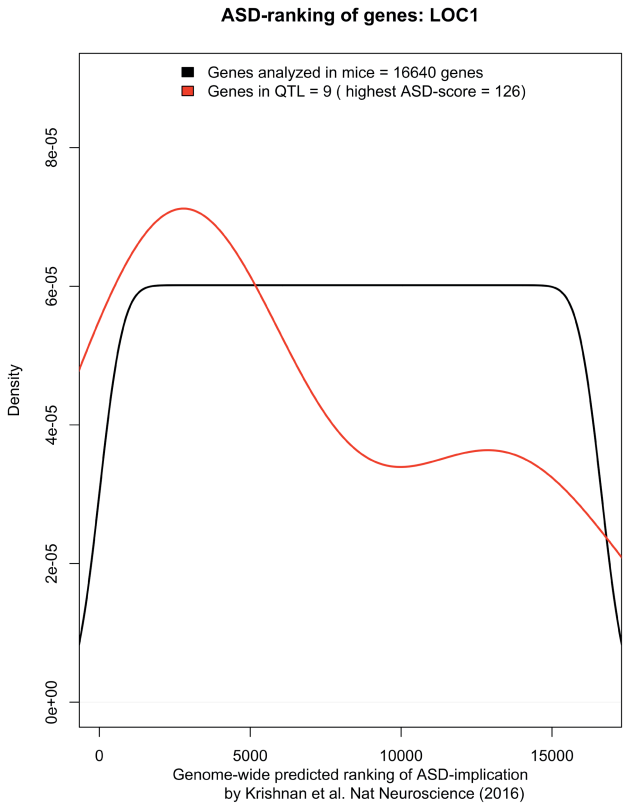

Figure S3. Characterization of genes in QTL by predicted ASD-implication. Proof of principle comparison of the 9 genes in the locomotor activity QTL on chromosome 1, to predicted ranking of all human genes by ASD-implication (Krishnan et al. Nat Neurosci 2016). Genes in this QTL show higher density for high-ranking genes compared to all genes with mouse-homolog (highest ASD-ranking gene ranked 124th, median 3476th). Equal or smaller median values were observed in fraction 0.021 of 10000 equally-sized randomly sampled gene sets.

Table S1. Quantitative trait loci (QTLs) for neurobehavioral traits in CC mice.

Phenotype	QTL	Location (Mb, build 37)	Max logP	Permuted P	Width (Mb)	Homologous genes
Digging	DIG1	X:105.265-126.750	6.19	P<0.001	21.5	26
	DIG2	X:163.579-165.659	6.14	P<0.001	2.1	7
	DIG3	X:140.042-158.193	4.89	0.012	18.2	63
Locomotor activity	LOC1	1:23.484-29.342	4.33	0.042	5.9	9
	LOC2	12:16.824-26.491	4.35	0.042	9.7	27
Exploratory patterns	EXPL1	2:173.252-179.848	4.69	0.012	6.6	27
	EXPL2	12:17.282-28.665	5.07	0.003	11.4	25
Brain weight	BRW1	4:127.229-128.178	4.33	0.034	0.9	2
	BRW2	13:45.602-55.115	4.54	0.021	9.5	69

General discussion

Enhancing the value of animal models for neurodevelopmental disorders

INTRODUCTION

Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder that affects about 1-2% of the worldwide population (Baron-Cohen et al., 2009; Blumberg et al., 2013; Kim et al., 2011; Mattila et al., 2011). Although awareness and recognition of ASD have increased over the past decades, and research on ASD has expanded, there are currently no medications available to improve the core symptoms of ASD. At this moment, therapeutic options are mostly restricted to behavioral interventions, which have proven successful in a subset of patients (Kasari et al., 2014; Rogers et al., 2014). Understanding underlying mechanisms from preclinical models has been increasingly recognized as a critical first step towards novel and improved treatments (de la Torre-Ubieta et al., 2016).

Studies into the mechanisms underlying ASD have long been complicated by the lack of known risk factors, poor access to brain tissue and the broad clinical definition of the disorder. Since the late 1970s, twin studies have consistently indicated that ASD has a large genetic component (Ronald and Hoekstra 2011). Therefore, a logical progression has become to unravel the large genetic component of ASD, and to develop animal models based on these genetic findings. Over the past decade, hundreds of genes have been associated with the ASD on the basis of rare inherited and *de novo* mutations. Together with the development of novel techniques to mutate these genes in rodents, these findings have allowed the development of genetic animal models to study the underlying mechanisms of ASD in a fixed genetic and environmental background.

While human genetic studies have contributed to the development of animal models for ASD, they have also underscored the complex nature of the disorder (de la Torre-Ubieta et al., 2016). Considering that ASD risk-genes have been identified mostly on the basis of rare inherited and *de novo* variants, it may be tempting to conclude that the genetic component of ASD mainly concerns rare genetic mutations. In contrast, however, recent studies into the genetic architecture of ASD indicate that *common* genetic variants play a major role in the genetic predisposition to the disorder (i.e. explaining ~50% of the total liability to ASD (Gaugler et al., 2014; Klei et al., 2012)). These variants are common throughout the general population and have small effects individually, but they can have major impact and causal to ASD when they act together (Gaugler et al., 2014). In comparison, rare genetic mutations can be found in up to 20% of affected individuals, but only explain about 5% of the liability to ASD (de la Torre-Ubieta et al., 2016). Taken together, these findings indicate that the development of ASD is highly dependent on genetic background variations, even in individuals in which a contributing rare inherited or *de novo* mutation has been identified.

Animal models for ASD have several important limitations that restrict the value of these models gaining better neurobiological understanding of ASD core symptoms, and hamper the development novel therapeutic strategies. First, they are mostly based on single rare genetic mutations tested in only one genetic background, while there is increasing evidence that findings from a single background are often not generalizable to other genetic backgrounds (Sittig et al., 2016). Secondly, the characterization of animal models often relies on comparative analysis and on the use of dichotomous outcomes (e.g. testing whether or not deletion of an ASD risk-gene leads to impaired social behavior). Since dichotomous outcomes are highly influenced by the "load" in the genetic background, such yes-or-no phenotypes only provide limited information about the true contribution of a developmental perturbation. Likewise, the true potential of a novel pharmacological compound remains unclear when tested in a single genetic background based on comparative analysis. Finally, most animal studies have focused on independent traits measured at adult age, while ASD is developmental disorder that emerges early in life. ASD is heterogeneous with respect to symptom manifestation, and the trajectories of repetitive and social behavior can be highly variable over time (Fountain et al., 2012).

To increase the value of animal models, the work presented in this thesis was aimed to:

1. Establish a longitudinal test battery for the characterization of mouse behavioral and cognitive domains across development.
2. Characterize these longitudinal trajectories in mice in a genetic model for ASD and developmental retardation.
3. Improve behavioral phenotyping strategies for animal models for ASD by assessing the impact of environmental novelty on behavioral testing.
4. Establish animal model approaches that take into account variations in genetic background as well as the polygenic nature of autistic-like traits in the human population.

These improvements could bridge the gap between preclinical animal studies and the heterogeneous human population, and may be of vital importance for the development of novel treatments to improve the core symptoms of ASD.

Characterization of behavioral traits across developmental stages

Most animal studies test behavioral phenotypes at adult stages, which contrasts to the developmental nature of ASD. To extend the use of mouse models into a wider developmental window, **Aim #1** of this thesis was to establish a longitudinal

test battery for the characterization of mouse behavioral and cognitive domains across development. To this end, we characterized the developmental trajectories of four mouse inbred strains from preadolescence into adulthood. These strains, C57BL/6J (C57), 129S1/SvImJ (129Sv), BTBR T+tf/J (BTBR) and A/J (AJ), were chosen based on known behavioral abnormalities in adult stages in the absence of gross abnormalities in their physical development. We observed that behavioral and cognitive changes can already be detected at the pre-adolescent period. These findings indicate that this longitudinal test battery provides sufficient sensitivity to detect behavioral and cognitive differences at different developmental stages, for example to characterize the behavioral development of genetic mouse models for neurodevelopmental disorders. Indeed, the added value of our longitudinal battery was recently demonstrated in a study involving *Cntnap2* gene knockout mice, which were characterized by extreme abnormalities in repetitive behavior, specifically in the adolescent and pre-adult stage, that would otherwise have remained undetected (Scott et al., 2017). These findings have direct implications to optimize the timing of pharmacological intervention studies, which require robust and replicable behavioral phenotypes (Brunner et al., 2015).

Our findings further revealed distinct developmental trajectories of repetitive behaviors, that may be relevant to those observed in the ASD patient population (Fountain et al., 2012). BTBR mice showed persistent high levels of repetitive grooming behavior during all developmental stages that was associated with the adult expression of cognitive rigidity, while C57 control mice displayed similar high levels of grooming behavior only during the preadolescent period (Molenhuis et al., 2014). These high levels of grooming behavior in C57 control mice during the preadolescent stage are consistent with observed high levels of repetitive behaviors early in typical child development (Turner, 1999). To validate the contribution of specific brain pathways to abnormal repetitive behaviors, a powerful future approach would be to link trajectories of behavior with longitudinal analysis of neurobiological abnormalities, in both animal models as well as the human population (Langen et al., 2014).

Limited impact of a single ASD risk gene

After having established a longitudinal test battery for the characterization of mouse behavioral and cognitive domains across development (**Aim #1**), we aimed to characterize these longitudinal trajectories in a genetic mouse model for ASD and developmental retardation (**Aim #2**). In this model, the *CNTN4* gene has been 'knocked out' that codes for a cell adhesion molecule and is strongly implicated in ASD (Glessner et al., 2009). Mutations of *CNTN4* have also been found in individuals

with other disorders such as anorexia nervosa (AN) (Wang et al., 2011), and it has been described that *CNTN4* plays a major role in the 3p.25-deletion syndrome, a rare genetic disorder that is characterized by developmental delay (Fernandez et al., 2008). Since cognitive rigidity is a common feature of both ASD and AN (Tchanturia et al., 2004), we aimed to characterize behavioral and cognitive developmental of *Cntn4*-deficient mice, as they could serve as a potential model for autistic-like development of specifically repetitive behaviors.

Against expectations from the association of *CNTN4* with ASD, the developmental neurological and cognitive screening of *Cntn4*-deficient C57BL/6J mice revealed no significant differences, and ASD-related behavioral domains were also unchanged. In contrast, the impact of *Cntn4*-deficiency limited to increased sensitivity to auditory stimuli, and enhanced learning in a spatial navigation task. We initially found an inconclusive result in the reversal-phase of a set-shifting task, however, based on additional experiments, we finally concluded that *Cntn4*-deficiency does not significantly affect cognitive flexibility. Yet, the suggestion of deficit in reversal learning remain remarkable, since *Cntn4* is most strongly expressed in the medio-dorsal thalamus, which is a brain region known to be critical for reversal learning in mice (Allen Institute for Brain Science and Mitra Labs, 2011; Parnaudeau et al., 2013).

The absence of autistic features in *Cntn4*-deficient mice was intriguing and merited further thoughts. A first explanation could be that *CNTN4* is highly important for the behavioral development in humans, while *Cntn4* does not play a similar role in mice. However, such divergence seems unlikely based on the degree of homology between the two genes, and because their expression patterns in the brain are highly similar. Homology implies functional similarity, and protein sequences of the human and mouse *Cntn4* are 95% identical (Yates et al., 2016), which is high compared to an average 85% identity between human and mouse protein sequences (Makalowski and Boguski, 1993), and similar to prominent ASD risk genes with evident behavioral effects in mice such as *Fmr1* (94% identity), *Cntnap2* (93% identity) and *Shank3* (95% identity). Similarities in brain gene expression patterns between mouse and human were indicated by high expression in thalamus and frontal cortex, and low expression in hippocampus and basal ganglia in both species (Allen Institute for Brain Science, 2017; The Broad Institute of MIT and Harvard, 2017; Zuko et al., 2013).

A second explanation may be an overestimation of the association of *CNTN4* with ASD and related disorders. For instance, it is becoming more likely that also other genes in the proximity of *CNTN4* might play a role in the clinical features of the so called 3p.25 deletion syndrome, the genomic location in which the *CNTN4* gene resides (Grozeva et al., 2014). Moreover, some of the associated copy-number variants in individuals with ASD appear to be upstream rather than inside the *CNTN4* gene (Glessner et al., 2009).

A third explanation for the lack of overt autistic features in *Cntn4*-deficient mice may be that disruption of *Cntn4* can contribute to changes in sensory behavioral responses and cognitive performance, but that this effect depends on genetic background variability. This notion is supported by recent studies into the genetic architecture of ASD.

Recent human genetic studies have indicated that rare genetic mutations can be found in up to 20% of affected individuals, while they only explain about 5% of the total liability to ASD (de la Torre-Ubieta et al., 2016). These numbers suggest that when a rare genetic mutation can be identified, it only explains an average 25% of the individual's genetic burden, while the remaining 75% remains to be explained by variations in the genetic background. These findings indicate that variations in genetic background play a major role in the development of ASD, even in individuals in which a rare inherited or *de novo* mutation has been identified. At the same time, studies in mice have also underscored that genotype-phenotype relationships observed in a single genetic background can often not be translated to other genetic backgrounds (Sittig et al., 2016). Such variable genotype-phenotype relationships could perhaps explain the limited impact of *Cntn4*-deifciency when tested only in a C57BL/6J background, as well as the discrepancies across human genetic studies into *CNTN4* (Murdoch et al., 2015). Overall, these results highlight the need for animal model approaches that take into account impact of variable genetic backgrounds, which specifically relates to **Aim #4** of this thesis.

Resistance to change – how to handle mouse models for ASD?

The value of preclinical animal studies into ASD can be increased by better modelling of genetic risk constellations, as well as by improving behavioral phenotyping strategies. Therefore, **Aim #3** of this thesis was to improve behavioral phenotyping strategies for animal models for ASD by assessing the impact of environmental novelty on behavioral testing. Since resistance to change is common feature in ASD, we hypothesized that exposure to environmental change could be used to potentiate the expression of behavioral phenotypes in animal models for ASD. Therefore, we first investigated the impact of unexpected or novel situations on existing gene knockout models for ASD. We found, based on systematic literature study, that behavioral abnormalities in single genetic knockout models for ASD are increased after transfer to another environment relative to baseline conditions.

To identify optimal conditions for future studies, we also tested behavioral activity across assays with variable degrees of novelty and of exposure times in a mouse model for ASD previously associated with novelty-induced hyperactivity, namely the BTBR mouse inbred strain. We found that short duration behavioral

assays with a high degree of novelty can help to precipitate rigid and inflexible behavior in animal models for ASD, and may facilitate the discovery of genotype-phenotype relationships in the context of ASD.

In addition to short behavioral assays, these findings also underscore the added value of longitudinal phenotyping strategies, as they allow to assess the precise difference between novelty-induced behaviors versus baseline expression within a single animal. Over the past decade, various home-cage systems have been developed that allow longitudinal behavioral monitoring without human interference (Loos et al., 2014; De Visser et al., 2006). An exciting future direction is to expand longitudinal behavioral profiling beyond the assessment of locomotor activity, for example by the use of automated behavior recognition software that now allows to detect individual variations in stereotyped behaviors as well as in social interactions (Boom et al., 2017; Weissbrod et al., 2013).

Modeling the quantitative nature of autistic traits

So far, most genetic animal models for ASD rely on single rare genetic mutations and comparative analysis in one genetic background (e.g. yes-or-no impairment of social behavior in a C57Bl/6J genetic background). There is increasing evidence, however, that behavioral differences observed in a single background are often not generalizable to other genetic backgrounds (Sittig et al., 2016). Another important limitation of the single-gene single-background approach was recently indicated by human genetic studies into the nature of autistic traits. These studies showed that almost all genetic risk factors for ASD can also be found in the general population, and that they confer quantitative rather than categorical effects on neurobehavioral traits (Robinson et al., 2016; Weiner et al., 2017). These findings indicate that a single genetic mutation is almost never sufficient to cause ASD, and confirm the notion that a clinical diagnosis of ASD is associated with the extreme tails of a quantitative trait distribution in certain behavioral traits. These studies indicate that autism is a quantitative phenomenon, meaning that a clinical diagnosis of ASD is almost always the consequence of multiple genetic (and non-genetic) effects combined (Figure 1A and B). The conception of autism as a quantitative phenomenon corresponds with large and robust behavioral differences observed in the polygenic BTBR inbred strain (i.e. **chapter 1**), in contrast to subtle and non-replicable differences that we observed in a single gene knockout model for ASD (e.g. **chapter 2**).

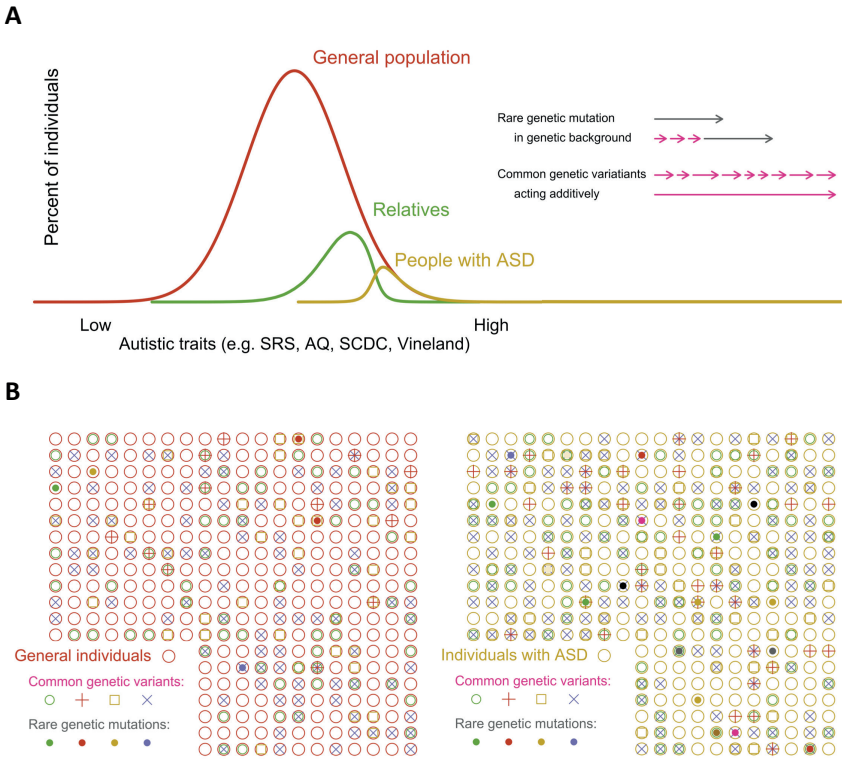

Figure 1. The quantitative nature of autistic-like traits in the general and clinical population (extremely simplified schematic representation). (**A**) Autistic-like traits are heritable and continuously distributed across the general population (red). The extreme tail of the population distribution is most likely to be associated with a clinical diagnosis (yellow), and relatives are more likely to have relatively high scores for autistic-like traits (green). Rare genetic mutations (purple) explain about 5% of the total liability to ASD (de la Torre-Ubieta et al., 2016), while multiple common genetic variants (pink) explain roughly 50% of the total liability to ASD (Gaugler et al., 2014). (**B**) Genetic risk factors for ASD can be found in the general population (red circles), as well as in individuals with ASD (yellow).

The conception of ASD as a quantitative phenomenon has important implications for animal studies into the biology of ASD. We reasoned that the translational value of animal model studies would be enhanced once the impact of developmental perturbations is studied in a quantitative framework across genetic backgrounds. Thus, to move animal models for ASD beyond single gene-single background designs, we aimed to design an experiment to systematically study the impact of genetic variation on quantitative behavioral trait distributions (**Aim #4**). For this purpose, we tested the potential of the Collaborative Cross (CC), a novel panel of mouse recombinant inbred lines with a unique level of genetic diversity derived from 5 classical laboratory strains and 3 wild-derived mouse inbred lines. Using this highly novel method, we performed a quantitative screen of neurobehavioral phenotypes relevant for ASD. In this heterogeneous population, we investigated the quantitative nature of mouse behavioral phenotypes commonly used in animal models for ASD, including a novelty-based assay derived from **Aim #3** of this thesis. Based on behavioral variation in 53 CC recombinant inbred lines, we found that ASD relevant traits such as stereotyped behaviors and locomotor activity during novelty were characterized by high heritability and continuous distribution across our CC sample, in contrast to classical tests of social recognition and grooming phenotypes. Next, to link heritable behavioral variation with genetic differences, we performed quantitative trait locus (QTL) mapping and identified QTLs that contained genes associated with corresponding phenotypes in human populations. These findings highlight the potential of the CC as a reference population that can be used to study the quantitative nature of ASD-like behavioral variation in mice. In future studies, this quantitative framework can be used to study the behavioral impact of genetic manipulations (e.g. combined novel gene-editing technologies such as CRISPR/Cas9), as well as therapeutic interventions across different genetic backgrounds.

Potential of H3R antagonism to modulate repetitive behaviors in humans

An illustration of the method derived from **Aim #4** is the ability to identify genetic effects across genetic backgrounds, as we identified a gene coding for the histamine receptor H3R in a CC quantitative trait locus for repetitive behavior patterns. This suggests that attenuation of the effects of the H3R gene can provide a powerful means to treat repetitive behavior symptoms in many different forms of ASD. Consistent with this hypothesis, manipulation of the H3R receptor has been shown to alter the expression of repetitive behaviors in both anatomical and pharmacological models (Baronio et al., 2015; Rapanelli et al., 2017; Wright et al., 2017).

Testing this hypothesis has come in sight as the first inverse agonist/antagonist acting on the histamine H3R receptor has recently been introduced in clinics, and was approved by the EMA for the treatment of excessive daytime sleepiness (Kollb-Sielecka et al., 2017). It is now possible to test H3R antagonism directly in the clinical ASD population since this compound, pitolisant, was recently approved for use in humans, and pharmacokinetics and safety profiles available (Wadman, 2013). Since sleep disturbances are frequent in people with ASD, this intervention may be especially effective for patients with repetitive behaviors and comorbid sleep problems (Humphreys et al., 2014). The attenuation of repetitive behaviors with H3R antagonism could mean a great improvement of the daily live of people with ASD and their families (Boyd et al. 2012). The association of the H3R in the heterogeneous CC population suggests that antagonism of this receptor may be beneficial to attenuate abnormal levels of repetitive behaviors in a variety of genetic backgrounds, although more research is needed to confirm this hypothesis.

The future of animal models for ASD: beyond reductionism and "deficit-based" thinking

Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder currently defined on the basis of behavioral symptoms. In nature, behavioral differences can be found between species, between subpopulations of the same species, and between individuals within a single population (Wilson, 1998). However, relative to other traits such as human height or forelimb structure in different mammals, only little is known about how genetics have shaped the evolution of behavioral differences (Weber et al., 2013).

Behavioral differences can have ecological relevance, and there is increasing evidence that that such differences not only exist *between* species, but also that they are common *within* populations (Coppens et al., 2010; Mousseau and Roff, 1987). Behavioral differences, or personality types, can play a positive role in the adaptive value of a population, and it has been shown that behavioral differences can emerge from natural selection and remain within a population even after many generations (Wolf et al., 2008). Combined with population-based studies into ASD as presented in this thesis, these observations suggest that ASD is likely due to an accumulation of neurobiological variations that are each also present in the general population, rather than caused by single brain "deficit" or "disorganization".

The expression of autistic traits in the general population is typically associated with a negative impact on daily-life functioning, since individuals in the extreme tails of the quantitative trait distributions are most likely to have a clinical diagnosis, and these people suffer from severe daily-life impairments (Figure 2). However, there is increasing

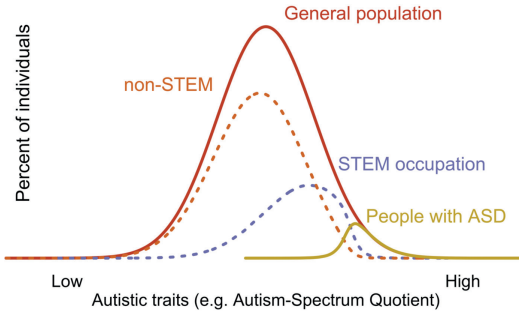

Figure 2. Individuals working in a career in Science, Technology, Engineering, and Mathematics (STEM) score higher on the Autism-Spectrum Quotient than individuals with a non-STEM occupation (Ruzich et al., 2015).

evidence that some of the behavioral variations associated with ASD may have benefits for the population as a whole. For example, individuals with higher scores on the Autism-Spectrum Quotient are more likely to work in so-called STEM careers (i.e. Computer & IT, Engineering, and Science & Technology (Ruzich et al., 2015)), and genome-wide polygenic risk-scores for ASD have been associated with positive outcomes such as increased verbal-numerical reasoning and better home organization (Krapohl et al. 2015; Hagenaars et al. 2016). These findings highlight a possible evolutionary principle, in which autistic traits are maintained within the population and have an advantage depending on the environment. They indicate that some genetic variation associated with ASD may have a positive impact for the population, but when they accumulate on the individual level they result in extreme daily-life impairments.

Thus, although autistic-like variations are present throughout the general population, this does not diminish the impact of ASD on the daily lives of affected individuals. However, it does have implications for the way we think about ASD and its underlying mechanisms. Towards better understanding of autistic phenomena, more fundamental knowledge is needed about individual behavioral differences within populations, and about how differences in brains and behaviors have been shaped by evolution. So far, most animal studies in the context of ASD aim to provide causal explanations through genetic or neural manipulations, and include a variety of neurobiological techniques and different types of results (Hyman, 2014; Krakauer

et al., 2017). As a consequence, however, behavior is commonly included only as a final add-on (e.g. yes-or-no impairment of (social) behavior), and fundamental understanding of behavioral variation often remains limited because of this.

More fundamental insights into brain-behavior relationships will be essential to gain true understanding of neurodevelopmental disorders such as ASD. Towards this end, behavioral-driven neuroscience might provide a powerful path forward, by focusing on naturally-occurring and ecologically relevant behavioral differences that are present in animal species. In this behavioral-driven approach, careful theoretical and experimental dissection of behavior provide understanding *before* applying new genetic tools or neural manipulations that can be used to test causality (Krakauer et al., 2017). Indeed, recently developed automated monitoring systems have the power to deliver behavioral profiles in great detail, including longitudinal observations in semi-natural environments.

Furthermore, wild-derived genetic resource populations are now available for a range of different species and will allow to study individual differences at an unprecedented level (Chesler, 2014; Greenberg et al., 2012; Lee et al., 2017; Shpigler et al., 2017; Williams and Auwerx, 2015). In such populations, the analysis of behavioral profiles with ecological relevance will provide a fruitful starting point to gain true understanding of behavioral differences. Only then specific molecular genetic or neuronal manipulations can be applied to confirm causality of variations in associated brain pathways. Such comprehensive understanding of natural diversity will provide a fundamental understanding of phenotypic variation, which will be essential to understand neurobiological variation underlying social behavioral differences. Ultimately, such fundamental insights will pave the way for the development of effective strategies for neurodevelopmental disorders such as ASD.

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Appendix

SAMENVATTING IN HET NEDERLANDS

Autismespectrumstoornis (ASS) is de naam voor een groep neurologische aandoeningen die worden gedefinieerd door verstoorde ontwikkeling van sociale interactie en communicatie, en door repetitief en stereotiep gedrag. Veel mensen met ASS hechten aan structuur en routines en hebben moeite om te gaan met kleine veranderingen en onverwachte situaties. ASS wordt gedefinieerd op basis van gedragssymptomen en treft 1-2% van de bevolking wereldwijd. Hoewel de erkenning van ASS in de afgelopen decennia sterk is gegroeid en onderzoek naar ASS is toegenomen, zijn er vooralsnog geen medicijnen om de kernsymptomen van ASS te verbeteren.

Op dit moment zijn therapeutische opties meestal beperkt tot gedragsinterventies, die succesvol zijn gebleken bij een subgroep van patiënten. De ontwikkeling van medicamenteuze opties zou mensen met ASS kunnen helpen, bijvoorbeeld om makkelijker om te gaan met onverwachte situaties of verandering. Preklinisch onderzoek naar de biologische mechanismen die ten grondslag liggen aan ASS wordt in toenemende mate gezien als een cruciale eerste stap richting betere behandelingen.

Lange tijd was onderzoek naar de biologie van ASS gecompliceerd door het ontbreken van bekende risicofactoren, beperkte toegang tot hersenweefsel en de brede klinische definitie van de stoornis. Tweelingstudies hebben sinds eind jaren 1970 laten zien dat ASS een grote genetische component heeft. Een logische stap is daarom de grote genetische component van ASS te ontrafelen en op basis van deze genetische bevindingen diermodellen te ontwikkelen. In het afgelopen decennium zijn honderden genen in verband gebracht met de ASS op basis van zeldzame genetische mutaties. Deze bevindingen – gepaard met ontwikkelingen in de biotechnologie – hebben de ontwikkeling van diermodellen mogelijk gemaakt om de onderliggende neurobiologische mechanismen van ASS te bestuderen in gecontroleerde laboratoriumcondities en om nieuwe behandelmethoden te testen.

Hoewel genetische studies bij mensen hebben bijgedragen aan de ontwikkeling van diermodellen voor ASS, onderstrepen deze studies in toenemende mate ook de complexe aard van deze ontwikkelingsstoornis. Recente studies naar de genetische architectuur van ASS laten zien dat ASS vrijwel nooit wordt veroorzaakt door één zeldzaam genetisch mutatie. In tegendeel, een klinische diagnose is vrijwel altijd het gevolg is van een opeenstapeling van meerdere genetische varianten, die elk ook veelvuldig voorkomen in de algemene "gezonde" bevolking. Deze genetische varianten hebben afzonderlijk kleine effecten en dragen ook bij aan normale gedragsverschillen tussen "gezonde" individuen. Echter, wanneer meerdere varianten samenvallen in één individu kunnen deze een grote impact hebben en leiden tot ASS.

De huidige diermodellen voor ASS hebben verschillende beperkingen die een beter begrip van de biologische grondslag van ASS in de weg staan en de ontwikkeling van therapeutische interventies belemmeren. Ten eerste zijn diermodellen vaak gebaseerd op één enkele zeldzame genmutatie die wordt getest in maar één genetische achtergrond, terwijl ASS meestal een optelsom is van een veelvoud genetische varianten (*normale variatie*) in combinatie met de genetische achtergrond. Ten tweede is de karakterisering van diermodellen vaak gebaseerd op het gebruik van dichotome uitkomstmaten (heeft een dier sociale neigingen, of überhaupt niet), terwijl dergelijke uitkomstmaten slechts beperkte informatie geven over de werkelijke bijdrage van een genetische mutatie of nieuwe farmacologische interventie. Ten slotte richten de meeste dierstudies zich alleen op individuele uitkomstmaten op volwassen leeftijd, terwijl ASS een ontwikkelingsstoornissen is die zich vroeg in het leven manifesteert. Het is bekend dat ontwikkelingstrajecten van repetitief en sociaal gedrag zeer variabel kunnen zijn in de tijd. Het werk gepresenteerd in dit proefschrift is gericht op het verhogen van de waarde van preklinisch onderzoek naar ASS.

Hoofdstuk 1 beschrijft de ontwikkeling van een longitudinale testbatterij voor de karakterisering van gedrags- en cognitieve domeinen in muismodellen over verschillende ontwikkelingsstadia heen. Hiertoe hebben wij de ontwikkelingstrajecten in kaart gebracht van vier inteeltstammen van muizen – van preadolescentie tot in volwassenheid. Deze stammen, C57BL/6J (C57), 129S1/SvImJ (129Sv), BTBR T+tf/J (BTBR) en A/J (AJ), zijn gekozen op basis van bekende gedragsafwijkingen op volwassen leeftijd in afwezigheid van grove afwijkingen in de fysieke ontwikkeling. Onze bevindingen onthulden verschillende ontwikkelingstrajecten van repetitief gedrag, die relevant kunnen zijn voor ontwikkelingstrajecten die zijn waargenomen in individuen met ASS. De resultaten laten ook zien dat gedrags- en cognitieve veranderingen al kunnen worden waargenomen in de pre-adolescente periode en dat onze longitudinale testbatterij voldoende gevoeligheid biedt om gedrags- en cognitieve verschillen in diverse ontwikkelingsstadia te kunnen detecteren, bijvoorbeeld in muizenmodellen voor ASS.

Hoofdstuk 2 beschrijft de toepassing van de longitudinale testbatterij in een genetisch muismodel voor ASS. In dit model is het CNTN4-gen verwijderd, dat normaal codeert voor een celadhesiemolecuul dat voorkomt in diverse hersenstructuren die betrokken kunnen zijn bij ASS. Mutaties van CNTN4 zijn gevonden bij personen met ASS maar ook bij mensen met andere mentale stoornissen zoals anorexia nervosa (AN). Omdat cognitieve rigiditeit een veel voorkomend kenmerk is van zowel ASS als AN, hebben wij de gedrags- en cognitieve ontwikkeling van Cntn4-deficiënte muizen in kaart gebracht, omdat deze zouden

kunnen dienen als een model voor autistisch-achtige ontwikkeling van repetitief gedrag. Tegen onze verwachtingen in liet de longitudinale screening van Cntn4-deficiënte muizen geen significante verschillen zien in de neurologische en cognitieve ontwikkeling – en ook ASS-achtige gedragsdomeinen waren onveranderd. De impact van Cnt4-deficiëntie was daarentegen beperkt tot verhoogde gevoeligheid voor auditieve stimuli en verbeterd leren in een ruimtelijke navigatietaak. Een mogelijke verklaring voor deze beperkte impact van Cntn4-deficiënte is de afhankelijkheid van de genetische achtergrond. Ook in de mens zijn er aanwijzingen dat de impact van CNTN4 mutaties sterk afhankelijk is van de genetische achtergrond. Al met al wijzen deze resultaten op de behoefte aan preklinische methodes die rekening houden met de invloed van genetische achtergrondvariatie.

De waarde van preklinische dierstudies naar ASS kan worden verhoogd door betere modellering van complex genetisch risico, maar ook door verfijnde strategieën voor gedragsfenotypering. Weerstand tegen verandering is een veelvoorkomend kenmerk van ASS. In **hoofdstuk 3** hebben wij daarom onderzocht of blootstelling aan verandering in de omgeving kan worden gebruikt om de expressie van gedragsfenotypen in diermodellen voor ASS te versterken. Resultaten van systematische literatuurstudie lieten zien dat gedragsafwijkingen in mono-genetische diermodellen voor ASS verhoogd zijn na overdracht naar een andere omgeving ten opzichte van basiswaarden. Additionele experimenten lieten zien dat gedragstesten met een korte duur én met een hoge mate van nieuwheid kunnen helpen bij het detecteren van star en inflexibel gedrag. Deze bevindingen kunnen de ontdekking van genotype-fenotype-relaties in de context van ASS vergemakkelijken in toekomstige studies.

Hoofdstuk 4 beschrijft een groot experiment om de impact van genetische diversiteit op gedrag in muizen op een systematische en meer kwantitatieve manier te bestuderen. Deze nieuwe benadering – in een unieke populatie van genetisch zeer diverse muislijnen – spiegelt de complexe genetische aard van ASS-achtige gedragsverschillen in de humane populatie. Met behulp van deze nieuwe aanpak voerden we een kwantitatief genetische screen uit voor muis gedragsfenotypen die relevant zijn voor ASS. De resultaten lieten zien dat relevante ASS-achtige kenmerken (zoals stereotiep gedrag en locomotorische activiteit tijdens nieuwe situaties) worden gekenmerkt door een hoge erfelijkheid en continue verspreiding in de muispopulatie. Dit was echter niet het geval voor klassieke tests van sociale herkenning en poetsgedrag (uitkomstmaten die tot op heden vaak worden bestudeerd in diermodellen voor ASS). Om de erfelijke gedragsvariatie te koppelen aan verschillen in het genoom hebben we vervolgens *quantitative trait locus (QTL) mapping* toegepast en gebieden kunnen identificeren die verantwoordelijk zijn voor de geobserveerde gedragsverschillen in stereotiep gedrag en locomotorische activiteit tijdens nieuwe situaties. Deze genetische

gebieden bleken ook genen te bevatten die geassocieerd zijn met overeenkomstige fenotypen in de mens. Deze bevindingen benadrukken het potentieel van het gebruik van deze referentiepopulatie, die kan worden gebruikt om de kwantitatieve aard van ASS-achtige gedragsvariatie in muizen te bestuderen. In toekomstige studies kan dit kwantitatieve raamwerk worden gebruikt om de gedragseffecten van genetische manipulaties te bestuderen (bijvoorbeeld gecombineerd met nieuwe biotechnologische technieken zoals CRISPR/Cas9). Daarnaast biedt deze aanpak de mogelijkheid om nieuwe therapeutische interventies te testen in *verschillende* genetische achtergronden, waardoor preklinische resultaten waarschijnlijk beter "vertalen" naar de (heterogene) menselijke populatie.

Gedragsverschillen komen in de natuur veelvuldig voor tussen verschillende diersoorten en subpopulaties van een diersoort. Er is echter nog maar weinig bekend over hoe genetica de evolutie van deze gedragsverschillen beïnvloedt. Tegelijkertijd is er toenemend bewijs dat gedragsdiversiteit niet alleen *tussen* soorten bestaat, maar dat ook veelvuldig voorkomt *binnen* populaties van dezelfde soort. Gedragsverschillen, of persoonlijkheidstypes, kunnen een positieve rol spelen in de adaptieve waarde van een populatie, en recent onderzoek laat zien dat dergelijke gedragsverschillen kunnen ontstaan door natuurlijke selectie en dat deze diversiteit zeer veel generaties behouden kan blijven. Deze bevindingen – in combinatie met populatie-gebaseerde studies naar ASS zoals gepresenteerd in dit proefschrift – suggereren dat ASS niet wordt veroorzaakt door een enkele "defecte" hersenstructuur, maar waarschijnlijker het gevolg is van een opeenstapeling van neurobiologische variaties die elk ook aanwezig zijn in de algemene "gezonde" bevolking.

Er zijn steeds meer aanwijzingen dat sommige gedragseigenschappen die samenhangen met ASS voordelen kunnen hebben voor de bevolking als geheel. Personen met relatief hoge scores van autisme-achtig gedrag werken bijvoorbeeld vaker in zogenaamde *STEM*-carrières (*Science, Technology, Engineering and Mathematics*). In Nederland komt ASS drie maal vaker voor in Eindhoven dan in bijvoorbeeld Utrecht en Haarlem, wat toegeschreven zou kunnen worden aan *hightech* miljardenindustrieën als ASML en Philips. Deze constatering heeft implicaties voor de manier waarop we over ASS en de onderliggende mechanismen denken. Op weg naar een beter begrip van autistische verschijnselen is daarom meer fundamentele kennis nodig over individuele gedragsverschillen binnen populaties, en over hoe verschillen in hersenen en gedrag zijn gevormd (en behouden blijven) door evolutie. Een dergelijk fundamenteel begrip van natuurlijke diversiteit zal essentieel zal zijn voor het begrijpen van neurobiologische variatie die ten grondslag ligt aan sociale gedragsverschillen. Uiteindelijk zullen dergelijke inzichten de weg banen voor de ontwikkeling van effectieve strategieën voor neurologische ontwikkelingsstoornissen zoals ASS.

LIST OF PUBLICATIONS

Molenhuis RT, Bruining H, Van Soldt P, Brandt M, Burbach JPH, Iraqi FA, Mott RA, Kas MJH. Modeling the quantitative nature of neurodevelopmental disorders using Collaborative Cross mice. *Manuscript under review.*

Ori APS, Bots MHM, Molenhuis RT, Olde Loohuis LM, Ophoff RA. A longitudinal model of human neuronal differentiation for functional studies of schizophrenia polygenic risk. *Manuscript under review, preprint available on bioRxiv.*

Molenhuis RT, Bruining H, Remmelink E, Loos M, Kas MJH. Mouse models for ASD respond differently to novelty. *Manuscript in preparation.*

Molenhuis RT, Bruining H, Kas MJH. Modelling autistic features in mice using quantitative genetic approaches. Advances in Anatomy, Embryology and Cell Biology. 2017

Molenhuis RT, Bruining H, Remmelink E, De Visser L, Loos M, Burbach JPH, Kas MJH. Limited impact of *Cntn4* mutation on autism-related traits in developing and adult C57BL/6J mice. Journal of Neurodevelopmental Disorders. 2016

Molenhuis RT*, De Visser L*, Bruining H, Kas MJH. Enhancing the value of psychiatric mouse models; differential expression of developmental behavioral and cognitive profiles in four inbred strains of mice. Eur. Neuropsychopharmacol. 2014 (* shared first author)

CURRICULUM VITAE

Remco Theodoor Molenhuis is geboren op 11 december 1989 te Nijmegen. Na het behalen van zijn VWO-diploma aan het Stedelijk Gymnasium Nijmegen, is hij Biomedische Wetenschappen gaan studeren met een minor in Filosofie aan de Universiteit Utrecht, wat leidde tot een bachelor diploma in 2011. Datzelfde jaar begon hij aan de onderzoeksmaster Neuroscience and Cognition aan dezelfde universiteit. Zijn eerste onderzoeksstage volgde hij in het laboratorium van dr. Martien Kas in het Universitair Medisch Centrum in Utrecht, gevolgd door een tweede stage in het laboratorium van prof. dr. Roel Ophoff in het UCLA Center for Neurobehavioral Genetics in Los Angeles. Na het behalen van zijn masterdiploma begon hij in 2013 aan een promotietraject onder supervisie van prof. dr. Martien Kas, prof. dr. Peter Burbach en dr. Hilgo Bruining in het UMC Utrecht Hersencentrum. Als onderdeel van dit traject deed hij tevens onderzoek aan de Sackler Faculty of Medicine in Tel Aviv, het Wellcome Trust Centre for Human Genetics in Oxford, de Vrije Universiteit in Amsterdam en het UCL Genetics Institute in London. De uitkomsten van dit werk zijn gepresenteerd in dit proefschrift. Gedurende zijn studie en promotietraject was hij betrokken in de medezeggenschap en lid van de Onderwijs- en Onderzoeksraad van het UMC Utrecht. In de eindfase van zijn promotietraject deed hij negen maanden onderzoek aan de afdeling gedragsfarmacologie van het Roche Innovation Center in Bazel. Sinds september 2018 is hij als post-doctoral fellow onderdeel van de onderzoeksgroep Neurobiologie van het Groningen Institute for Evolutionary Life Sciences, waar hij verder gaat met onderzoek naar individuele variatie en repetitief gedrag.

Remco Theodoor Molenhuis was born in Nijmegen, the Netherlands on 11 December 1989. After graduating from the Stedelijk Gymnasium Nijmegen in 2008, he studied Biomedical Sciences with a minor in Philosophy at Utrecht University, leading to a bachelor's degree in 2011. In the same year, he enrolled in the research master Neuroscience and Cognition at the same university. His first scientific internship was in the lab of dr. Martien Kas in Utrecht, followed by a second internship in the lab of prof. dr. Roel Ophoff at the UCLA Center for Neurobehavioral Genetics in Los Angeles. After obtaining his master's degree, he started his PhD under supervision of prof. dr. Martien Kas, prof. dr. Peter Burbach en dr. Hilgo Bruining at the Brain Center Rudolf Magnus in Utrecht in 2013. As part of his PhD trajectory, he also conducted research at the Sackler Faculty of Medicine at Tel Aviv University, the Wellcome Trust Center for Human Genetics in Oxford, the Free University in Amsterdam and the UCL Genetics Institute in London. The results of this work are presented in this thesis. Throughout his studies he was also involved in employee participation and served as a member of the UMC Utrecht Research and Education Council. At the end of his PhD trajectory he completed a 9-month Roche Internship for Scientific Exchange (RiSE-program) in the behavioral pharmacology group at the Roche Innovation Center in Basel, Switzerland. In September 2018 he started as a post-doctoral fellow in the Neurobiology research group at the Groningen Institute for Evolutionary Life Sciences, where he will continue his research into individual variation and repetitive behaviors.

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Kas-family in Utrecht:
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