GENETIC DISSECTION OF BEHAVIORAL PHENOTYPES
LOST&FOUND IN TRANSLATION

Genetische Ontleding van Gedragsfenotypen
Verloren&Gevonden tijdens Translatie

(met een samenvatting in het Nederlands)

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CHAPTER 1

INTRODUCTION

SCOPE OF THE THESIS

Most psychiatric disorders are highly variable in their clinical presentation. For instance, the typical 'Rain man' representation of autistic behavior with extravagant mathematic skills next to social inaptness does not apply to the autistic boy with epilepsy who is retarded and does not engage in any social contact at all. Or, the mumbling bearded homeless man does not seem to resemble the physics professor with a broadened deluded mind who is also diagnosed with schizophrenia. The diverse clinical pictures observed among psychiatric patients are probably a reflection of many different underlying neurobiological and environmental mechanisms [1].

Psychiatric disorders so far lack clear biological markers, in contrast to other complex diseases such as diabetes mellitus or myocardial infarction for example. No biochemical features or anatomical properties have yet been identified as reliable indicators of psychiatric morbidity. The blood-brain barrier and the anatomical seclusion of the brain are important factors that obstruct studies into human brain functioning.

Yet, a new dawn is fading since multiple genetic abnormalities have been associated to psychiatric disorders [2-6]. Some of these genetic findings may be strong "blueprints" for later behavioral outcome, if they "distinctly" disrupt development across different genetic and environmental backgrounds [3,4]. The advancements in the knowledge about the human genome are astounding [7], yet a major challenge is to put this knowledge into clinical perspective. A shift from clinical psychiatric descriptions to genetic behavioral phenotypes (i.e. observable traits or characteristics caused by altered gene expression) will be increasingly pursued. However, inter-individual variability in behavior is the resultant of a highly complex interaction of many different genetic and non-genetic factors. Research is required to find out how to disentangle specific genetic contributions to behavior and vice versa.

This thesis aims to show that the exploration of human genetic disorders and animal genetic models can bring understanding of the causes and mechanisms of common psychiatric disorders [8 9]. The first part of this thesis are studies on genetic behavioral phenotypes in boys with Klinefelter syndrome, a human genetic disorder characterized by one or more extra X-chromosomes [10]. The second part describes studies that specify genetic behavioral phenotypes using advanced mouse genetic models and techniques [9].

PSYCHIATRIC GENETICS

Psychiatric genetic studies were presaged by the results of twin studies in which high monozygotic twin concordance rates (i.e. the presence of the same disorder in both members of a pair of twins) were found [11,12]. Heredity (whether a first-degree relative has either disease) had been identified as the greatest risk factor for several major psychiatric disorders, which promised great success for psychiatric genetic efforts.

On 15 February 2001, *Nature* published a 62-page paper entitled "Initial sequencing and analysis of the human genome" [13] which allowed a first look at the contents of the human genome. Today, the human genome is known to contain approximately 21,000 genes. Tremendous progress has been booked in the discovery of genes underlying diseases. At the same time, these discoveries have also shown the complex and polygenic nature of common diseases such as major psychiatric disorders.

Initial progress in genetic studies was achieved through family studies that explored genetic linkage: the tendency of certain genetic loci (the specific location of a gene or DNA sequence on a chromosome) to be inherited together [14]. This was followed by genome wide association studies (GWAS). GWAS involve testing a comprehensive catalogue of genetic variants in cases and controls to find those variants associated with a disease [15]. GWAS mostly rely on the common disease-common variant hypothesis (CD/CV), which posits that common genetic variants (classically defined as >1% prevalence of 'allele frequency') could have a role in the causation of common disease. It is assumed that the vast majority of genetic variants in the population are due to common variants; therefore those variants that enhance disease susceptibility will include many of these common variants except if the variants have had a negative effect on reproductive fitness. Three key results have emerged from genetic linkage and association studies [7]:

1. Most traits or diseases can be influenced by a large number of genetic loci
2. The vast majority of the common variants only have moderate effect, increasing risk by 10-50% (notably lower for most variants implicated in psychiatric disease)
3. The discovery of many large collections of genes that can modulate a phenotype has begun to reveal cellular pathways

Many family linkage and GWAS studies have also been conducted in major psychiatric disorders such as autism, schizophrenia, depression, bipolar disease and ADHD [16]. In addition to common variants, a new form of rare variants has been identified through genome wide assays that may provide new etiological clues. Larger deletions or duplications of stretches of DNA, also called copy number variants (CNV), occur across the human genome also in healthy individuals [17]. An intriguing minority of newly identified CNVs seems to arise from recent or de novo (i.e. not inherited) mutations and these have been especially been implicated in autism and schizophrenia [18,19]. Estimates are that up till 20% of the autism population is due to these rare CNV mutations, however every individual CNV accounts for at most of 0.5% of the population [20]. With the prevalence of autism being about 1/100, only 1/50000 individuals in the general population will have autism due to one of these rare variants.

Furthermore, the penetrance of most rare CNVs seems variable and most of these 'causative' variants also seem to occur in healthy individuals. They are also not disease specific as most of these rare CNVs act across different disorders [3,5,21,22].

Overall, psychiatric genetic studies so far have been informative to understand the genetic architecture, but the difficulties to replicate common variants and the rarity of most causal variants have obstructed insights into specific phenotypes and molecular mechanisms.
Nonetheless, the prospect of rather frequent rare causative CNVs in developmental psychiatric disorders has not been without consequence. It has led to a more explicit appreciation of the heterogeneity of psychiatric disorders, especially of autism and schizophrenia.

GENETIC DISORDERS IN NEUROBEHAVIORAL STUDIES

Many believe that especially autism is in fact the representation of a wide range of developmental disorders that share abnormalities in social interaction, communication and repetitive behaviors though in different combinations and of different severity [3,5,21,23]. This has led to a revival of interest in the behavioral phenotypes of naturally occurring rare genetic disorders that are associated with neurodevelopmental problems [21,24]. These rare disorders are generally caused by a spectrum of rare mutations or structural genomic abnormalities (CNVs as described above), including whole chromosome abnormalities [25]. To date between 6.000 and 7.000 rare diseases have been identified and many of those have manifestations of intellectual disability, developmental disorders or other behavioral phenotypes [8]. Most of these disorders share particular dysmorphic or other characteristic features, which enhance the chance of clinical detection and thus allow the inclusion of larger numbers of subjects for study [24].
Familiar examples of genetic disorders with behavioral impact are Down syndrome due to an extra copy of chromosome 21 and Rett syndrome due to specific mutations in the MECPP gene [26,27]. Although most genetic disorders are variable in their neurodevelopmental profile, some of them seem to have clinically distinct behavioral phenotypes. The probability of such genetic phenotypes can be illustrated by Rett syndrome (RTT). A more homogeneous profile of autistic symptoms together with non-autistic symptoms has led to the description of the RTT genetic subphenotype that is formally classified in the DSM-IV-TR as an autism subtype. RTT is a progressive neurodevelopmental disorder that manifests in girls during early childhood [28,29]. Mutations in *MECP2* gene are found in more than 95% of classic RTT cases [30,31]. Both specific autistic and non-autistic features characterize RTT [28,29]. Importantly, RTT specific autistic features are also observed in the general population of autistic individuals though probably in a much lower frequency. This example illustrates that genetic disorders may serve to find more precise behavioral genotype-phenotype relationships.

KLINEFELTER SYNDROME: XXY

Klinefelter syndrome is such a genetic disorder that has been reinvented over the past decade as an interesting human genetic model for abnormal behavioral development. Originally it was thought to be an endocrine disorder when it was discovered, but later the chromosomal cause was recognized. The original publication of dr Harry Klinefelter "Syndrome characterized by gynaecomastia, aspermatogenesis without a-Leydigism, and increased excretion of follicle stimulating hormone" dates from 1942 [32].

The genetic origin of the syndrome was not recognized until 1959 in Jacob's Nature article: "A case of human intersexuality having a possible XXY sex-determining mechanism" [33].

From 1941 to 1942 dr Harry Klinefelter went for one year to Boston to work at the Massachusetts General Hospital. Working under the supervision by Fuller Albright he described a group of nine men with "gynecomastia, aspermatogenesis without aleydigism, and increased excretion of follicle-stimulating hormone", the first description of what would be called the Klinefelter syndrome. Klinefelter described his findings as follows: "Albright's Saturday morning clinics were famous throughout the Massachusetts General Hospital. At the first one I attended, I saw a tall black boy named George Bland who had gynecomastia and very small testes (1.0–1.5 cm in length). When I asked Dr. Albright what this was all about, he said he did not know but that he would be happy for me to work on it. During the rest of the year, we found 8 other patients with this same condition and reported the series at the endocrine meetings in 1942. Dr. Albright was charitable enough to let me put my name first on the paper that was published later in 1942 in the Journal of Clinical Endocrinology. The title, "A Syndrome Characterized by Gynecomastia, Aspermatogenesis without Aleydigism, and Increased Excretion of Follicle-Stimulating Hormone," was so long that the syndrome came to be known by my name, though it was really just another of Dr. Albright's diseases. A few years after the syndrome was described, Heller and Nelson reported that the gynecomastia was not a necessary part of the syndrome, though it occurred in about 75% of the patients. The hallmarks of the syndrome, therefore, are small testes, sterility, and increased excretion of follicle-stimulating hormone.
Fourteen years after the original description of the syndrome, 2 groups independently discovered that the buccal mucosal cells of these patients contained an extra chromatin mass, or were chromatin positive. A few years later, Jacobs and Strong found that these chromatin-positive patients had 47 chromosomes, with an extra X chromosome, and were XXY. "

About 80% of Klinefelter cases are due to the congenital numerical chromosome aberration 47,XXY; the remaining 20% have higher-grade chromosome aneuploidies (48,XXXY; 48,XXYY; 49,XXXXY), 46,XY/47,XXY mosaicism, or structurally abnormal X

chromosomes [10]. Recent studies estimate the prevalence of KS 1:640 [34,35], which makes it the most prevalent aneuploidy in males and the most frequent cause of male infertility.
It should clearly be noted that many patients with Klinefelter's syndrome remain undiagnosed. Abramsky and Chapple calculated that 10% of expected cases were identified prenatally and 26% were diagnosed in childhood or adult life because of hypogonadism, gynaecomastia, or infertility, leaving 64% undiagnosed [36]. A large Danish national registry study confirms that Klinefelter's syndrome is widely underdiagnosed, with less than 10% of the expected diagnoses made before the age of puberty [37].

XXY CLINICAL MANIFESTATIONS

Klinefelter Syndrome is a complex disorder with highly variable endocrinological, morphological and cognitive manifestation of altered X-chromosomal gene expression. The 'prototypic' man with KS has traditionally been described as tall, with sparse body hair, gynecomastia, small testes and decreased verbal intelligence [38]. Yet, the clinical picture of XXY males may range from severe signs of androgen deficiency, or even a lack of spontaneous puberty, to normally virilised males who only consult a doctor because of their infertility.

XXY PATHOPHYSIOLOGY

The numerical chromosome aberrations in Klinefelter's syndrome arise by non-disjunction either during meiotic divisions occurring in germ-cell development or in early embryonic mitotic cell divisions. Incorrect meiotic divisions are predominant. In autosomal trisomies, paternal non-disjunctions account for only about 10% of all cases; however, paternal errors contribute more frequently to sex-chromosome aneuploidy. The 47,XXY condition has been extensively studied, and about half of all cases are paternally derived [39]. It is not yet known how supernumerary X chromosomes lead to androgen insufficiency. The underlying molecular mechanisms remain unknown. The presence of an extra X chromosome seems to predestine germ cells to a shorter life. In various animal species and in human beings, a normal complement of primordial germ cells is present in the fetal testes of XXY males, but these cells degenerate at an accelerated rate during childhood [40,41].
The X chromosome contains about 2000 genes compared to the Y chromosome containing 7only 8 genes, out of the estimated 21,000 genes in the human genome [42,43]. Expression of extra copies of genes on the X chromosome leads to so called gene dosage effects as opposed to gene deficiencies through mutations, deletions or monosomies. Several genetic mechanisms have been proposed to explain the variability of KS phenotypes [44].

X- INACTIVATION PATTERNS Random inactivation of one of both X chromosomes (XCI) is though to occur to prevent overexpression of X linked genes in KS in comparison to euploidic XY males [42]. Skewed inactivation is usually defined as above 80%

activation/inactivation of one allele. It has been detected in a variable percentage of KS cases and has been studied whether skewing could affect KS phenotypes (table 1). The XCI pattern has not vet clearly shown to affect KS phenotypes, but this might be due to the wide range of features and samples that have been studied (e.g. psychological and anthropometric measures, hormones). Whether XCI is skewed more often in KS than in females also remains to be determined [44].

CAG REPEAT LENGTH OF ANDROGEN RECEPTOR GENE The human androgen receptor gene (AR, previously also HUMARA, located in Xq11.2–q12) is of double interest concerning genotype-phenotype correlations in KS. The AR contains a highly polymorphic trinucleotide repeat (CAG)n in exon 1 [45] with the normal length varying between 9 and 36/37 repeats [46]. Expanded repeats of AR cause the neurological disorder of X-linked spinobulbar muscular atrophy [47]. The (CAG)n repeat is also correlated with physiological androgen effects in healthy men. It has pharmacogenetic implications as well, because testosterone treatment effects seem to be modulated by its number [48]. The calculation of a so-called 'X-weighted mean' taking both into account has been introduced and correlations with clinical features have been described [49]. In KS four studies have also investigated such correlations with varying results (table 1).

PARENT-OF-ORIGIN EFFECTS Differential gene expression on the basis of the origin of paternal or maternal alleles is called imprinting. This could theoretically be of influence in KS as mentioned above nearly half of the KS cases have been shown to be of paternal origin through non-disjunction in paternal meiosis, where most other trisomies nearly always arise from errors in maternal meiosis [39]. To date, six studies have analyzed parent-of-origin effects on different KS phenotypes with inconsistent results (table 1) [50-55].

Table 1. Summary of studies reporting parental origin of the supernumerary X chromosome, X inactivation, androgen receptor (AR) (CAG)n repeat length, or combinations associated with KS phenotype, adapted from Tuttelmann et al. [44].

Study	KS Subjects (n-age)	Outcome measures	Genetic analysis	Results
Zitzmann *et al*. (2004) [56]	77: 18–65 years	Anthropo- and sociometrical data, features of hypogonadism (gynecomastia, etc.), hormones	X inactivation, *AR* (CAG)n	*AR* (CAG)n positively correlated with body height and predictive for gynecomastia and smaller testes; *AR* (CAG)n inversely correlated with bone density, stable partnership and higher education
Zinn *et al*. (2005) [55]	35: 0.1–39 years	Anthropometric measurements including penile length and testicular volume	Parental origin, X inactivation, *AR* (CAG)n	*AR* (CAG)n inversely correlated with penile length
Stemkens *et al*. (2006) [52]	61: 2–56 years	Anthropometric and psychomotor development, IQ	Parental origin	Impaired speech and motor development problems more often in paternal X cases
Ross *et al*. (2006) [51]	11: 19–54 years	Psychotic symptoms	Parental origin, X inactivation	No association
Wikström *et al*. (2006) [53]	14: 10–13.9 years	Pubertal development, growth, testicular volume, hormones	Parental origin, iso/hetrodisomy, X inactivation, *AR* (CAG)n	Paternal origin of X chromosome associated with later onset of puberty; longer *AR* (CAG)n with later reactivation of pituitary-testicular axis
Ross *et al*. (2008) [50]	50: 4.1–17.8 years	Cognitive and motor development	Parental origin, X inactivation, *AR* (CAG)n	No associations
Zeger *et al*. (2008) [54]	55: 2.0–14.6 years	Anthropometric measurements including penile length and testicular volume; hormones	Parental origin	No association

XXY BEHAVIORAL STUDIES

Many genes that affect mental functioning have been shown to reside on the X chromosome, which may well explain the occurrence of neurobehavioral deficits in KS [57]. However, there have been different appreciations of the behavioral consequences of Klinefelter syndrome since its discovery. The literature on cognitive, psychological and behavioral features in individuals with KS has developed in three phases.

PHASE 1: "SUPERNUMERARY X PSYCHOPATHS." XXY men were originally associated in the 1950s and 1960s with psychopathology, sexual perversions and fire setting behavior. Studies among prisoners claimed that Klinefelter patients are at risk to become "psychopaths". These associations were later refuted, especially as additional genetic studies demonstrated the high prevalence of 47,XXY in the "normal" population [58-60].
PHASE 2: "FOLLOWING KLINEFELTER FROM BIRTH." Several longitudinal studies followed small cohorts of KS patients (ranging from about 10 to 20 subjects) identified by cytogenetic screening of large numbers of consecutive births [61-69]. The strengths of these studies include the selection of subjects from birth cohorts, prospective design, frequent psychological assessments and long-term follow-up.
PHASE 3: "SPECIFYING XXY BEHAVIORS." A third group of studies has emerged after the completion of the prospective studies, and described more specific aspects of the psychological and behavioral characteristics in males with KS including neuropsychological studies and studies correlating cognitive outcomes to various genetic factors [54,55,70-72].

Reviews on the neurobehavioral phenotype in KS indicate three domains of cognitive and/or behavioral dysfunctioning that are predominant in KS [22,69,72-75]: language problems, neuropsychological deficits of executive functioning (EF) and social problems.

LANGUAGE PROBLEMS Language difficulties have been identified in 70–80% of children with KS starting at an early age [50]. This finding has been relatively robust, also shown in prospectively ascertained samples [62,65,68,76,77]. Most of the studies have primarily identified expressive speech and language difficulties, but receptive language deficits have also been noted. Language deficits have been shown to persist into adulthood [78].

NEUROPSYCHOLOGICAL DEFICITS Most studies have shown that general cognitive ability in patients with KS falls in the average to low average range, with significant discrepancies driven by deficits in the verbal conceptual domain, rather than the nonverbal reasoning or spatial domain [69,77-80]. Clinical observations of hypo-assertiveness and inattentiveness led to the impression that cognitive processes important for the executive system are disturbed in KS [73]. The executive system is a theorized cognitive system in psychology that controls and

manages other cognitive processes. Executive dysfunctioning therefore may lead to deregulation of thought, emotion and behavior, including inappropriate and inflexible social behavior.
Empirical studies of EF in KS are still fairly sparse and have addressed different aspects of EF and also in different age groups [73]. Deficits on verbal EF task including working memory have been shown in adults with KS, while performance on non-verbal EF tasks seemed unaffected [81,82]. Only three earlier studies have investigated EF in samples of children and adolescents with KS [50,83,84], mainly with reports of deficits in regulation of attention and reduced speed of information processing.

SOCIAL PROBLEMS Boys with Klinefelter syndrome are generally described as quiet, shy, hypoactive, and unassertive "*with a dislike for rough games and easy crying when bullied by other children*" [64]. Adults with 47,XXY also rate themselves as more sensitive, apprehensive, and insecure compared to their peers [80]. Later studies confirmed that males with 47,XXY have deficits in social interactions possibly contributed by language-based learning difficulties, social cognition impairments, and language delay [70,71]. Notably van Rijn et al. conducted several meaningful studies to describe the cognitive mechanism behind social maladjustment in Klinefelter syndrome [70,71,85-87]. Adult men with 47,XXY have deficits in perception of social-emotional cues as revealed by their impairment in recognizing facial expressions of anger. As a result of their misperception of nonverbal signals and/or facial affect recognition, problems in social functioning may emerge. Furthermore, males with 47,XXY experience increased levels of emotional arousal, yet they are less able to identify and verbalize their own emotions, in comparison to the general population [87].

XXY PSYCHIATRIC MANIFESTATIONS

Psychiatric problems have not deserved much attention in KS. Since its discovery, KS has been linked to psychotic and social developmental psychopathology, but these associations were mostly based upon incidental case reports or retrospective cohort studies. Van Rijn et al. were the first to highlight that social difficulties and vulnerability for autistic traits exist in adults with 47,XXY and not only in the domain of social communication skills but also in the other domains including attention switching, attention to detail, communication, and imagination [71]. Earlier individual case reports had also indicated an association between KS and autism spectrum disorders [84,88-90]. High levels of schizotypal traits and schizophrenia symptoms have also been shown in males with 47,XXY [70,91,92]. Strangely enough no psychiatric surveys have been conducted in under aged subjects with KS, which would be expected given the high prevalence of developmental learning, language and social problems.

XXY GENETIC BEHAVIORAL PHENOTYPES

Over 60 years of studies in Klinefelter have clearly proven that KS is associated with particular psychological and behavioral impairments. A further challenge now is to find traits and mechanisms in KS that translate the link between the supernumerary X chromosome and the behavioral phenotype. Such insights will also be relevant for other developmental disorders and provide insight into X-chromosomal mechanisms in behavior. Such research requires an in-depth analysis of detailed behavioral and cognitive traits using sensitive *scales* that measure a broad range of traits in relation to genetic and non –genetic factors in KS.

ANIMAL STUDIES OF PSYCHIATRIC DISORDERS

The example of Klinefelter syndrome illustrates the complexity of finding more exact genotype-phenotype relationships relevant to psychiatric disorders. It shows that the hunt for core behavioral phenotypes in relation to a genetic alteration in humans is clouded through many factors such as general level of cognitive function, socioeconomic status and exposure to adverse events. As for other complex human disorders, this has been a major drive to develop animal models of aberrant behavior [9,93,94]. The power of using animal models in neurobehavioral research lies in the unique possibility to investigate causal mechanisms under controlled environmental and genetic conditions [9,95]. *Drosophila* (fruitfly), *Caenorhabditis elegans* (nematode worm) and *Danio rerio* (zebrafish) are often used as molecular or cellular models, whereas rodents are preferred when the brain and behavioral aspects of the phenotype are investigated. The mouse (*mus musculus*) has become the prime organism of choice for modeling human brain disease [96]. The mouse shares the complex genomic and neuroanatomical organization of mammals and there is wealth of molecular tools that are available to manipulate gene function [97]. Humans have been interested in the inheritance of traits in mice for at least 3,000 years. Already, the Chinese lexicon written in 1100 BC has a word for spotted mice [98,99]. Interest gradually spread to the West, via the Imperial courts of Japan(!), and reports on the inheritance of mouse coat color were published in Europe in the eighteenth century [99]. The mouse was used as a laboratory animal as early as 1664 in studies of the properties of air-[100]. Modern mouse genetics started around 1902 [100]. The guidelines for generating inbred mouse strains, first published in 1952, state [101]: *"A strain shall be regarded as inbred when it has been mated brother sister (hereafter called b×s) for twenty or more consecutive generations (F20), and can be traced to a single ancestral breeding pair in the 20th or a subsequent generation. Parent×offspring matings may be substituted for b×s matings provided that, in the case of consecutive parent×offspring matings, the mating in each case is to the younger of the two parents."*

VALIDITY OF ANIMAL MODELS

Genetic analysis in animal models has tended to focus on the analysis of genetically engineered mice in which one or more genes have been turned off through a targeted mutation [93,102]. Most of these genetic animal models involve either human disease candidate genes or genes that affect important systems in brain function such as neurotransmitter systems. The validation of animal models for the heterogenic and complex psychiatric disorders has been proven complex.

Animal models for psychiatric disorders ideally should fulfill three criteria of validity for animal models:

1. construct validity (etiology is the same as in humans)
2. predictive validity (response to intervention is comparable to humans)
3. face validity (phenotype appearance is the same as in humans)

Most translational animal models for psychiatric disorders cannot achieve construct validity, as the putative neurobiological mechanisms are generally unknown [9,103,104]. Predictive validity implies that a new model or testing paradigm needs to be responsive to existing pharmacological interventions, which does not allow the identification of new targets that in fact aim to improve upon existing often imperfect treatments. It is also difficult to obtain animal models that at the level of face validity fully mimic the presentation of psychiatric disorders.

Thus, the traditional concept of an animal model of human disease was to find an animal (possibly under a defined set of conditions or experimental manipulation), which mimics all aspects of a human disease. The validity issues have led to considerable skepticism on such a prospect. No single gene knockout or selection line is likely to represent the full genotypic and phenotypic complexity of any human disease. For instance, the translation of cognitive processes between humans and animal models is difficult as intellectual disability in a human does not have a true equivalent in animal models.

Kas et al. have proposed that, in order to identify shared genotype–phenotype relationships in animal and humans, the same gene should affect analogous phenotypes in both species considering species-specificity in the expression of this phenotype [9]. This approach depends on conserved gene function, the presence of functional polymorphisms in that gene in both species, and thirdly on the choice of an appropriate analogous phenotype in both the model species and in humans. The choice of phenotype in the model organism is critical and species-specific conditions should be taken in consideration. It seems more feasible to study an animal that models some portion of the disease that may correspond to the contribution of a limited number of loci involved. This requires a dissection of behavioral domains into readily measurable components.

CROSS-SPECIES GENETIC MAPPING OF BEHAVIOR

As a consequence, an increasing appreciation of the properties of mouse inbred strains that have been established over the last century has occurred. Over 450 inbred strains of mice have been described and characterized, offering a wealth of different genotypes and phenotypes for genetic and other studies. Inbred strain combinations have been used to set up a cross or segregating population to enhance genetic mapping purposes, in particular quantative trait (QTL) mapping. Genetic mapping of is the statistical study of the alleles that occur at a locus and the traits that they contribute to. Quantitative traits refer to phenotypes (characteristics) that vary in degree and can be attributed to polygenic effects, i.e., product of two or more genes, and their environment. Quantitative trait loci (QTLs) identify a particular region of the genome as containing a gene that is associated ('mapped') with the quantitative trait being assayed or measured.
Mapping regions are shown as intervals across a chromosome, where the probability of association is plotted for each genetic marker used in the mapping experiment. When a QTL is found, it is often not the actual gene underlying the phenotypic trait, but rather a region of DNA that is closely linked with the gene. For organisms whose genomes are known such as for mice, the challenge is to exclude genes in the identified region whose function is known with some certainty not to be connected with the trait in question

Mouse Genetic Reference Populations (GRPs) with more optimal genetic properties have been developed to enhance genetic mapping. A mouse genetic reference population consists of a set of genetically well-characterized lines that are often used over a long period of time to study a multitude of different phenotypes. Once a GRP has been genotyped, subsequent studies can focus on the analysis of interesting and important phenotypes and their joint and independent relations. Many of these same GRPs have been phenotyped extensively for more than 25 years, resulting in rich sets of phenotypes. Thus, a GRP is an ideal immortal resource for systems genetics because of the relative ease with which vast amounts of diverse data can be accumulated, analyzed, and combined [95]. GRPs provide the ability to study multiple phenotypes and substantial numbers of genetically defined individuals under one or more environmental conditions. When accurate phenotypes from 20 or more lines in a GRP have been acquired it becomes practical to explore and test the genetic correlations between that trait and any previously measured trait in the same GRP. This explains the use of the term "reference" in GRP. GRPs are also ideal for developmental and behavioral studies because the same genetic individual can be phenotyped at multiple stages. There are several types of GRPs such as recombinant congenic (RCC) strains and consomic or chromosome substitution strains (CSS).

An intriguing prospect is to perform genetic mapping of so called naturally occurring behaviors [9]. These are behaviors that are essential in every species for survival and

adaptation, such as food seeking, mating and social coherence. It is likely that the genetic regulations of these behaviors are conserved in evolution. Disruption of these systems is likely to result in substantial mal-adaptation and behavioral disturbance, which in humans may lead to psychiatric disorders. Therefore, genetic mapping of naturally occurring behaviors in mice might elucidate highly conserved genetic mechanisms that are relevant for psychiatric disorders in humans. Once these conserved genes are identified, their subsequent evaluation as a single locus trait may elucidate fundamental mechanisms in psychiatric pathogenesis.

OVERVIEW OF STUDIES IN THE PRESENT THESIS

CHAPTER 2 describes a general survey of psychopathology in 51 boys with Klinefelter syndrome. This study aims to show that boys with KS are at substantial risk for developmental psychopathology

CHAPTER 3 investigates the specificity of phenotypes in autistic subjects with KS and 22q11 deletion syndrome through statistical comparison of both disorder autistic symptom profiles with the profiles of genetically heterogeneous autism spectrum disorders.

CHAPTER 4 investigates cognitive executive functioning in boys with KS and its relation to social functioning. This study aims to investigate the relationship between cognitive control and social adaptation in a genetic disorder.

CHAPTER 5 hypothesizes that the parent-of origin of the X chromosome influences psychopathogy in KS. It is assumed that X chromosomal imprinting can influence autistic and schizotypal symptomatology.

CHAPTER 6 searches for core cognitive features in KS by cross-species comparison of recognition deficits in mice with a supernumerary X chromosome to recognition deficits in boys with KS.

CHAPTER 7 is a large study that describes the genetic mapping of temporal components of social memory in mice using chromosome substitution strains. The aim is to find genes that regulate aspects of social cognition in mice and to investigate their possible role in disorders of social behavior in humans, such as autism and schizophrenia.

CHAPTER 8 is a general discussion.

CHAPTER 9 summarizes the findings of the studies.

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CHAPTER 2

PSYCHIATRIC CHARACTERISTICS IN A SELF-SELECTED SAMPLE OF BOYS WITH KLINEFELTER SYNDROME

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ABSTRACT

Klinefelter syndrome is the most frequent chromosomal aneuploidy with a prevalence of 1 in 700. Klinefelter syndrome has been widely associated with cognitive impairment and language problems. No previous studies have systematically investigated the association of Klinefelter syndrome with psychiatric disorders in children and adolescents. To our knowledge, the only data available are from psychiatric inventories of adults with Klinefelter syndrome. The objective was to explore the extent of psychiatric morbidity in children with Klinefelter syndrome.

Fifty-one subjects with Klinefelter syndrome aged 6 to 19 years were included through the Dutch Klinefelter association and 2 university medical centers. The sample was screened using structured and standardized assessment procedures covering the full range of psychiatric problems and disorders. In addition, all boys were formally evaluated for the presence of a language disorder.

A wide range of classifications could be applied, with language disorder (65% [33 of 51]) as the most prevalent disorder, followed by attention-deficit disorders (63% [32 of 51]) and autism spectrum disorder (27% [14 of 51]). Behavioral impairment was most evident among cases classified as autism spectrum disorder and psychotic disorder (12% [6 of 51]).

Children with Klinefelter syndrome seem to be at risk for problems in social and language development, as well as for problems in regulation of emotion and behavior. This is reflected in the broad spectrum of psychiatric classifications applicable in the present selected sample. Health care professionals should be aware of an increased a priori possibility of psychiatric problems when confronted with a child with Klinefelter syndrome.

INTRODUCTION

In previous decades, there has been ongoing concern about the cognitive and behavioral phenotype of Klinefelter syndrome (KS) [1]. KS is caused by 1 or more supernumerary X chromosomes in males. A recent study suggested that the prevalence of KS has increased over previous decades from 1.09 to 1.72 per 1000 male births based on newborn surveys [2]. Many cases remain undiagnosed because of substantial variation in clinical presentation [1]. Only 10% of subjects with KS are diagnosed prenatally, with another 25% diagnosed during childhood or adulthood, leaving 65% undiagnosed [3,4].

In adults, the cognitive defects in KS seem specific and are not caused by a global decline in intellectual functioning [5]. Most men with KS perform normally on nonverbal abilities and overall intelligence but are specifically impaired on measures of language skills [6,7]. The majority of males with KS (up to 70%–80%) suffer from a language disorder (LD) of some form at all ages. Significant impairments are frequently observed in higher order aspects of expressive language, especially in deficits with word retrieval, expressive grammar, verbal processing speed, and executive abilities [5,8,9]. Furthermore, social cognition in adult KS is often marked by inadequate emotional arousal [10].

A recent study expanded on the description of the cognitive phenotype of KS in a sample of 50 children [11]. Specific language, academic, attention, and motor abilities were impaired in this sample. Vocabulary and meaningful language understanding were unaffected, although higher linguistic competence was impaired. Younger children displayed deficits in sustained attention without impulsivity.

To date, no systematic studies have investigated whether such cognitive and learning problems are associated with psychiatric disorders in children and adolescents with KS. Systematic psychiatric surveys were explicitly advocated in a National Institutes of Health 2003 report on "The expansion of the Klinefelter phenotype and the identification of new research directions" [12].

Data on psychiatric screenings in adult KS emphasize the need for such screenings in minors with KS. A psychiatric screening among 31 adult subjects with KS showed a high prevalence of psychosis (6.4%) and depression (19.4%) [13]. A survey of hospital admissions among adults with KS in Denmark (N = 832) showed that the hazard ratio (HR) for admission for psychiatric disorders was second highest of all diagnosis groups (HR: 3.65), in particular for psychoses (HR: 4.97) [14].

With this present study we aimed to provide insight into what psychiatric problems might occur during development once the diagnosis of KS has been made. A screening in 51 self-selected school-aged children with KS was performed using structured assessment procedures to encompass a wide range of psychiatric problems and disorders. To confirm the earlier established association of KS with language problems, all boys were evaluated for the presence of a LD according to *Diagnostic and Statistical Manual of Mental*

Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria. Furthermore, the relationship of IQ with psychiatric problems was evaluated.

METHOD

PATIENTS The sample consisted of 51 boys (mean age: 12.2 years [range: 6–19 years]; 27 subjects aged <12 years, 17 subjects aged 12–16 years, and 7 subjects aged 16–19 years). The inclusion criteria for the current study demanded an age between 6 and 20 years, no history of closed head injury or neurologic illness, sufficient comprehension of the Dutch language, and a diagnosis of KS confirmed by standard karyotyping. Mosaic forms of the syndrome were allowed (n = 4), whereas higher aneuploidies than 47,XXY were excluded because of their association with profound mental retardation [15].

The Dutch Klinefelter association and 2 centers for clinical genetics and pediatrics situated in the center of the Netherlands were involved in recruiting the children for this study. A newsletter presented on the Internet or in writing informed parents and children of the aim and methods of the study. Parents and children had to apply actively for participation in the study by contacting the research team.

Subsequently, they were sent written information about the selection criteria and the implications of participation in the study. The newsletter encouraged parents and children to participate regardless of any problems present. They were invited for assessment if they met the inclusion criteria. The Dutch Central Committee on Research Involving Human Subjects approved the research protocol. Written informed consent was obtained from participants (if >12 years of age) and their parents or guardians.

Half of the boys (26 of 51) were diagnosed with KS through prenatal amniotic fluid investigation. The boys diagnosed with KS after birth (25 of 51) had different combinations of symptoms that had led to cytogenetic testing. The majority of the group diagnosed after birth suffered from marked hypogonadism (22 of 51) in combination with learning problems (9 of 25) and/or unspecified behavioral problems (9 of 25). Other reasons for suspicion on KS were tall stature with severe allergies or asthma. At the time of the assessments, 14 boys were treated with testosterone and 4 received other medication (atypical antipsychotics [n = 1], antiepileptics [n = 1], and stimulating compounds [n = 2]).

PROCEDURE AND MEASURES Assessments were conducted at the Department of Child and Adolescent Psychiatry of the University Medical Centre Utrecht. One investigator (Dr Bruining) performed all psychiatric interviews and observations of the subjects. He is a senior resident in child and adolescent psychiatry. Fully trained child psychologists conducted the psychological examinations (eg, IQ tests).

Initially, a comprehensive medical and behavioral history was obtained from the children. All interviews were videotaped. The classification for LD was applied on the basis of the DSM-IV-TR criteria with the exception that we allowed a concurrent pervasive

developmental disorder. Individual measures of language development and academic achievement were obtained from preexisting records. Language disorders were not subtyped (eg, mixed receptive-expressive and expressive LDs). One combined category of LDs is listed in the Results. The Kiddie-Sads-Present and Lifetime Version (K-SADS-PL) [16] interview was administered to assess diagnoses in the domains of affective, psychotic, anxiety, and behavioral disorders. The interview is a semi-structured diagnostic interview designed to assess current and past episodes of psychopathology in children and adolescents according to the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* and the DSM-IV-TR criteria. Probes and objective criteria are provided to rate individual symptoms.

The presence of an autism spectrum disorder (ASD) was assessed using the Autism Diagnostic Interview, Revised (ADI-R) [17]. No subtypes of ASDs (eg, pervasive developmental disorder-not otherwise specified [NOS], Asperger syndrome, autism) were allocated subordinate to a general ASD diagnosis, because no replicable measures exist for such phenotypic refinements. The interviewer (Dr Bruining) was certified for the assessment of the ADI-R. The ADI-R focuses on the 3 core or so-called content domains of autism (ie, social interaction, communication, and stereotyped behaviors) [17]. ADI-R items are coded and converted to numerical scores for these domains and also for an "age-of-onset" domain. A classification of an ASD is applied when scores in all domains are met or when scores are met in 2 core domains and meet criteria on the age-of-onset domain, but are 1 point away from meeting autism criteria in the remaining core domain. Reliability of the ADI-R in a population with mild to moderate mental retardation has been established [18]. Furthermore, the ADI-R proved valuable in studies with populations with genetic syndromes such as the 22q11 deletion and Angelman and Prader Willi syndromes[19-21].

IQ was assessed by using the Dutch adaptation of the Wechsler Intelligence Scale for Children and in some cases the Wechsler Adult Intelligence Scale, resulting in average total IQ (TIQ) scores with verbal and performance scale scores [22].

After the completion of all the tests and interviews, the videotapes of all subjects and the outcomes were discussed in a consensus meeting headed by the head of the department (Dr van Engeland). The consensus meeting served to control for procedural mistakes and to verify whether the classifications through the K-SADS-PL interview and ADI-R were in agreement with our clinical judgment.

No classifications were applied for any disorder without threshold scores in the ADI-R or K-SADS-PL interviews. Current DSM-IV-TR guidelines prescribe not to apply particular diagnoses next to an ASD (eg, attention-deficit/hyperactivity disorder [ADHD], LD, schizophrenia, and schizoaffective disorder). However, we assessed all classifications separately regardless of comorbidity rules to provide a complete representation of

psychopathology and psychiatric symptoms within the selected group. The concurrence of the most prevalent classifications in the cohort is described in "Results."

RESULTS

Table 1 summarizes the psychiatric classifications for the sample with subsequent intelligence scores.

The DSM-IV-TR criteria for the combined group of expressive or mixed receptive-expressive LDs were met in 65% (33 of 51) of the participants.
The application of the K-SADS-PL interview yielded the following results: 63% (32 of 51) of the subjects were classified as having ADHD, of which 43% (22 of 51) could be classified as having the predominantly inattentive subtype, and 20% (10 of 51) were classified as having the combined subtype.
Twenty-four percent (12 of 51) of the subjects had suffered from a depressive episode.

Table 1. *Classifications according to DSM-IV*

DSM IV diagnosis		Prevalence in present study % (number)	Prevalence in general population %		TIQ	VIQ	PIQ
Language disorder		65 (33)	2-19 [23]		78	77	85
ADHD	IS	63 (32)	43 (22)				
	CS		20 (10)	5.3 [24]	80	79	85
ASD		27 (14)	0.6-1.6 [25]		77	75	82
Depressive disorder		24 (12)	9.5 [26]		85	84	90
Generalized anxiety		18 (9)			83	82	89
Separation anxiety		14 (7)			84	79	92
Psychotic disorder		8 (4)			85	83	91
Schizophrenia		2 (1)	0.14-0.46 [27]		73	79	72
Schizoaffective		2 (1)			101	101	103
Whole cohort		*100 (51)*			*80*	*78*	*86*

Prevalence in general population: e.g. percentages for underaged populations, TIO=average total IQ, VIQ= average verbal IQ, PIQ= average performance IQ, ASD = Autism Spectrum disorder, ADHD = Attention Deficit/Hyperactivity Disorder, IS=inattentive subtype,-CS=combined sybtype, NOS= not otherwise specified.

Eighteen percent (9 of 51) fulfilled the criteria for generalized anxiety disorder in the present or the past, and 14% (7 of 51) had suffered from separation anxiety disorder at a younger age.
Psychotic disorder NOS was found in 8% (4 of 51) of the subjects. These boys showed psychotic symptoms, accompanied by a circumscribed period of clear distress in which an obvious change of behavior was observed. Another 2 subjects (4% [2 of 51]) suffered from multiple psychotic episodes with chronic distress and decline in functioning, despite intensive inpatient and outpatient treatment. One of these 2 boys fulfilled the criteria of a schizoaffective disorder. The other boy met the criteria for schizophrenia, disorganized

subtype. Psychotic symptoms were reported in 45% (23 of 51) of the subjects. These were predominantly auditory hallucinations and paranoid delusions. The psychotic symptoms by themselves did not cause obvious distress or decline in general functioning, except in subjects with a full psychotic disorder (ie, psychotic disorder NOS, schizophrenia, and schizoaffective disorder).

The ADI-R revealed that 27% (14 of 51) of the boys met the threshold for an ASD. In all of these cases, the diagnosis of an ASD was confirmed in the consensus meeting.

Figure 1 gives an overview of the symptom scores in the 3 core domains measured in the ADI-R.

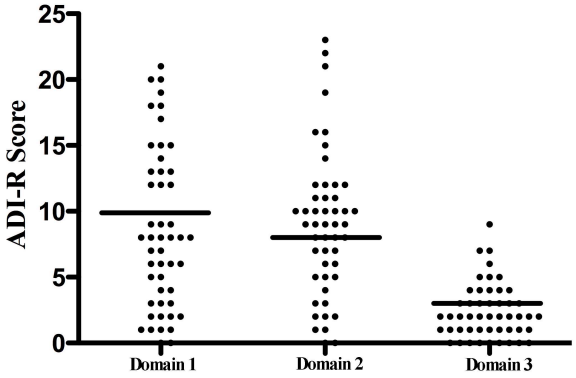

Figure 1. *ADI-R scores in the sample for all 3 core domains. Horizontal lines depict ADI-R autism cutoff values for each domain. Domain 1: qualitive abnormalities in social reciprocity (cutoff = 10); domain 2: qualitive abnormalities in communication (cutoff = 8); domain 3: restricted, repetitive, and stereotyped patterns of behavior (cutoff = 3)*

Ten subjects had scores above the cutoff values in all 3 core domains of the ADI-R, 11 had scores above the cutoff values in 2 domains, and 13 subjects in 1 of 3 ADI-R core domains, leaving 17 individuals with no autistic threshold scores in any of the domains. The autism threshold for the communication domain was most frequently exceeded, in 61% (31 of 51) of the cases, when assessing the domains separately. All 6 boys with KS who were diagnosed with a psychotic disorder (eg, psychotic disorder NOS, schizophrenia, schizoaffective disorder) also met the criteria for ASD.

All subjects with ASD displayed noticeable behavioral impairment before the age of 5 years. Psychotic disorders did not manifest before the age of 12 years. The onset of the other classifications was not related to age and was evenly distributed in the sample (6–19 years).

In all subjects with an ASD classification, the parents had sought professional guidance for behavioral and/or social problems. The parents generally perceived individual LDs and ADHD as mild impairment, and only a minority had requested psychiatric consultation. However, the majority of subjects, 69% (35 of 51), were attending special education facilities at the time of assessme87nt.

Mean TIQ score was 80 (range: 59–121), mean performance IQ score was 86 (range: 61–125), and mean verbal IQ score was 78 (range: 55–112). Of the 25 individuals with TIQ

scores of <80, 72% (18 of 25) had at least one psychiatric classification according to the DSM-IV-TR, in comparison to 21 of the 26 participants (21 of 26 [81%]) with TIQ scores of >80. IQ measures did not correlate with specific types of psychiatric classifications (multivariate analysis of variance). **Table 2** shows the co-occurrence of the 3 most prevalent classifications found (ie, ADHD, LD, and ASD).

Table 2. *Clustering of Language Disorder, ADHD and ASD.*

	LD		No LD		Total
	+ ASD	*-ASD*	*+ ASD*	*- ASD*	
ADHD	7	11	5	9	32
No ADHD	2	13	0	4	32
Total	9	24	5	13	51

LD = Language disorder, ASD= Autism Spectrum disorder, ADHD= Attention Deficit/Hyperactivity Disorder (subtypes combined to one group)

Twelve of the 14 boys with an ASD also met the criteria for ADHD, and 9 of 14 boys with ASD also suffered from a LD. Furthermore, 7 boys met the criteria for all 3 disorders (ASD, ADHD, and LD). Twenty-two individuals were comorbid for LD and ADHD without a diagnosis of ASD. The 4 subjects with a mosaic karyotype also met the criteria for ≥1 psychiatric classification, including ASD and depressive disorder.

DISCUSSION

Several psychiatric classifications could be applied in this sample of 51 boys with KS. In concordance with the literature, a high prevalence of LDs (65%) was found. Further prevalent psychiatric classifications were ADHD (63%) and ASD (27%), with markedly higher prevalences than described for the normal population. Intelligence of the subjects did not affect the prevalence of classifications within the sample, with average IQ scores in the sample slightly lower than described in other studies.

The psychiatric outcome was not influenced by age except with psychotic disorders, which were exclusively observed in subjects aged 12 years or older. This is in accordance with the literature stating that psychoses at a younger age are extremely rare [28].

Classifications of ADHD, ASD, and LD seemed to cluster in our sample of boys with KS. Language disorders have been frequently associated with ASD without KS . An overlap of ADHD with ASD in samples of other children with psychiatric disorders has been described before [29,30].

All children and adolescents in the present study with a psychotic disorder NOS (n = 6) were comorbid for ASD. In general, earlier studies have described an overlap of autistic symptoms with early features of schizophrenia or psychotic disorder [31,32]. Specific association between ASD and psychotic risk in adult KS is supported by the findings of van Rijn et al [33,34].Their cohort of 32 adult men with KS showed high scores on the Autism Spectrum Questionnaire [35],an instrument that measures autistic traits, as well as elevated

scores on the Schizotypical Personality Questionnaire [36], an indicator of genetic vulnerability to schizophrenia. In addition, van Rijn et al described high levels of social cognitive deficits and subsequent social behavioral dysfunction in the same KS cohort [10,34]. They suggested that the social cognitive deficits could be associated with high risk for autism and schizophrenia in KS. The concurrence of ASD and KS has incidentally been described before in 7 case studies [37-41]. Schizophrenia in adults with KS has been reviewed and studied by DeLisi et al [42,43]. The prevalence of KS with schizophrenia in their review of cytogenetic screenings in adult psychiatric cohorts (numbers ranging from 60–6000) was ~4 to 6 times higher compared with the general population rate. However, Mors et al did not find an association of KS with schizophrenia [44]. Future studies should provide insight into the structure and development (eg, longitudinal studies) of psychiatric problems in children with KS.

Limitations of the present study should be taken into account. The sample consists of self-selected children who were already diagnosed with KS, and the findings cannot be generalized to all children and adolescents with KS. In addition, it must be understood that the classifications in our research sample were primarily derived by the use of standard instruments. The classifications found do not naturally reflect requested help by the parents, notably in the cases of individual LD and ADHD. The advertisements for the current study aimed to invite participants regardless of any problems or illness present. However, it is likely that parents who experienced problems in the upbringing of their children were more willing to participate in the study. The knowledge of parents and teachers of the child's KS karyotype might influence the environmental expectations and attitudes toward these children, possibly resulting in a biased observation from our part. In this sample, however, no clear differences in prevalence of psychiatric problems were found between the group diagnosed with KS before birth and the groups diagnosed later in life (results not shown).

Although structured procedures and quantification of symptom scores were used, it would have been preferable if the child psychiatrists had been blind to the genetic make-up of the child. This is practically difficult given obvious somatic features in some cases of KS.

With regard to the specific diagnosis of an ASD, the administration of a standardized observation in addition to the structured interview method of the ADI-R, such as the procedure according to the Autism Diagnostic Observation Schedule [45], would have strengthened the assessment procedures.

The present study reveals that normal functioning in children and adolescents with KS might be challenged because of the risk of serious developmental psychopathology. Impairments in the areas of communication, socialization, and disorganized behaviors may be present in boys with KS. This makes them liable to isolation and emotional problems that may require guidance and treatment. Clinicians should be aware of the possibility of KS in a child with psychiatric problems. Specific behavioral problems might trigger suspicion of KS. A language-based learning disability with physical features of KS should

justify karyotyping in a child given the high prevalence of the syndrome, especially if social problems and difficulties with behavior regulation are present. Pediatricians should have a low threshold for obtaining a karyotype in children with autism and/or ADHD, particularly in boys who are tall.

It must be emphasized that only 10% to 25% of the expected diagnoses of KS are made before puberty [3,4]. It is as yet unknown whether the large contingent of subjects with undiagnosed KS is largely unaffected by psychiatric disorders. On the basis of the findings in the present self-selected sample, we recommend that all children with KS should be examined by a child psychiatrist, which may prevent delay of treatment and may possibly protect these children from adverse environmental influences. Screening for behavioral and cognitive/learning problems in all children with KS is recommended, preferably before the age of 10 years. Early detection could improve the prognosis of psychiatric problems, some of which may be alleviated by medication or other timely interventions.

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CHAPTER 3

DISSECTING THE CLINICAL HETEROGENEITY OF AUTISM SPECTRUM DISORDERS THROUGH DEFINED GENOTYPES

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ABSTRACT

The etiology of autism spectrum disorders (ASD) is largely determined by different genetic factors of variable impact. This genetic heterogeneity could be a factor to explain the clinical heterogeneity of autism spectrum disorders. Here, a first attempt is made to assess whether genetically more homogeneous ASD groups are associated with decreased phenotypic heterogeneity with respect to their autistic symptom profile.

The autistic phenotypes of ASD subjects with 22q11 deletion syndrome (22q11DS) and ASD subjects with Klinefelter Syndrome (KS) were statistically compared to the symptom profile of a large (genetically) heterogeneous ASD sample. Autism diagnostic interview-revised (ADI-R) variables were entered in different statistical analyses to assess differences in symptom homogeneity and the feasibility of discrimination of group-specific ASD-symptom profiles.

The results showed substantially higher symptom homogeneity in both the genetic disorder ASD groups in comparison to the heterogeneous ASD sample. In addition, a robust discrimination between 22q11-ASD and KS-ASD and idiopathic ASD phenotypes was feasible on the basis of a reduced number of autistic scales and symptoms. The lack of overlap in discriminating subscales and symptoms between KS-ASD and 22q11DS-ASD suggests that their autistic symptom profiles cluster around different points in the total diagnostic space of profiles present in the general ASD population.

The findings of the current study indicate that the clinical heterogeneity of ASDs may be reduced when subgroups based on a specific genotype are extracted from the idiopathic ASD population. The current strategy involving the widely used ADI-R offers a relatively straightforward possibility for assessing genotype-phenotype ASD relationships. Reverse phenotype strategies are becoming more feasible, given the accumulating evidence for the existence of genetic variants of large effect in a substantial proportion of the ASD population.

INTRODUCTION

Autism spectrum disorders (ASDs) delineate a group of behaviorally-defined disorders including autism, PDD-NOS, and Asperger syndrome.

Many efforts are being made to address the clinical heterogeneity of ASDs. At the same time, the diversity of genetic findings in the past decade indicate that ASDs should also be considered genetically heterogeneous [1,2]. This raises the question to what extent the clinical heterogeneity can be explained by the underlying genetic heterogeneity of ASDs. In this study we will address this issue through the assessment of the homogeneity of the ASD phenotype in genetically more homogenous samples.

In addition to the growing number of genetic ASD susceptibility loci with small effect sizes, recent studies have described new "causative" genetic variants in ASDs that are assumed to have a large impact on ASDs [3,4]. They are thought account for about 10–20% of ASD cases [1,4,5]. These risk variants are likely to show incomplete penetrance and imperfect segregation with disease as most variants have also been observed in non-autistic controls [5,6]. Furthermore, several ASD variants have been shown to cause brain disorders other than ASD, including schizophrenia, mental retardation and epilepsy [1,6]. This combination of incomplete penetrance and pleiotropic phenotypes could indicate that these loci cause a global disruption in brain development, making it more vulnerable to develop a range of different brain disorders. Efforts are required to distinguish distinct aspects of those brain disorders that are caused by these genetic variants with large effect, versus aspects that result from various other (environmental and/or genetic) hits.

A logical starting point would be to assess whether at all, ASD cases ascertained for a particular genetic variant display distinct autistic characteristics. This model can be considered probable when cases carrying the same genetic variant are found to share particular (combinations of) symptoms in higher frequencies than most cases in the idiopathic ASD population. The probability of genetic ASD subphenotypes can be illustrated by Rett syndrome (RTT). A more homogeneous profile of autistic symptoms together with non-autistic symptoms has led to the description of the RTT genetic subphenotype that is formally classified in the DSM-IV-TR as an ASD subtype. RTT is a progressive neurodevelopmental disorder that manifests in girls during early childhood [7,8]. Mutations in *MECP2* gene are found in more than 95% of classic RTT cases [9,10]. Patients with RTT appear to develop normally up to 6–18 months of age. Deceleration of head growth is often the presenting symptom. This is often accompanied by general growth retardation, weight loss, and a weak posture and ataxia [7]. Social withdrawal and loss of language become apparent at early age. Most patients progressively develop stereotypic hand wringing or washing movements. Other frequent autistic features include expressionless face, hypersensitivity to sound, lack of eye-to-eye contact and unresponsiveness to social cues [8]. This illustrates that both specific autistic and non-autistic

features characterize RTT. Importantly, the RTT autistic features are also present among the general population of autistic individuals though probably in a much lower frequency.
The modest recurrence of most identified large risk variants so far precludes the inclusion of adequate carrier numbers to evaluate the specificity of the autistic subphenotype per variant. Each of these variants on its own represents only a small proportion (at most 1–2%) of the ASD population [4,11]. Genetic disorders such as RTT that are frequently associated with ASD have associated features such as congenital malformations or somatic disorders that enhance the chance of clinical detection. Therefore, a focus on ASD subjects ascertained for particular a well defined genetic disorder enables the inclusion of larger numbers. Importantly, similar to the newly discovered genetic variants of large effects, most genetic disorders are associated with ASD only in a fraction of affected subjects, thus the defining variants in these disorders also display incomplete penetrance. This warrants a focus on subsets of individuals with a particular genetic disorder that are diagnosed with ASD which could possibly precipitate the impact of a particular variant on autistic symptomatology [1].
As a proof of concept we studied the ASD phenotype of ASD subjects with 22q11 deletion syndrome (22q11DS) and Klinefelter syndrome (KS, 47 XXY). 22q11DS and KS subjects *without* an ASD classification were excluded. 22q11DS and KS are relatively frequent disorders affecting 1–2,000–4,000 and 1–700 respectively [12-14]. Both are clinically defined genetic disorders like RTT and increased rates of ASD have been described in both 22q11DS and KS subjects [15-17]. The presence of both disorders has also previously been described among populations of subjects with ASD [18-20].
The structure of the ASD phenotype associated with 22q11DS and KS was compared to a large a large genetically heterogeneous sample of ASD subjects in different statistical analyses involving standard autistic measurements. The analyses aimed to assess differences in symptom homogeneity and the feasibility of differentiation of group-specific ASD-symptom profiles.

METHOD

PATIENTS The Dutch Central Committee on Research Involving Human Subjects had approved the research protocol. Patient associations and centers for clinical genetics, and pediatrics were involved in recruiting the children for the original psychiatric surveys out of which the subjects for the present study were selected (see below). A newsletter presented on the web or in writing, had informed parents and children of the aim and methods of the study. Parents and children of had to apply actively for participation in the study by contacting the research team. Subsequently they were sent written information about the selection criteria and the implications of participation in the study. They were invited for assessment if they met the inclusion criteria. Written informed consent was obtained from

participants (if older than 12 years of age) and their parents or guardians according to the declaration of Helsinki.

22q11DS or KS subjects were selected for this study if they had been diagnosed with an ASD via previous surveys on general psychopathology on children with 22q11DS (n = 90) and Klinefelter Syndrome (KS) sample (n = 51), all 47, XXY none higher aneuploidies, no mosaics). (See [16,17] for more extensive details on recruitment and further characteristics of the patient samples). This survey had resulted in an ASD classification in 14 of the 51 KS boys (14/51 = 27%) and 39 of the 90 22q11DS children (39/90 = 43%). The classification of an autism spectrum disorder (ASD) was made on the basis of DSM-IV-TR standardized interviews and the Autism Diagnostic Interview (ADI-R) [21]. Videotapes of all subjects and the DSM-IV-TR/ADI-R outcomes were discussed in a consensus meeting headed by the head of the department. The consensus meeting served to control for procedural mistakes and to verify whether the classifications through the DSM-IV-TR and ADI-R interviews were in agreement with the clinical judgment. All 22q11DS and KS subjects with an ASD met ADI-R thresholds and DSM-IV-TR criteria.

A genetically heterogeneous ASD sample was recruited as part of a genetic study of autism and from a clinical sample of patients referred to the department of child and adolescent psychiatry for diagnostic reasons. Thus, these subjects were unascertained for their genotype and should therefore represent a reference sample of maximal genetic heterogeneity. Inclusion criteria were: age four years or older, no severe medical or neurological illness, IQ>40. The final sample consisted of 372 verbal subjects. Study participants ranged in age from 4 to 20 years. Similar to the ASD cases obtained from the 22q11 and XXYDS cohorts, all subjects out of the heterogeneous ASD sample had been evaluated in consensus meetings to confirm ASD diagnosis through the interviews and all subjects met ADI-R thresholds.

IQ had been assessed by means of the Dutch versions of the Wechsler scales (WPSSI WISC III and WAIS) [22-24] in the KS and 22q11DS sample and in a significant part of the ASD heterogeneous group (65%). IQ scores of the heterogeneous ASD sample that could not be assessed with the Wechsler scales have been assessed with the RAVEN Progressive Matrices [25] the Mullen Scales of Early Learning [26] or the Snijders-Oomen non-verbal intelligence test-Revised [27]. No difference in intelligence level between those with and those without ASDs had been found in both the 22q11DS and KS samples in the original psychiatric surveys (see [16,17]).

PROCEDURE AND MEASURES ADI-R subscale and symptom variables were entered in the statistical analyses for phenotype comparisons. The ADI–R is an established 'gold standard' in diagnostic/phenotypic evaluations of autism. It is an extensive clinical interview administered to the parents. The interview focuses on the three core or so called "content" domains of autism (i.e. qualitative abnormalities in social interaction (S),

qualitative abnormalities in communication (C) and stereotyped and repetitive behaviors (R) [21]. ADI-R items are coded for these domains and also for an "age of onset" domain. A classification of an autism spectrum disorder is applied when scores in all domains are met or when scores are met in two core domains and meet criteria on the "age of onset" domain, but are one point away from meeting autism criteria in the remaining core domain. Reliability of the ADI-R in a population with mild to moderate mental retardation has been established [28]. The ADI-R may also be used to assess profiles of autistic symptomatology [29,30]. The ADI–R algorithm is composed of 37 symptom "items". These items were originally selected as a minimum for optimal ASD classification [21]. ADI-R labels consist of 2 to 5 items and are directly related to the DSM-IV-TR criteria of an Autistic Disorder. As a result, 12 ADI-R labels are used. Each ADI-R domain consists of 4 labels, eg S1-4, C1-4 and R1-4. Items are coded as 0 (ASD behavioral symptom specified not present), 1 (specified behavior not sufficient to code "2") or 2 (specified ASD symptom present). Maximum label scores thus range from 4–10. An overview of the description of the ADI-R items, labels and the ADI-R domains of the algorithm is provided in **Tables S1** and **S2**.

STATISTICAL ANALYSES Symptom homogeneity was operationalized as the (inverse of the) mean number of ADI-R algorithm items within each ASD group on which the subjects scored clearly in the autistic range, i.e. ADI-R item score = 2. Thus, per subject, the number of items with score = 2 were counted. A lower number of ADI-R items that reached the autism criterion can be considered indicative of a relative reduction in symptom heterogeneity. Differences between groups in number of ADI-R items on which the autism criterion was reached were analyzed by means of a univariate analysis of variance, with post hoc Bonferroni multiple comparisons.

Discriminant analyses (DA) were performed to determine whether the genetic disorder ASD subsamples could be differentiated from the heterogeneous ASD sample on the basis of ADI-R variables. The analyses addressed the question to what extent ADI-R label and/or ADI-R symptom item profiles could successfully discriminate 22q11DS or KS ASD profiles from the heterogeneous ASD sample profile (i.e., 22q11DS+ASD vs. heterogeneous ASD, and KS+ASD vs. heterogeneous ASD). In addition, three group DAs were performed to explore the separation of the three groups by means of 2 discriminant functions.

For all discriminant analyses, stepwise Wilks' lambda was used, with probability of F for entry and removal of variables set at 0.5 and 0.10 respectively. For classification the within-group covariance matrices were used and prior probability was set to equal for all groups.

RESULTS

We compared the autism symptom profile of 39 subjects with 22q11DS and ASD and 14 subjects with KS and ASD to a large genetically heterogeneous sample of 372 ASD

subjects (further referred to as the heterogeneous ASD sample). Autism diagnostic interview-revised (ADI-R) algorithm variable scores were entered in the analyses to evaluate differences in symptom homogeneity and the feasibility of ASD genetic subphenotype discrimination. **Table 1** shows the basic characteristics of the 22q11DS-ASD, KS-ASD and heterogeneous ASD samples. Correlations in the heterogeneous ASD sample between IQ scores and the different ADI-R outcome scores were virtually negligible ($-0.10<r<0.10$). Therefore IQ was considered irrelevant to the outcome of the statistical comparisons with the genetic disorder ASD samples.

Table 1: *Sample characteristics.*

		Gender			
Sample	**N**	**F**	**M**	**Age** ± SD	**TIQ** ± SD
22q11DS-ASD	39	18	21	13.2 ± 2.6	66.1 ± 13.3
KS-ASD	14	0	14	13.7 ± 3.0	81.5 ± 13.0
Heterogeneous ASD	372	56	316	10.6 ± 3.7	98.7 ± 18.7

KS-ASD = Klinefelter syndrome with Autism Spectrum Disorder, 22q11DS-ASD = 22q11 deletion syndrome with Autism Spectrum Disorder. F= Female, M=Male, TIQ= average total IQ score.

SYMPTOM HOMOGENEITY Symptom homogeneity was operationalized as the (inverse of the) mean number of ADI-R algorithm symptom (called "items" in the ADI-R, see **Tables S1** and **S2**) scores within each ASD group on which the subjects scored clearly in the autistic range, i.e. ADI-R item score = 2. The mean number of ADI-R items on which the subjects with ASD reached the autism criterion scores differed significantly between the genetic disorder (22q11DS-ASD and KS-ASD) ASD samples and the heterogeneous ASD sample [$F(2,423) = 24.13$, $P<.0001$, $\eta_p^2 = .102$] which indicates increased symptom homogeneity in both the genetic disorder ASD samples. Post-hoc multiple comparisons with Bonferroni correction, showed that the average number of ADI-R items that reached the ADI-R autism criterion score (eg, score = 2) in the 22q11DS (10.28 items, SD = 4.46) and the KS (11.07 items, SD = 5.44) subjects with ASD was much lower ($P<.00001$, resp. $P<.002$) than in the heterogeneous ASD sample (16.59 items, SD = 6.14) (see **Figure 1**).

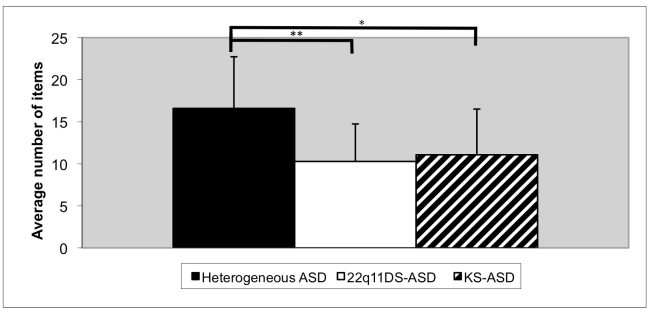

Figure 1. *Mean number of ADI-R items reaching autistic criterion (ADI-R score = 2). * P<0.002, ** P<0.0001, univariate analysis of variance.*

Discriminant analyses (DA) were performed to determine whether the genetic disorder ASD subsamples (22q11DS and KS with ASD) could be differentiated from the heterogeneous ASD sample on the basis of ADI-R label scores and ADI-R item scores, respectively. The success of the predictive ability of extracted ADI-R variables in the DAs is reflected in classification matrices that show the number and percentage of correctly and incorrectly identified subjects.

22Q11DS-ASD VERSUS HETEROGENEOUS ASD Discriminant analysis (DA) involving ADI-R domain subscale (called "labels" in the ADI-R, see Table S1) scores extracted labels S3, C1, R3, and R4 which resulted in a correct classification of 80% of the 22q11DS subjects and 78% of the heterogeneous ASD subjects. Box's M test for equal population covariance matrices was not significant ($P = 0.35$). Wilks' lambda = 0.83, χ^2 (5) = 74.5, $P<.0001$. In the DA of the autism diagnostic interview-revised (ADI-R) algorithm items, 12 items were extracted. This resulted in a correct classification of 95% of the 22q11DS subjects and 93% of the heterogeneous ASD subjects (**Table 2**). Box's M test for equal population covariance matrices was not significant (P = 0.08). Wilks' lambda = 0.58, χ^2 (12) = 217.7, $P<.0001$.

Table 2. *Classification matrix of the discriminant analyses involving ADI-R labels and items respectively.*

Sample		No of labels extracted	Correctly predicted % (n)	No of items extracted	Correctly predicted % (n)
22q11DS-ASD vs HeterogeneousASD	n = 39	4	80 % (31)	12	95 % (37)
	n =372		78 % (290)		93 % (346)
KS-ASD vs HeterogeneousASD	n = 14	1	86 % (12)	3	71 % (10)
	n = 372		65 % (242)		80 % (298)

KS-ASD VERSUS HETEROGENEOUS ASD In the DA of KS-ASD versus heterogeneous ASD involving ADI-R labels, only label A2 was extracted which resulted in a correct classification of 86% of the KS subjects and 65% of the heterogeneous ASD subjects. Box's M test for equal population covariance matrices was not significant ($P = 0.91$). Wilks' lambda = 0.97, χ^2 (1) = 10.4, $P = .001$. In the DA involving the autism ADI-R items, only 3 out of 37 items were extracted. This resulted in a correct classification of 71% of the KS subjects and 80% of the heterogeneous subjects with ASD (**Table 2**). Box's M test was not significant ($P = 0.63$). Wilks' lambda = 0.91, χ^2 (3) = 34.50, $P<.001$. **Table 3** and **Table 4** state the description of the extracted labels and items respectively with the discriminant coefficients for the DA of KS-ASD versus heterogeneous ASD.

Table 3. *Description of extracted ADI-R labels with discriminant function coefficients for the discriminant analyses of 22q11DS-ASD and KS-ASD versus heterogeneous ASD.*

Sample	Label	Description	Function
22q11DS-ASD	S3	Lack of shared enjoyment	.417
	C1	Lack of, or delay in, spoken language and failure to compensate through gesture	.669
	R3	Stereotyped and repetitive motor mannerisms	.274
	R4	Preoccupations with part of objects or non-functional elements of material	.315
KS-ASD	S2	Relative failure to initiate or sustain conversational interchange	1.000

Domain S = Qualitative Abnormalities in Reciprocal Social Interaction, Domain C = Qualitative Abnormalities in Communication, Domain R = Restricted, Repetitive, and Stereotyped Patterns of Behavior.

Table 4. *Description of extracted ADI-R items with discriminant function coefficients for the discriminant analyses of 22q11DS-ASD and KS-ASD versus heterogeneous ASD.*

Sample	Item	Item description	Domain	Function
22q11DS-ASD	38	Neologisms/Idiosyncratic Language	C	.215
	43	Nodding	C	.270
	45	Conventional/Instrumental Gestures	C	.229
	49	Imaginative Play With Peers	S	.192
	50	Direct Gaze	S	.230
	51	Social Smiling	S	.214
	52	Showing and Directing Attention	S	.313
	57	Range of Facial Expressions Used to Communicate	S	-.609
	58	Inappropriate Facial Expressions	S	.221
	67	Unusual Preoccupations	R	-.648
	68	Circumscribed Interests	R	.352
KS-ASD	34	Social Verbalization/Chat	C	.215
	53	Offering to Share	S	.270
	62	Interest in Children	S	.229

Domain S = Qualitative Abnormalities in Reciprocal Social Interaction, Domain C = Qualitative Abnormalities in Communication, Domain R = Restricted, Repetitive, and Stereotyped Patterns of Behavior.

A three-group DA (ie 22q11-ASD vs. KS-ASD vs. heterogeneous ASD) involving ADI-R items resulted in a correct classification of 92.3% of the 22q11DS-ASD subjects, 78.6% of the KS-ASD subjects, and 76.4% of the heterogeneous ASD subjects (see **Table S3**) on the basis of 12 extracted items. Box's M test was not significant ($P = 0.25$). Wilks' lambda for function 1 = 0.55, χ^2 (24) = 250.86, $P<.0001$, and for function 2 Wilks' lambda = 0.90, χ^2 (11) = 42.04, $P<.0001$. The 3-group percentages were similar to the results of the individual 22q11DS-ASD and KS-ASD to heterogeneous ASD ADI-R item comparisons. **Figure 2** is the plot of the individual discriminant coefficients of the 3-group discriminant analysis. It illustrates that 22q11DS-ASD is predominantly discriminated from heterogeneous ASD by function 2, KS-ASD from heterogeneous ASD by function 1.

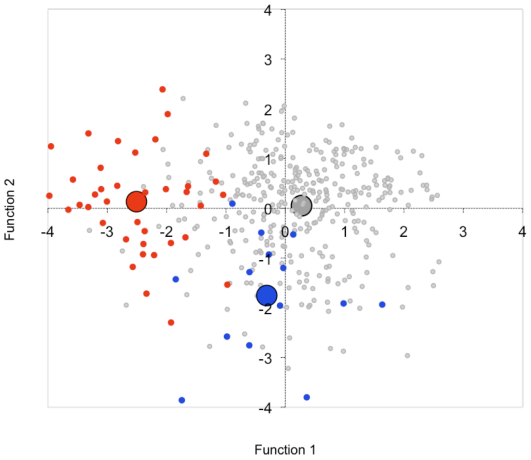

Figure 2. *Plot of individual subject canonical function coefficients of the 3-group discriminant analysis for heterogeneous ASD (grey dots) versus 22q11DS-ASD (red dots) versus KS-ASD (blue dots), the larger dots represent the group centroids. 22q11DS-ASD is predominantly discriminated from heterogeneous ASD by function 1, KS-ASD from heterogeneous ASD by function 2.*

Table S4 states the description of the extracted items with the discriminant coefficients for the 3-group DA. No additional items were extracted for the three-group comparison than had been extracted in both separate 22q11DS-ASD and KS-ASD versus heterogeneous ASD comparisons. In addition, 4 items that were extracted in the 2-group comparisons were not extracted for the 3-group comparisons (items 38, 49 and 51 out of the 22q11DS-ASD versus Heterogeneous ASD sample and item 62 out of the KS-ASD versus heterogeneous ASD comparisons). **Table S5** provides an overview of all the extracted ADI-R in the different DAs. **Table S6** contains a verifying calculation regarding the stability of the DA results.

DISCUSSION

Our results indicate that the clinical heterogeneity of ASDs might be reduced when subgroups based on a specific genotype are extracted from the overall genetically heterogeneous ASD population. A substantially lower autistic symptom variance was shown in both the 22q11DS-ASD and KS-ASD samples in comparison to the heterogeneous ASD sample. Further analysis revealed that the symptom profiles of the two studied genetic ASD disorders could be robustly discriminated from the heterogeneous ASD profiles through a limited number of autistic subscales and symptoms. In our opinion, these results support the notion of the existence of genetic subphenotypes within the ASD population. The lack of overlap in the discriminating ADI-R labels and items between KS-ASD and 22q11DS-ASD could indicate that both syndromes represent ASD profiles that cluster around different specific points in the total ASD diagnostic space, which was also suggested by the results of the three-group analysis. It should be emphasized that genetic disorder ASD subphenotypes are expected to overlap with profiles present among heterogeneous samples, as all are diagnosed according to the same DSM-IV-TR/ADI-R criteria. The premise of an overlap of ASD subphenotypes between carriers and non-

carriers of large impact variants raises interesting questions. It could be speculated that autistic symptom profiles out of the heterogeneous ASD population that overlap with symptom profiles related to specific genotypes can possibly point to convergent etiologies.

Several limitations should be addressed. The data were gathered from different studies, however, all studies were performed by the authors of this paper, and the diagnostic instruments were identical between the studies. The 22q11DS-ASD and KS-ASD subjects were not selected form an original ASD sample, but derived from psychiatric surveys among children with 22q11DS and KS samples. It would have been preferable if all subjects had been recruited in the same way. However, this would require unfeasibly large ASD samples to extract a sufficient number of 22q11-DS and KS-ASD cases. Although the ASD subjects with 22q11DS and KS and the heterogeneous ASD subjects fulfilled the same DSM-IV-TR clinical and ADI-R criteria, the average clinical threshold for suspecting ASD could have been different in the heterogeneous sample. Therefore, ascertainment bias cannot be ruled out. However, we did not aim to validate the association of 22q11DS and KS with ASD but rather investigated whether specific genotypes can confer specific autistic symptom profiles.

We do realize that reliability for the ADI-R is typically at the level of overall diagnosis, or subdomain area and that the ADI-R was not originally designed as a dimensional measure. Nonetheless we found the largest contrast between the different groups at the level of individual symptom items, while the DA involving ADI-R label subscales also delivered better results than expected by chance.

The sample sizes in this study preclude conclusions towards the nature of the discrimination symptoms. Similar assessments in other larger ASD cohorts are required to prove whether the current approach is feasible for "reversed phenotype" efforts. Additional measures such as the Social Responsiveness Scale or the Autism Diagnostic Observation Schedule (ADOS) can possibly aid to enhance specificity of phenotype descriptions [31,32].

The current findings could suggest that the KS and 22q11DS genotypes do not seem to merely augment heterogenic and complex genetic susceptibility, i.e. lower the ASD threshold in an aspecific way. Rather, our results could suggest that the strong influence of a specific genetic variant leads to an ASD subphenotype that is relatively specific with an increased within-group symptom homogeneity in comparison to the heterogeneous ASD population. Based upon these observations we hypothesize that the increased symptom homogeneity is mainly driven by the effect of one (or limited) genetic pathway(s). In contrast, the phenotype observed in the general ASD population is most likely mediated by the interplay of various combinations of all culprit causative genetic pathways, and therefore associated with larger ASD symptom heterogeneity. This consideration was emphasized in recent overview of advancements in genetic studies of complex traits: "For a substantial number of common diseases the newly identified pathways suggest that molecular subphenotypes may exist; that is, although a number of different pathways might

potentially be involved in the development of a particular disease when all cases are considered, in any individual with the disease only one or a subset of these pathways might be involved" [33]. Other ASD subphenotypes related to newly identified genetic variants (e.g. 1q21 duplication or deletion, the 22q13.3 deletion and the duplication of the 15q11–13 region) may be identified when properly studied. This could ultimately lead to a dissection of the ASD phenotype into a proportion of "genetic subtypes", and a remaining group of ASD patients in whom the ASD phenotype is the resultant of a more complex interaction between common genetic variants and environmental factors.

In conclusion, the current findings support the possibility that reduced genetic heterogeneity can be associated with reduced ASD symptom heterogeneity. The method of the current study using symptom variance and discrimination analysis involving the widely used ADI-R offers a relatively straightforward possibility for assessing genotype-ASD relationships. The assessments aim to initiate further reverse phenotype strategies, especially given the accumulating evidence for the existence of genetic variants of large effect in a substantial proportion of the ASD population.

Table S1. *ADI-R algorithm items sorted by labels and domains.*

	S: Qualitative Abnormalities in Reciprocal Social Interaction
	S1: Failure to use nonverbal behaviors to regulate social interaction
50	Direct Gaze
51	Social Smiling
57	Range of Facial Expressions Used to Communicate
	S2: Failure to develop peer relationships
49	Imaginative Play With Peers
62	Interest in Children
63	Response to Approaches of Other Children
64	Group Play with Peers (age < 10.0)
65	Friendships (age $\geq$ 10.0)
	S3: Lack of shared enjoyment
52	Showing and Directing Attention
53	Offering to Share
54	Seeking to Share Enjoyment With Others
	S4: Lack of socioemotional reciprocity
31	Use of Other's Body to Communicate
55	Offering Comfort
56	Quality of Social Overtures
58	Inappropriate Facial Expressions
59	Appropriateness of Social Responses
	C: Qualitative Abnormalities in Communication
	C1: Lack of, or delay in, spoken language and failure to compensate through gesture
42	Pointing to Express Interest
43	Nodding
44	Head Shaking
45	Conventional/Instrumental Gestures
	C4: Lack of varied spontaneous make-believe or social imitative play
47	Spontaneous Imitation of Actions
48	Imaginative Play
61	Imitative Social Play
	C2(V): Relative failure to initiate or sustain conversational interchange
34	Social Verbalization/Chat
35	Reciprocal Conversation
	C3(V): Stereotyped, repetitive or idiosyncratic speech
33	Stereotyped Utterances and Delayed Echolalia
36	Inappropriate Questions or Statements
37	Pronominal Reversal
38	Neologisms/Idiosyncratic Language
	R: Restricted, Repetitive, and Stereotyped Patterns of Behavior
	R1: Encompassing preoccupation or circumscribed pattern of interest
67	Unusual Preoccupations
68	Circumscribed Interests
	R2: Apparently compulsive adherence to nonfunctional routines or rituals
39	Verbal Rituals
70	Compulsions/Rituals
	R3: Stereotyped and repetitive motor mannerisms
77	Hand and Finger Mannerisms (score highest of 77/78)
78	Other Complex Mannerisms or Stereotyped Body Movements
	R4: Preoccupations with part of objects or non-functional elements of material
69	Repetitive Use of Objects or Interest in Parts of Objects
71	Unusual Sensory Interests (score highest of 69/71)

Table S2. *ADI-R algorithm items sorted by number.*

Item no	Item description	Domain label
31	Use of Other's Body to Communicate	S4
33	Stereotyped Utterances and Delayed Echolalia	R3
34	Social Verbalization/Chat	B2
35	Reciprocal Conversation	B2
36	Inappropriate Questions or Statements	B3
37	Pronominal Reversal	B3
38	Neologisms/Idiosyncratic Language	B3
39	Verbal Rituals	R2
42	Pointing to Express Interest	B1
43	Nodding	B1
44	Head Shaking	B1
45	Conventional/Instrumental Gestures	B1
47	Spontaneous Imitation of Actions	B4
48	Imaginative Play	B4
49	Imaginative Play With Peers	S2
50	Direct Gaze	S1
51	Social Smiling	S1
52	Showing and Directing Attention	S3
53	Offering to Share	S3
54	Seeking to Share Enjoyment With Others	S3
55	Offering Comfort	S4
56	Quality of Social Overtures	S4
57	Range of Facial Expressions Used to Communicate	S1
58	Inappropriate Facial Expressions	S4
59	Appropriateness of Social Responses	S4
61	Imitative Social Play	B4
62	Interest in Children	S2
63	Response to Approaches of Other Children	S2
64	Group Play with Peers (age < 10.0)	S2
65	Friendships (age > 10.0)	S2
67	Unusual Preoccupations	R1
68	Circumscribed Interests	R1
69	Repetitive Use of Objects or Interest in Parts of Objects	R4
70	Compulsions/Rituals	R2
71	Unusual Sensory Interests (score highest of 69/71)	R4
77	Hand and Finger Mannerisms (score highest of 77/78)	R3
78	Other Complex Mannerisms or Stereotyped Body Movements	R3

Table S3. *Classification matrix of the 3-group discriminant analysis of heterogeneous ASD versus KS-ASD versus 22q11DS-ASD.*

	Correctly predicted % (n)	**Incorrectly predicted % (n)**		
Diagnostic groups		**Heterogeneous**	**22q11DS**	**KS**
Heterogeneous ASD	76.4 % (285)		6.4 % (24)	17.2 % (64)
22q11DS-ASD	92.3 % (36)	2.6 % (1)		5.1 % (2)
KS-ASD	78.6 % (11)	21.4 % (3)		0.0 % (0)

Table S4. *Description and discriminant function coefficients of ADI-R items extracted in the 3-group discriminant analysis of heterogeneous ASD versus KS-ASD versus 22q11DS-ASD.*

			Function	
Item	**Item description**	**Domain**	1	2
34	Social Verbalization/Chat	C	.130	.383
43	Nodding	C	.260	-.404
45	Conventional/Instrumental Gestures	C	.207	.233
50	Direct Gaze	S	.269	-.131
52	Showing and Directing Attention	S	.274	-.410
53	Offering to Share	S	.092	.677
57	Range of Facial Expressions Used to Communicate	S	-.561	.071
58	Inappropriate Facial Expressions	S	.266	.033
62	Interest in Children	S	.141	.417
67	Unusual Preoccupations	R	-.636	.154
68	Circumscribed Interests	R	.387	.113
6971	Repetitive Use of Objects or Interest in Parts of Objects, or Unusual Sensory Interests	R	.340	-.079

Table S5. *Overview of extracted ADI-R items with subsequent labels in the different discriminant analyses. DA1= 22q11DS-ASD versus heterogeneous ASD. DA2= KS-ASD versus heterogeneous ASD. DA3= 3-group comparison of 22q11DS-ASD versus KS-ASD versus heterogeneous ASD*

Item no	**Domain**	**Item description**	**DA1**	**DA2**	**DA3**
34	C	Social Verbalization/Chat		x	x
38	C	Neologisms/Idiosyncratic Language	x		
43	C	Nodding	x		x
45	C	Conventional/Instrumental Gestures	x		x
49	S	Imaginative Play	x		
50	S	Direct Gaze	x		x
51	S	Social smiling	x		
52	S	Showing and Directing Attention	x		x
53	S	Offering to Share		x	x
57	S	Range of Facial Expressions Used to Communicate	x		x
58	S	Inappropriate Facial Expressions	x		x
62	S	Interest in Children		x	x
67	R	Unusual Preoccupations	x		x
68	R	Circumscribed Interests	x		x
6971	R	Repetitive Use of Objects or Interest in Parts of Objects / Unusual Sensory Interests	x		x

Table S6. *Results for stability calculations of DAs. ADI-R items extracted in the 4 additional DAs, sorted by correspondence/item number. To verify whether the stepwise analysis of the three groups DA did provide stable solution, the stepwise DA to separate the three groups has been repeated another four times, with half of the sample of subjects with heterogeneous ASD. Thereto the sample was divided in four quarters, called Q1 to Q4. The DA has been performed with inclusion of the heterogeneous subsamples Q1+Q2, Q3+Q4, Q1+Q3, and Q2+Q4. The 'solutions' are compared with each other and with the result of the DA presented in the paper that included the total sample of subjects with heterogeneous ASD. The comparison is focused on the number and type of items that are extracted. Table S6 shows that the solutions are highly similar. The number of items extracted varies between 10 and 15. There are 8 items that appear in each DA, there is 1 items that appears in 3 DAs, there are 7 items that appear in 2 DAs, there is 1 items appearing in only one DA. Comparing the four solutions with the results of the original DA presented in the paper shows that all of the 12 items extracted in this DA, all show up in one or more one of the other DAs. Eight items of the original DA show up in all other analyses, the other four items of the original DA appear in at least two of the other DAs.*

		Samples				
Item	**Item description**	**Q1+Q2**	**Q3+Q4**	**Q1+Q3**	**Q2+Q4**	**ALL**
43	Nodding	x	x	x	x	x
50	Direct Gaze	x	x	x	x	x
53	Offering to Share	x	x	x	x	x
57	Range of Facial Expressions Used to	x	x	x	x	x
58	Inappropriate Facial Expressions	x	x	x	x	x
67	Unusual Preoccupations	x	x	x	x	x
68	Circumscribed Interests	x	x	x	x	x
6971	Repetitive Use of Objects or Interest in Parts of Objects/ Unusual Sensory Interests	x	x	x	x	x
52	Showing and Directing Attention	x		x	x	x
45	Conventional/Instrumental Gestures	x			x	x
49	Imaginative Play With Peers	x		x		
51	Social Smiling		x	x		
62	Interest in Children		x	x		x
70	Compulsions/Rituals	x			x	
7778	Hand and Finger Mannerisms / Other Complex Mannerisms or Stereotyped Body Movements	x			x	
34	Social Verbalization/Chat	x			x	x
38	Neologisms/Idiosyncratic Language	x				

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CHAPTER 4

EXECUTIVE FUNCTION DEFICITS AND SOCIAL PROBLEMS IN KLINEFELTER SYNDROME (47, XXY)

Submitted

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ABSTRACT

Klinefelter syndrome (47,XXY) is associated with problems in social functioning and high risk for psychopathology. Studying neurocognitive functions that might be associated to social dysfunction in Klinefelter might therefore help to further unravel the mechanisms underlying social developmental problems and developmental psychopathology. In the present study the relation between social dysfunction and core domains of cognitive control: attention, inhibition and mental flexibility, is evaluated.

45 boys with Klinefelter syndrome (mean age 11, 7 yrs, range 6-18 yrs.) were assessed for attention, inhibition and mental flexibility by subtests of the Amsterdam Neuropsychological Tests (ANT) and compared to a population sample of age matched control boys. Social dysfunction was assessed by the Autism Diagnostic Interview-revised (ADI-R) in boys with Klinefelter syndrome to index the social problems on different domains of symptoms that are associated with autistic disorders. Boys with Klinefelter syndrome demonstrated less well developed attention regulation, inhibition and mental flexibility. Attention regulation abilities and inhibition appeared to be associated with the level of social communication problems and the inflexibility of behavior (rigidity and stereotypical behavior). Findings suggest that cognitive control dysfunctions in Klinefelter syndrome are associated with the risk for social developmental problems and autism spectrum symptomatology.

INTRODUCTION

Klinefelter syndrome (47,XXY) is the most frequent aneuploidy in males with 1:700 affected [1]. The neurobehavioral phenotype of KS is related to a mixture of hypoandrogenism and X chromosomal gene dosage effects [2]. These result in variable language-based learning problems, specific cognitive dysfunction, and social difficulties with generally only mild intellectual disability [3]. These problems seem accompanied by an increased vulnerability to a range of psychiatric disorders [4,5].

Naturally occurring genetic disorders that are associated with neurodevelopmental problems can provide further insight into neurocognitive mechanisms underlying behavioral and emotional problems. Various genetic disorders show specific neurocognitive deficits in attentional-executive skills, even when global intellectual abilities are judged to be within normal limits or when individuals do not present with sufficient features to fulfill criteria for a psychiatric diagnosis. It is important to identify such manifestations both for etiological research and for clinical management.

The phenotypic variability of KS offers an opportunity to study the relationship of different components of neurocognitive functioning and behavioral outcome.

Originally, clinical observations of hypoassertiveness, inattentivenes and problems in regulation of behavior have led to the impression that executive functioning (EF) is disturbed in KS [3,6]. Executive functions are essential in flexible adaptive functioning in complex situations like social situations, and in inhibition of irrelevant thoughts and actions. Executive dysfunctions therefore may lead to deregulation of thought, emotion and behavior, including inappropriate and inflexible social behavior. Based on a recent review of neurocognitive deficits in KS it is concluded that further studies are needed to define a more precise nature of EF dysfunctions in KS [3]. Empirical studies of EF in KS are still fairly sparse and have addressed different aspects of EF in different age groups [3]. Boone and colleagues showed deficits on verbal EF tasks that were associated with verbal intelligence level, while performance on non-verbal EF tasks was unaffected in adults [7]. In addition, Fales et al. found deficits in tasks of verbal working memory in 21 adult men with KS [8]. Kompus et al. (2011) concluded that inhibition might be selectively disturbed while attention might be intact, based on an evaluation of the performance of 27 men with KS [9]. Studies that investigated EF in samples of children and adolescents with KS described reduced speed of processing, impaired attention abilities, but intact conceptual thinking [6,10,11].

Only recently, studies have begun to systematically evaluate whether cognitive problems in KS coincide with psychopathology. Psychiatric surveys of boys with KS have shown high prevalence of ADHD, psychosis, and ASD [5,12]. High levels of autistic symptomatology were also found in subjects lacking a formal diagnosis of ASD [5]. Studies in adult men with KS have shown increased rates of affective and psychotic symptoms and disorders [4,13-15]. Van Rijn et al. reported high levels of distress during social interaction and high levels of autism traits in men with Klinefelter syndrome [16]. Most notably, Van Rijn et al. reported

high levels of schizotypal symptoms within a group of mostly adult men with KS to be associated with executive dysfunction, in particular disinhibition and mental inflexibility [15,17]. No earlier studies have assessed behavioral consequences of cognitive dysfunction in children with KS.

The aim of the present study is first to evaluate important domains of cognitive regulation in children and adolescents with KS by focusing on inhibition, mental flexibility, and attention regulation. Second we wanted to evaluate if these EF dysfunctions are associated with increased social maladaptation as is reflected in autistic symptom domains. Ultimately, executive dysfunction may serve as a marker for a compromised development of social skills in KS.

METHOD

SUBJECTS Forty-five boys with Klinefelter syndrome participated in the study (mean age 11, 7 yrs, range 6-18 yrs.). The present study was part of a large study on cognition and psychopathology in Klinefelter syndrome at the two cooperating departments of the University of Leiden and the University of Utrecht. Written information was available at several centers for clinical genetics and pediatrics as well as on the webpage of the Klinefelter patients association to invite parents and children to participate in the study. Parents and children had to apply actively for participation in the study by contacting the research team and were encouraged to participate regardless of the presence of any psychological or behavioral problems. Parents and children above the age of 12 gave their written informed consent. KS was confirmed by standard karyotyping, all boys had a 47, XXY karyotype, none were mosaics, seven boys were on androgen substitution treatment. For every boy with KS 2 age-matched normal control boys were included, that were recruited from regular schools, resulting in a control group of 90 boys.
The research project was approved by the ethical committee of the University Medical Centre Utrecht and was in accordance with the declaration of Helsinki.

PROCEDURE Assessment of EF in boys with KS was performed at the department of Child and Adolescent Psychiatry of the University Medical Centre of Utrecht. Normal controls performed the tests at their school. All children were tested during morning hours in a quit room to avoid distraction. At the same time, parents were interviewed. The tests were performed in a fixed order by experienced child neuropsychologists. Preceding each test, short practice trials were run to verify that the subjects understood the instructions and acted accordingly.

MEASURES Attention regulation and EF were measured by several subtasks of the Amsterdam Neuropsychological Tasks (ANT) program [18]. Several studies have demonstrated satisfactory psychometric properties of the ANT paradigms. The chosen

paradigms have successfully been used in subjects with impaired frontal functioning and attention problems [19].

ATTENTION REGULATION Attention regulation is measured by a task for baseline speed which has been used also by others as a measure of intensity (alertness) of attention [20] (**Figure 1**).

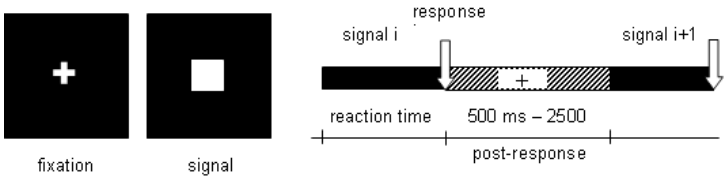

Figure 1. *Fixation stimulus and imperative signal, and timing between signals in the Baseline speed task.*

On the screen a (fixation) cross is continuously projected. This cross changes into a block unexpectedly. As soon as a block is present the button must be pressed. The cognitive level of this task is therefore limited to detection of the mere presence of the stimulus. This task consists of two parts: for the left and the right index finger (n = 32 trials, both parts). Outcome parameters are mean reaction time and within-subject standard deviation of the 32 reaction times (fluctuation), per part.

SUSTAINED ATTENTION Sustained attentional control is measured by a sustained attention task. 600 signals are presented in 50 series of 12 consecutive patterns that contain 3, 4 of 5 dots in a pseudo random sequence (**Figure 2**). The subject is asked to press the "yes" button only when a 4-dot pattern (target signal) appears. The "no" button must be pressed when the 3- and 5-dot patterns (non-targets) appear. Whenever the subject commits an error, auditory feedback (beep signal) is given. The outcome parameters are mean completion time per series (tempo) and the within-subject standard deviation of the 50 series completion times (fluctuation in tempo). The latter parameter is taken as the primary marker of sustained attention skills.
Since all pattern types are presented equally frequent, the target/nontarget ratio is 1:2. As a result, the paradigm induces a growing response bias for the 'no-answer' because on average, the 'no'-button should be pressed twice as frequently as the 'yes'- button. The no-answer becomes the 'pre-potent' response which should be inhibited on the presentation of target signals. It is therefore predicted that the % of misses will be larger than the % of false alarms and this difference score is operationalized as a measure of (sustained) inhibition skill.

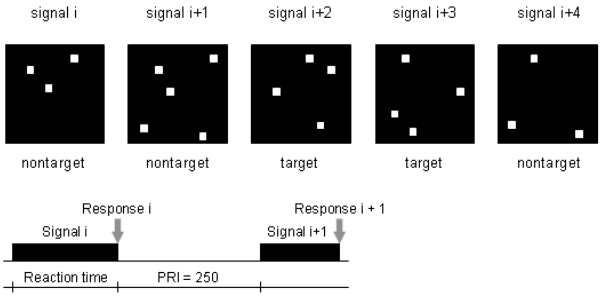

Figure 2. *Example of signals and timing between signals in the sustained attention task. PRI=Post-Response Interval*

Mental flexibility and inhibition Mental flexibility is measured by the attentional set shifting task (**Figure 3**).The task consists of three parts of 40, 40 and 80 trials respectively.

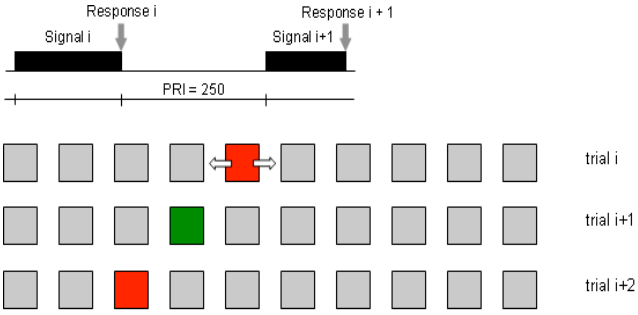

Figure 3*. Timing between signals and an example of two consecutive trials in part 3 of the task. In trial (i+1) the block has jumped to the left and has turned green: the correct response is to press the left button (compatible response. In trial (i+2) the block has jumped to the left and the color changed to red: the correct response is now to press the right button (incompatible response). PRI=Post-Response Interval.*

In this task, a bar consisting of 10 gray squares is positioned centrally on the screen. In part 1, one of the blocks is a green one. After the start of the test, this block jumps to the right and to the left in a random order. The subject must follow the movement of the block. When the block jumps to the left, the subject must press the left button, when it jumps to the right the subject must press the right button. Ergo this is the 'fixed compatible' stimulus-response (SR) mapping condition. During part 2, a red block jumps in similar fashion along the bar but now the subject should 'mirror' the movement of the jumping block: when the block has jumped to the left, the right button must be pressed, when the block has jumped to the right, the left button must be pressed. This part is called the 'fixed incompatible' condition requiring the inhibition of pre-potent responses. In part 3, the subject is presented with trials in a random order, in which the block after the jump (to the right or to the left) is green or red. This is the 'random compatibility' condition: the subject should switch response sets on (color) cue which requires attentional flexibility. Inhibition is operationalized contrasting the performance of part 1 (compatible responses) with part 2 (incompatible responses).

Flexibility is operationalized contrasting the performance of part 3 (only compatible responses) with part 1 (idem). The model predicts that these task manipulations result in slower and less accurate processing.

AUTISTIC TRAITS Autistic symptoms were measured by the Autism Diagnostic Interview-Revised (ADI-R) [21]. The ADI–R is an established 'gold standard' for assessing a categorical diagnosis of autism, but may also be used to assess profiles of autistic symptomatology [21]. It is an extensive clinical interview administered to the parents [21]. The ADI-R measures elements of autistic behaviors by each item. The ADI–R algorithm contains a total of 37 items. The ADI-R items within each core domain are grouped into ADI-R labels (or sub-domains). Items are scored as 0 (behavioral symptom specified not present), 1 (specified behavior not sufficient to code ''2'') or 2 (specified symptom present). The 3 domains originally defined by the ADI-R are also referred to as the 'content' domains of autism (i.e. qualitative abnormalities in social interaction (S), qualitative abnormalities in communication (C) and stereotyped and repetitive behaviors (R). The 3 ADI-R domains consists of 4 labels, and each label consists of 2 to 5 items, which are directly related to the DSM-IV-TR criteria of an Autistic Disorder. However, multiple studies have found evidence for a different 'empirical' symptom structure than the DSM triad [22-27]. These alternative factor analyses provide a useful measurement structure for studies that aim to explore the relationship between symptom domains and, for instance, autistic susceptibility genes or genetic disorders. Notably, one of these factor analyses has been successfully replicated in 2 independent large samples [22,23]. This resulted in 3 latent factors based on the ADI-R symptomatology of autism, which have a slightly different item structure than that of the theory based DSM triad. In this factor structure impaired social communication contains information about poor verbal and nonverbal social communicative interchange. Impaired make-believe and play is comprised of a lack of play skills in individual activities and in relationship with peers. Stereotyped language and behavior consists of stereotyped characteristics in speech and behavior. We used these three empirically derived factors instead of the classic ADI-R domains model for correlations with EF functions to maximize the detection of the relationship between neurocognitive deficits and behavioral manifestations.

STATISTICS Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 14.0. Group differences on the baseline speed and sustained attention task were analyzed using multivariate analyses of variance with reaction time and fluctuation in reaction time, respectively tempo and fluctuation in tempo, as dependent variables. If data inspection showed outliers and invalid response patterns, individuals were excluded from analyses per subtask. Differences in inhibition skills during sustained attention were analyzed by repeated measures analyses of variance with Group as between subjects (BS) factor, Error Type (misses vs. false alarms) as within subject (WS) factor, and % of errors

as dependent variable. For the set shifting task data, inhibition was analyzed using a similar design with inhibition (compatible-part 1 vs. incompatible-part 2) as WS factor. Separate runs were made for reaction time en % of errors as dependent variables respectively, and number of errors. Mental flexibility was also analyzed similarly in two runs with SR-mapping condition (fixed: flexibility not required-part 1 vs. variable: flexibility required-part 3) as WS factor. In all analyses age was entered as a covariate. The alpha-level of significance was set at p=0.01. Effect sizes were estimated using partial eta squared (η_p^2), and interpreted as small ($\eta_p^2 \approx 0.01$), medium ($\eta_p^2 \approx 0.06$), and large ($\eta_p^2 > 0.13$), following Cohen (1988). Partial correlations, controlling for age, were used to explore the relation between social problems and autistic traits and those executive function indices that appear to differentiate between groups (p=.05). Individual ADI-R factor scores were entered in these analyses as autistic traits.

Results

Mean age of the boys with KS (12.09 ± 3.67 years) and of the control group (11.37 ± 3.20 years) did not differ significantly [t(1,132)=1.091, p=.277).

ATTENTION INTENSITY/ATTENTION REGULATION The multivariate analysis of speed and fluctuation in speed on a task for baseline speed was significant [F(2,130)=12.99, p<.0001, η_p^2=.167]. Compared to normal controls, the boys with Klinefelter were both slower [F(2,131)=25.60, p<.0001, η_p^2=.164] and demonstrated less stability in speed [F(2,131)=10.18, p=.002, η_p^2=.072] (**Figure 4**, left panel).

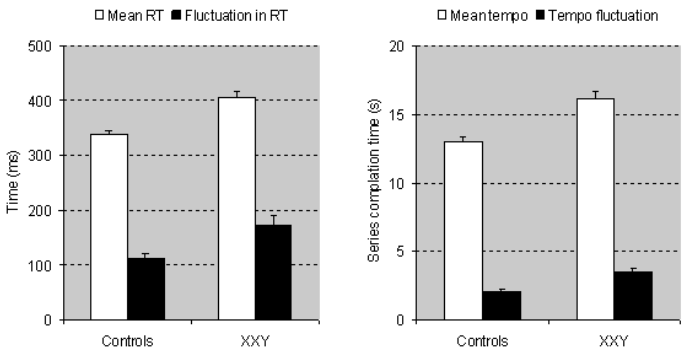

***Figure 4**. Speed and speed stability on baseline speed task (left panel) and sustained attention task (right panel) as a function of Group (mean values ± SE) Sustained attention.*

The multivariate analysis of tempo and fluctuation in tempo on a task for sustained attention was significant [F(2,131)=16.67, p<.0001, η_p^2=.203]. Compared to normal controls, the boys with Klinefelter demonstrated a lower tempo [F(1,131)=18.92, p<.0001, η_p^2=.125] combined with a (much) larger fluctuation in tempo [F(1,131)=32.95, p<.0001, η_p^2=.200] (**Figure 4**, right panel).

INHIBITION Boys with KS made more errors on the sustained attention task than normal controls [$F(1,132)=15.69$, $p<.001$, $\eta_p^2=.106$]. The percentage of errors on target signals (misses) was larger than the percentage of errors on non-target signals for all children [$F(1,132)=9.60$, $p=.002$, $\eta_p^2=.068$], indicating the effect of the induced response bias. Group interacted with Error type [$F(1,132)=6.97$, $p=.009$, $\eta_p^2=.050$], reflecting that boys with KS made disproportionately more misses than false alarms compared to control children. This outcome suggests the existence of a deficient inhibition of pre-potent responses in boys with KS (**Figure 5**, left panel).

Boys with KS made more errors on Part 1 and 2 of the set shifting task than normal controls [$F(1,130)=28.27$, $p<.0001$, $\eta_p^2=.177$]. The percentage of errors increased for all children when they were required to make incompatible responses [$F(1,131)=18.08$, $p<.0001$, $\eta_p^2=.121$]. In addition, Group interacted with Response type [$F(1,130)=10.64$, $p=.001$, $\eta_p^2=.075$] reflecting that differences between groups increased when incompatible responses had to be produced. This outcome suggests the existence of a deficient inhibition of prepotent responses in boys with KS (**Figure 5**, right panel).

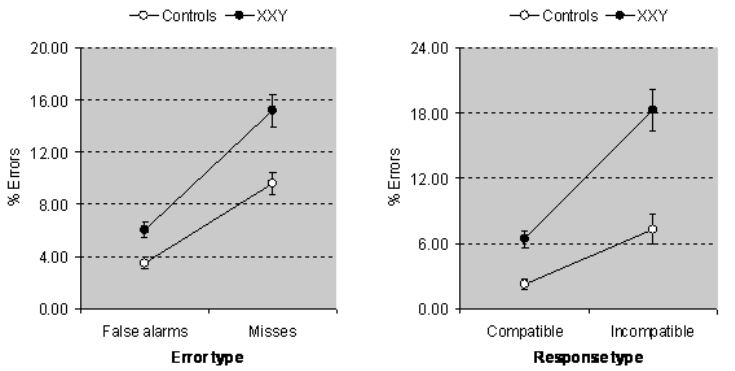

Figure 5. *Inhibition errors during sustained attention (left panel), and in the set shifting task (right panel) as a function of Group and Error type and response type, respectively (mean values ± SE).*

MENTAL FLEXIBILITY Boys with KS made more errors on Part 1 and 3 of the set shifting task than normal controls [$F(1,130)=52.45$, $p<.0001$, $\eta_p^2=.287$]. The percentage of errors increased for all children when they were required to make compatible responses under random SR-mapping conditions (part 3) [$F(1,131)=28.16$, $p<.0001$, $\eta_p^2=.178$]. Moreover, Group interacted with SR-mapping condition [$F(1,130)=24.17$, $p<.0001$, $\eta_p^2=.157$] reflecting that differences between groups increased when mental flexibility was required. This outcome suggests the existence of a deficient flexibility in Boys with KS (**Figure 6**).

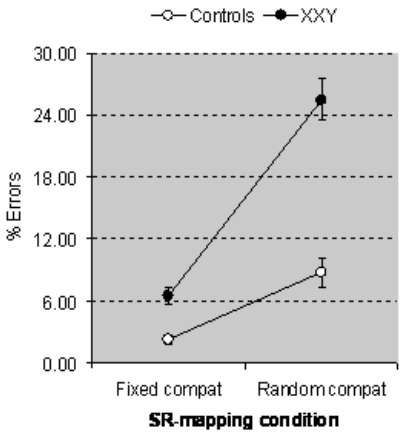

Figure 6. *Flexibility errors in the set shifting task (right panel) as a function of Group and SR-mapping condition (mean values ± SE).*

EF AND AUTISTIC SYMPTOMS Attention intensity/attention regulation. Instability in speed during baseline attention was significantly correlated to stereotypical language and behavior (r=.294, p=. 039). Percentage of error during sustained attention correlated significantly with the overall ADI score, indicating that higher social behavioral problem scores coincide with more difficulty in inhibition (r=.33, p=.022).With respect to specific domains of autistic traits, percentage of error during sustained attention was significantly associated to impaired social communication (r=.381, p=.009). Percentage of misses as well as false alarms were significantly correlated to this domain of autistic traits (misses: r=.349, p=.016; false alarms: r=.341, p=.018). Percentage of misses was also related to level of stereotypical behavior and language (r=.319, p=.025). Inhibition during mental flexibility was also correlated to stereotypical behavior and language (r=.331; p=.023). No meaningful correlations between mental flexibility and autistic traits were found in the KS group.

DISCUSSION

In this study boys with KS demonstrate increased response variability reflecting decreased alertness (lower intensity of attention). The large fluctuation in speed of processing found in boys with KS during time-on-task indicates a sustained attention deficit. Both results point to poor attention regulation.

Boys with KS also show a disproportionate increase in error rate when response bias is high (sustained attention task) and when they have to inhibit pre-potent responses (Shifting set task). Both outcomes indicate inhibition problems. Moreover, they also demonstrate a disproportionate increase in error rate when flexibility demands are high (Shifting set task), which indicates poor mental flexibility. These failures in executive functioning are also referred to as deficits in cognitive control. They are likely to have consequences for the quality of various aspects of behavioral control especially during situations that ask for adaptive control, e.g. social situations.

Therefore, the second aim of this study was to evaluate if the EF deficits contribute to social behavioral problems as these are frequently observed in KS. Based on an earlier study it is reported that boys with KS display abundant autistic symptomatology, and in some cases (27 %) fulfilling the criteria for an autistic spectrum disorder [5]. Autistic symptoms in the present cohort of boys with KS seem associated with EF problems. Especially deficits in attention regulation and inhibition seem to coincide with social communication abnormalities and stereotypical behavior and language in the present sample of boys with KS.
Lower alertness as expressed in less intense processing of information in KS may result in less optimal orientation on relevant information that is necessary for adequate adaptation to the changing environment which might especially result in social adaptation problems. Inhibition problems refer to the inability to adequately process incoming information to adjust behavior to environmental demands. Stereotypical behavior and language might especially express inhibition problems.

The present study has several limitations. Selection bias has occurred as the selection of participants was based on a procedure in which parents and children had to volunteer to participate. Selection bias is hard to avoid as the awareness of being affected with Klinefelter syndrome already interferes with objectivity for developmental issues. As a result, the findings of this study might not be representative for the children with Klinefelter syndrome in general. Further, sustained attention function and EF are likely to be related to intellectual functioning. We choose not to control for general levels of intelligence in the present study because this might have resulted in underestimation of problems in attention control and EF within this group. Androgen treatment did not affect the EF scores when we compared the boys with (n=7) and without current androgen substitution. It is possible that the window of development (age) interferes with the level of EF dysfunctions. Future longitudinal studies should aim to describe the developmental course of EF in KS.

In the present study it was found that attention regulation, inhibition and mental flexibility is impaired in KS, despite the fact that the group of boys was not selected based on their autism symptoms. Although the representativeness of this specific group of boys for the KS population might be questioned, the relationship between EF dysfunction and the risk for social problems and autistic psychopathology was affirmed.
Many studies have assessed EF dysfunction in the context of autism spectrum disorders. The clinical heterogeneity of autistic spectrum disorders complicates to specify more precise relationships between aspects of EF and social dysfunctioning. The current study further illustrates that the relationship of a genetic disorder with EF and susceptibility for ASD could provide an opportunity to more precisely dissect relationships between cognitive control and the risk for developmental psychopathology.

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CHAPTER 5

THE PARENT-OF-ORIGIN OF THE EXTRA X CHROMOSOME MAY DIFFERENTIALLY AFFECT PSYCHOPATHOLOGY IN KLINEFELTER SYNDROME.

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ABSTRACT

Several genetic mechanisms have been proposed for the variability of the Klinefelter syndrome phenotype such as the parent-of-origin of the extra X chromosome. Parent-of-origin effects on behavior in KS can possibly provide insights into X-linked imprinting effects on psychopathology that may be extrapolated to other populations. Here, we investigated whether the parent-of-origin of the supernumerary X chromosome influences autistic and schizotypal symptom profiles in KS.

Parent-of-origin of the X chromosome was determined through analysis of the polymorphic CAG tandem repeat of the androgen receptor gene. Autistic traits (Autism Diagnostic Interview—Revised) were measured in a younger KS sample (n = 33) with KS and schizotypal traits (Schizotypal Personality Questionnaire) were assessed in an older KS sample (n = 43). Scale scores on these questionnaires were entered in statistical analyses to test parent-of-origin effects.

The results show that parent-of-origin of the X chromosome is reflected in autistic and schizotypal symptomatology. Differences were shown in the degree of both schizotypal and autistic symptoms between the parent-of-origin groups. Furthermore, the parent-of-origin could be correctly discriminated in more than 90% of subjects through Autism Diagnostic Interview-Revised scales and in around 80% of subjects through Schizotypal Personality Questionnaire scales.

These findings point to parent-of-origin effects on psychopathology in KS and indicate that imprinted X chromosomal genes may have differential effects on autistic and schizotypal traits. Further exploration of imprinting effects on psychopathology in KS is needed to confirm and expand on our findings.

INTRODUCTION

Klinefelter syndrome (KS; 47,XXY karyotype and variants) is somatically and behaviorally characterized by a marked variation in severity of the phenotype [1,2]. KS is the most common chromosomal aberration in men with .1% to .2% of the male population affected [3-5]. It has been proposed that besides gene–environment interactions specific genetic factors may contribute to the phenotypic variability in KS, including preferential X-inactivation patterns and the parent-of-origin of the supernumerary X-chromosome [6-8].

The parent-of-origin might influence the KS phenotype via differential expression of paternal versus maternal X-alleles, that is, parental imprinting [7,9]. In contrast to other trisomies that originate from errors at maternal meiosis, KS is a notable exception because nearly half of the cases derive from paternal nondisjunction [10]. Therefore, KS could be an interesting condition to study imprinting effects through the assessment of parent-of-origin effects of the extra X chromosome. The multiple neurodevelopmental and behavioral difficulties observed in KS could be due to the disproportionally high number of genes on the X chromosome that are involved with mental function [11-13]. Men with KS often present with language-based learning problems, cognitive dysfunction, and social difficulties [1,14,15]. These problems are accompanied by an increased vulnerability to a wide range of psychiatric disorders in some, but not all, men with KS [16-20]. This wide variability in psychiatric morbidity could enhance the chance of detecting parent-of-origin effects on psychopathology in KS. Behavioral studies in KS have shown an increased prevalence of autistic and schizotypal traits, in some cases to the level of autism and schizophrenia[7,16,18,21,22]. Therefore, KS is an interesting condition to study X-linked imprinting effects on autistic and psychotic symptomatology in subjects with the same genetic disorder.

The role of epigenetic mechanisms such as genomic imprinting in psychiatric pathogenesis is an evolving theme. Probably the most studied example of imprinted genes in psychiatric disease is the 15q11-13 region. The Prader–Willi (PWS) and Angelman (AS) syndromes are clinically distinct developmental disorders with frequent psychotic and autistic symptoms, respectively, that are caused by genetic defects in the imprinted domain at chromosome 15q11-q13, resulting in the loss of paternal (PWS) or maternal (AS) gene function [23,24]. Crespi *et al.* have proposed that autistic and psychotic disorders represent opposing imprinted conditions[25-28]. They suggest that the etiologies of psychotic spectrum conditions commonly involve genetic and epigenetic imbalances in the effects of imprinted autosomal genes, with a bias toward increased relative effects of maternally expressed imprinted genes. In contrast, they hypothesize that autistic spectrum conditions are associated with increased relative effects of paternally expressed imprinted genes.

With respect to X chromosomal genes, no clear imprinting effects have been described in humans. Skuse *et al.* have proposed that the parent-of-origin of the X-chromosome in monosomy X (Turner syndrome, TS) affected performance on measures of social cognition

with better performance by females with paternally derived X chromosomes [29]. Earlier studies have assessed parent-of-origin effects on language and cognition in KS. Speech and language impairments were found to be more pronounced in subjects with paternal origin of the extra X chromosome in one study [7], whereas parent-of-origin effects, using similar measures, were not observed in other studies [30-32]. In addition, a previous study did not show a parent-of-origin effect on the prevalence of psychiatric disorders in a sample of 20 adult men with KS, including psychosis [17].

The goal of this study was to assess whether the parent-of-origin contributes to the variability of autistic and schizotypal symptomatology in subjects with KS. Autistic and schizotypal trait scores were analyzed separately. We assessed both parent-of-origin group differences in each set of trait scores and feasibility of using trait scores to discriminate parent-of-origin groups. Data on trait scores were available from two previous studies. Autistic traits had been assessed in a sample of boys and adolescents (here referred to as the younger sample) with KS in an earlier survey on developmental psychopathology [18]. Schizotypal traits in a sample of adolescents and adults with KS (here referred to as the older sample of adults) were available through an earlier study on schizophrenia spectrum symptomatology [21].

METHOD

The Dutch Central Committee on Research Involving Human Subjects approved the research protocols. Written informed consent was obtained according to the declaration of Helsinki. The current samples were extracted from larger samples recruited as part of a larger research program that studies the neurobehavioral consequences of KS. Both samples had been recruited via the Dutch Klinefelter association and two centers for clinical genetics and pediatrics situated in the center of the Netherlands A newsletter encouraged to participate regardless of any existing problems (recruitment details are described elsewhere) [18,21]. Exclusion criteria for both Klinefelter and control subjects were neurologic conditions that impair speech and motor development (other than through KS) or a history of head injury with loss of consciousness, recent history of substance abuse, or intellectual disability (IQ < 70). There were no mosaic forms of KS among the participants; all had the full 47,XXY karyotype.

PATIENTS The younger KS sample consisted of 33 subjects with KS (mean age 12.0 years, SD 3.6, range 6–19 years). Determination of parent-of-origin of the extra X chromosome in this sample showed that in 19 subjects, the extra X chromosome originated from the mother (maternal group) and in 14 subjects from the father (paternal group). Age was significantly higher in the maternal group than in the paternal group of the younger sample ($p = .03$, t test). At the time of the assessment, 8 younger subjects were treated with testosterone (three

from the maternal group, five from the paternal group). Total IQ scores did not differ between maternal and paternal groups (p = .54, t test).

Measures of autistic traits in the younger sample were compared with a control sample of 29 healthy siblings (14 females, 15 males) of patients with velocardiofacial syndrome (22q11 DS) children, ranging in age from 9 to 19 (mean = 14.1; SD = 2.5). This sample originated from a study of phenotypes in individuals with velocardiofacial syndrome with and without autism [33]. These subjects were older (p = .01, t test) than the KS subjects in the younger sample.

The older sample consisted of 43 subjects (mean age 31.1, SD 14.4, range 10–68 years). This sample was part of prior sample that had been recruited for a study into schizophrenia spectrum pathology [21]. Determination of parent-of-origin of the extra X chromosome in this sample showed that in 25 subjects the extra X chromosome originated from the mother (maternal group) and in 18 subjects from the father (paternal group). At the time of assessment, 15 men (60%) from the maternal group and 11 (61%) from the paternal group were treated with testosterone supplements. Age and total IQ scores did not differ between maternal and paternal groups group (p = .42 and p = .38 respectively, t test).

The older sample was compared with a normal control sample that was recruited using advertisements in local newspapers or was drawn from a database in our department. None of the control subjects had a history of psychiatric illness as confirmed with the Mini International Neuropsychiatric Interview Plus [34]. Mean age did was not different between adults with KS and controls (p = .12, t test). **Table 1** shows the basic characteristics of the younger and older sample.

Table 1. *Age and TIQ in the Younger and Older KS Sample.*

	Younger sample				**Older sample**			
	Co	*M*	*P*	*MvsP*	*Co*	*M*	*P*	*MvsP*
n	29	19	14		75	25	18	
Age	14.1	10.5	14.1	0.03	26.7	37.2	28.9	0.42
SD	±2.6	±2.9	±3.4		±14.9	±15.7	±12.5	
TIQ	102.3	81.8	79	0.54	na	90.9	87.8	0.38
SD	±16.7	±14.1	±12.1			±9.5	±14.8	

M vs. P gives p values for t test comparisons of maternal origin vs. paternal origin group comparisons
KS, Klinefelter syndrome; maternal, maternal origin; paternal, paternal origin of extra X-chromosome; TIQ, total IQ score.

PROCEDURE AND MEASURES

Technical details regarding the determination of parent-of-origin are stated in the Supplementary data.

AUTISTIC TRAITS The ASD traits in the younger sample were measured by the Autism Diagnostic Interview—Revised (ADI-R) [35]. The ADI-R is an established gold standard for

assessing a categorical diagnosis of autism but may also be used to assess profiles of autistic symptomatology [33,36]. It is an extensive clinical interview administered to the parents. The interview consists of three core or "content" domains of autism (i.e., qualitative abnormalities in social interaction [S], qualitative abnormalities in communication [C], and stereotyped and repetitive behaviors [R]) [35]. The ADI-R measures autistic behaviors that are called items. The ADI-R algorithm contains 37 items. The ADI-R Diagnostic Algorithm (as opposed to the ADI-R Current Behavior Algorithm), which measures behaviors between the ages of 4 and 5 years, was used in this study. The ADI-R items within each core domain are grouped into ADI-R labels (or subdomains). Each ADI-R domain consists of four labels (e.g., S1-4, C1-4, and R1-4), and each label consists of 2 to 5 items, which are directly related to the DSM-IV-TR criteria of an autistic disorder. Items are scored as 0 (ASD behavioral symptom specified not present), 1 (specified behavior not sufficient to code as 2), or 2 (specified ASD symptom present). Maximum label scores thus range from 4 to 10.
The three ADI-R domain scores and 12 ADI-R label scores were entered in the current analyses as autistic traits. An overview of the description of the ADI-R items, labels, and the ADI-R domains of the algorithm is provided in Supplementary Tables S1 and S2. Table S2 in Supplement 1 states the individual ADI-R algorithm items sorted by labels and domains.

SCHIZOTYPAL TRAITS The older sample was investigated for schizotypal traits through the Schizotypal Personality Questionnaire (SPQ) [37]. The SPQ is a self-report measure of schizotypal traits that has been shown to be normally distributed in the general population. Factor analytic studies have revealed three dimensions of schizotypy: 1) *positive schizotypy* (e.g., referential thinking and delusional atmosphere), 2) *negative schizotypy* (e.g., constricted affect and social anxiety), and 3) *disorganization* (odd speech and eccentric behavior) [38]. These three dimensions are clusters of traits derived from 10 individual SPQ labels, which are assigned to the clusters according to the analyses of Vollema *et al.* [39]. Total score for each label is based on a range of questions that are answered with "yes" (one point) or "no" (zero points). The SPQ is regarded as a sensitive instrument to measure subclinical schizophrenia-like traits or symptoms [39].

STATISTICAL ANALYSIS Multivariate analyses of variance (MANOVA) with parent-of-origin as between subjects factor (normal controls, maternal KS, paternal KS) and ADI-R domain and label scores as dependent variables, respectively, were performed to test differences between groups on ASD traits. Similar MANOVAs as for autistic measures cited earlier were performed with SPQ dimension and label scores as dependent variables, respectively. A Helmert contrast was applied to compare the controls with the total KS samples (contrast 1) and the maternal KS sample with the paternal KS sample (contrast 2) in one run. Partial

eta squared (η_p^2) indicates effect sizes, with $\eta_p^2 \sim .03$ representing a weak effect, $\eta_p^2 \sim .06$ representing a moderate effect, and $\eta_p^2 \geq .14$ representing a large effect [28]. Whereas MANOVA was used to test differences between group means, discriminant analysis, direct method, was used to evaluate to what extent autistic and schizotypal trait scores could differentiate on the individual level between the maternal and paternal younger and older subsamples. For classification, the within-group covariance matrices were used, and prior probability was set to equal for all groups. The success of the predictive ability of the ADI-R domain/label and SPQ dimension/label scores for parent-of-origin is reflected in the classification matrix that shows the number and percentage of correctly identified subjects. The threshold for significance in the analyses was set at $p = .05$.

RESULTS

PARENT-OF-ORIGIN AND AUTISTIC SYMPTOMS First, our main hypothesis was tested, whether the parent-of-origin groups differed in the degree of autistic trait scores. Age and testosterone treatment were irrelevant to the outcome of autistic ADI-R scores because these were measured according to the ADI-R algorithm, which assesses behaviors between ages 4 and 5 years.

The multivariate effect of group on the ADI-R domain scores was significant [$F(6,116) = 11.27$, $p < .0001$, $\eta_p^2 = .368$], with univariate F tests significant for all domains ($p < .0001$ and $\eta_p^2 \geq .407$). The first contrast (controls vs. total KS sample) revealed significant differences for all domains ($p < .0001$). The second contrast (maternal vs. paternal KS) revealed no significant differences ($p \geq .054$). **Figure 1** indicates that the paternal group scores higher than the maternal group on the domains of impairments in social interaction (S) and impairments in communication (C), whereas on the domain of repetitive and stereotyped behaviors (R), the maternal group scores higher. No domain differences reached significance, although differences in domain S approached significance ($p = .054$).

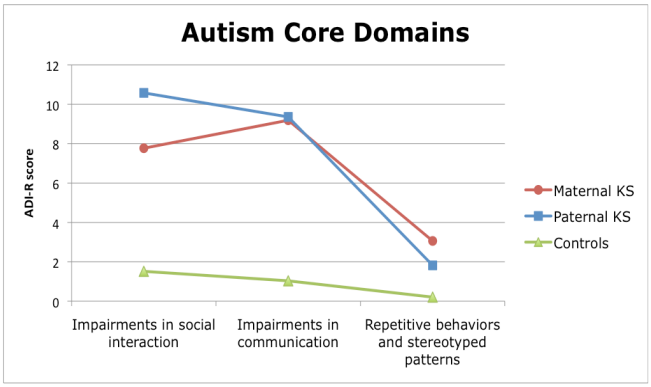


Figure 1. *Average Autism Diagnostic Interview—Revised (ADI-R) domain scores for parent-of-origin groups in the younger Klinefelter syndrome (KS) versus control samples. The three domains have different scoring ranges because they consist of 15, 13, and 6 items.*

The multivariate effect of group on the ADI-R labels scores was significant [$F(24,98) = 4.56$, $p < .0001$, $\eta_p^2 = .528$], with univariate F tests significant for all labels ($p < .0001$ and $\eta_p^2 \geq .268$) for all labels except R2 ($p = .038$, $\eta_p^2 = .105$) and R3 ($p = .020$, $\eta_p^2 = .125$). The first contrast (controls vs. total KS sample) revealed significant differences for all labels except R3 ($p = .055$). Significance levels reached $p < .0001$ for all other labels except R2 ($p = .02$). The second contrast (maternal vs. paternal KS) revealed that paternal KS subjects scored higher on S3 (Lack of shared enjoyment; $p = .018$) and C4 ($p = .043$; stereotyped, repetitive or idiosyncratic speech), whereas maternal KS subjects scored significantly higher (i.e., showed great impairment) on C1 ($p = .016$; Lack of, or delay in, spoken language and failure to compensate through gesture). **Figure 2** gives an overview of the ADI-R label scores.

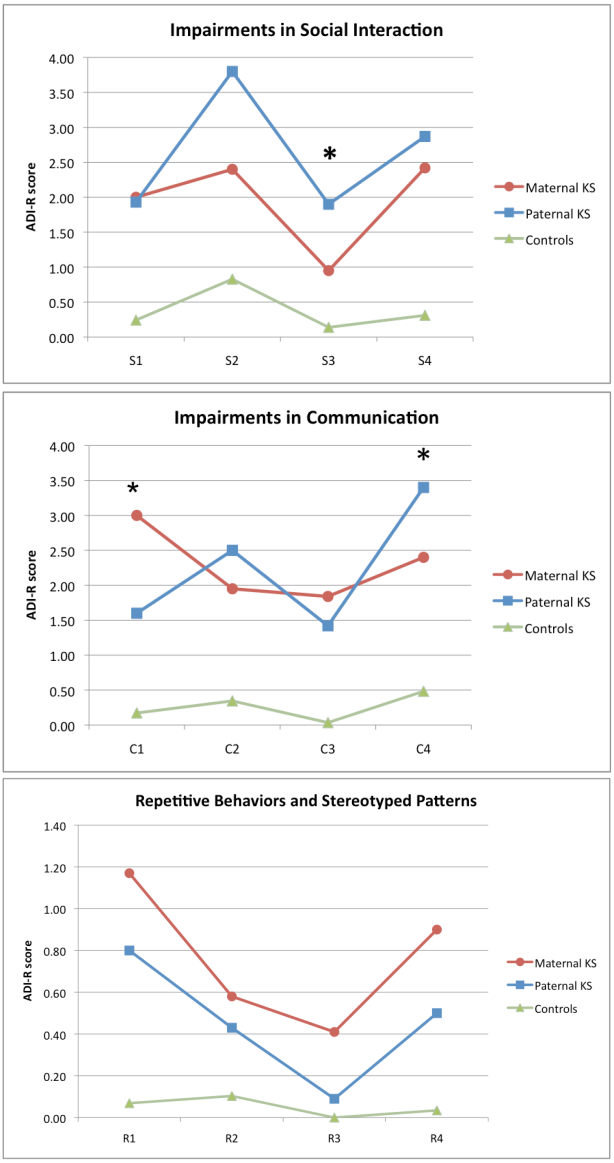


Figure 2. *Average Autism Diagnostic Interview—Revised (ADI-R) label scores for different parent-of-origin groups in the younger Klinefelter syndrome sample. *p < .05, Helmert contrast analyses of maternal versus paternal group scores.*

Discriminant analysis involving the ADI-R domains (Wilks' lambda = .741, $\chi^2(3) = 8.83$, $p = .032$) resulted in a correct classification of 78.9% of the members of the maternal group and 78.6% of the members of the paternal group, misdiagnosing four and three subjects per group, respectively. Discrimination analysis involving the ADI-R labels resulted [Wilks' lambda = .397, $\chi^2(12) = 23.11$, $p = .027$] in a correct classification of no less than 94.7% of the members of the maternal group and 92.7% of the members of the paternal group, only misdiagnosing one subject in each group.

PARENT-OF-ORIGIN ORIGIN AND SCHIZOTYPAL SYMPTOM PROFILES As part of our main hypothesis, we further tested whether the parent-of-origin subgroups differed in schizotypal trait scores. Testosterone treatment did could not explain group effects on total schizotypal scores in the older sample (Mann–Whitney test: 60% of maternal subjects vs. 61% paternal subjects receiving treatment, $Z = -.09$, $p = .92$). The multivariate effect of group (maternal vs. paternal vs. controls) on the SPQ main dimension scores was significant [$F(6.228) = 7.9303$, $p < .0001$, $\eta_p^2 = .173$], indicating that the groups comprising the older sample differed in schizotypal dimension scores.

The univariate between subjects effects were significant for all dimensions with p varying between < .0001 ($\eta_p^2 = .33$) and .001 ($\eta_p^2 = .118$), with the maternal group scoring highest and the controls scoring lowest on all dimensions (**Figure 3**). The first difference contrast (KS vs. control) showed significantly higher scores for the total KS sample on all SPQ dimension ($p \leq .004$). The second difference contrast (maternal vs. paternal group) showed a significant difference for the dimension Disorganization ($p = .032$) but not for the other two dimensions (Positive/Negative schizotypy, $p = .33$).

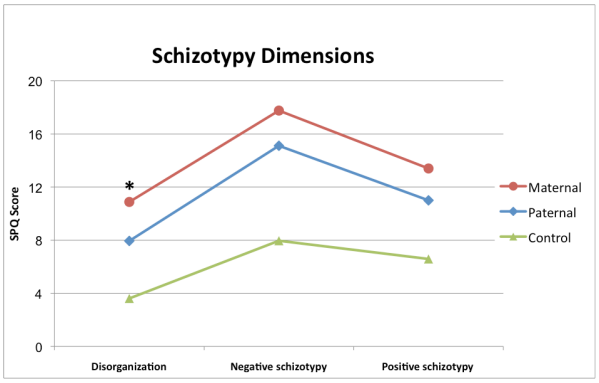


Figure 3. *Schizotypal Personality Questionnaire (SPQ) dimension scores for the parent-of-origin groups in the older Klinefelter syndrome sample. *p < .05, Helmert contrast analyses of maternal versus paternal group scores.*

The same significant multivariate effect of group (maternal vs. paternal vs. controls) held for individual SPQ labels [$F(20.214) = 4.03$, $p < .0001$, $\eta_p^2 = .273$], indicating that the groups differed in scores. The univariate between subjects effects were significant for all labels, except for Delusional ideas ($p = .16$), with p varying between < .0001 ($\eta_p^2 = .355$) to < .016 ($\eta_p^2 = .070$). The first difference contrast (KS vs. controls) showed significantly higher scores for the total KS sample on all SPQ labels with $p \leq .005$, except for Delusional ideas ($p = .06$) and Magical thinking ($p = .022$). The second and most important difference contrast (maternal group vs. paternal group) showed a trend toward significance for Magical thinking ($p = .094$) and significant differences on Excessive social anxiety ($p = .049$) and Odd or eccentric behavior ($p = .010$), with maternal scores scoring higher than paternal scores on 8 of 10 subscales, an equal score for delusional ideas and a marginally lower score for ideas of reference (**Figure S1** in Supplementary data).

Discriminant analysis involving the SPQ labels resulted in a correct classification of 80.0% of the members of the maternal group and 77.8% of the members of the paternal group, misdiagnosing only five (of 25) and four (of 18) subjects per group, respectively.

DISCUSSION

This study is the first to describe parent-of-origin effects on psychopathology in KS. The results show that the parent-of-origin of the extra X chromosome is reflected in the level of autistic symptoms and schizotypal traits. Multivariate effects of group (maternal vs. paternal vs. controls) on both schizotypal and autistic traits were found, indicating that the parent-of-origin groups differed in scores on both traits. Furthermore, significant differences on individual schizotypal and autistic trait scores were shown between maternal and paternal groups. The significantly higher scores on schizotypal traits were consistently associated with maternal origin. The significant differences in autistic traits were not consistent. One was associated with maternal origin and others with paternal origin. Further analysis revealed that the parent-of-origin of the extra X chromosome could be discriminated through specific autistic and schizotypal trait profiles. More than 90% correct classification of parental groups was feasible on the basis of the autistic trait profile in the younger sample and around 80% correct classification of the parental groups on the basis of the schizotypal profile in the older sample. Our results indicate that parent-of-origin effects on autistic and psychotic psychopathology exist, but further conclusions are precluded until other studies have provided evidence of similar imprinting effects.

Both the autistic and schizotypal measurements did not seem to be influenced by age. However, recall bias in the retrospective ADI-R interview might be greater in older subjects because of the longer time passed since the 4- to 5-year age period used to measure behaviors, especially if testosterone treatment at a later age has influenced behaviors. A relative strength is that we showed imprinting effects in two largely independent samples. In comparison with other studies on KS, our sample sizes were substantial but seem underpowered for more detailed comparisons.

It should be noted that maternal nondisjunction during meiosis I leads to uniparental heterodisomy (two different X chromosomes from the same parent—in this case, the mother), although an error during meiosis II results in uniparental isodisomy (duplicate of one maternal X chromosome in the child; **Figure 4**).

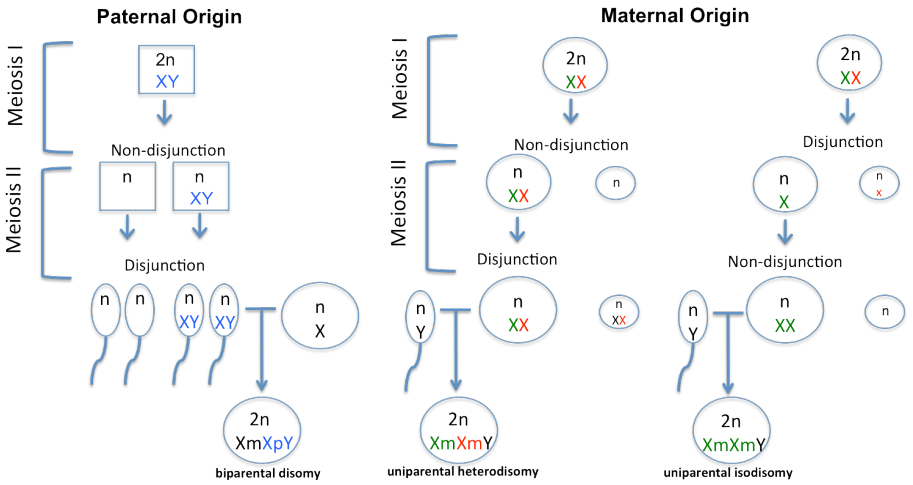
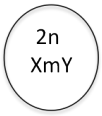


Figure 4. *Different errors during gametogenesis leading to Klinefelter syndrome. Maternal origin is due to uniparental heterodisomy (two different X chromosomes from the same parent) via nondisjunction in meiosis I or uniparental isodisomy (duplicate of one maternal X chromosome) via nondisjunction in meiosis II. Paternal origin is due to biparental disomy via nondisjunction in meiosis I (one paternal and one maternal X chromosome). Smaller round structures next to oocytes and ovums in female meioses designate first and second polar bodies.*

In our samples, we were able to determine the phase of meiotic nondisjunction in 29 of the total 44 maternal subjects. Evaluation of the androgen receptor polymorphic allelic differences showed heterodisomy in 15 cases and uniparental isodisomy in 14 cases. These numbers were too small for further comparisons, but the hetero- versus isodisomic nature of the extra X chromosomes could also be of influence on the KS phenotype, which would add another level of complexity in assessing parent-of-origin effects in KS.

It is not known whether, in turn, the parent-of-origin influences the complex gene dosage and inactivation patterns in KS. It has been well documented that 15% of the human X-linked genes are known to stably escape inactivation, and 10% of genes have a heterogeneous expression, that is, these genes are inactivated in certain women and expressed on the inactive X chromosome in others [40]. These findings suggest that genes that escape inactivation as well as those within pseudoautosomal regions (i.e., the homologous DNA sequences of nucleotides on the X and Y chromosomes) are likely to cause gene dosage differences in KS. Analogous to a figure depicting X chromosomal gene dosage in euploidic individuals by Davies *et al.* [41], **Figure 5** depicts the possible relationships between imprinting status and X-inactivation in KS.



	Y homologue	No Y homologue	Y homologue	No Y homologue	Y homologue	No Y homologue
Inactivation first X						
Paternally expressed	XmXpY=2	XmXpY=1	XmXmY=1	XmXmY=1	XmY=1	XmY=0
Maternally expressed	XmXpY=1	XmXpY=0	XmXmY=2	XmXmY=0	XmY=1	XmY=0
Inactivation second X						
Paternally expressed	XmXpY=1	XmXpY=0	XmXmY=1	XmXmY=0	n/a	n/a
Maternally expressed	XmXpY=2	XmXpY=1	XmXmY=2	XmXmY=1	n/a	n/a
No inactivation						
Paternally expressed	XmXpY=2	XmXpY=1	XmXmY=1	XmXmY=0	XmY=1	XmY=0
Maternally expressed	XmXpY=2	XmXpY=1	XmXmY=3	XmXmY=2	XmY=2	XmY=1

Figure 5. *Theoretical genes dosages for X-linked imprinted genes in different parent-of-origin forms of Klinefelter syndrome and euploidic males, depending on their direction of imprinting, X-inactivation status, and the presence or not of a functionally equivalent Y homologue. In the example of XmXpY, Xm is the "first" X-chromosome, Xp is the "second" X-chromosome. For instance, "Paternally expressed" and "Inactivation of the second X" for XmXpY results in an active (first) Xm, which is not expressed when paternal genes are preferentially expressed, i.e., gene dosage = 1 when a homologues gene can be expressed from the Y chromosome, and gene dosage = 0 when there is no Y homologues gene.*
Figure is analogous to a figure depicting X chromosomal gene dosage in euploid individuals by Davies et al. [41] *.*

It has been speculated that elevated expression of X-linked genes in KS cause a liability for psychosis rather than ASD [19]. This is based mainly on the observation of similar deficits in verbal cognition and brain anatomy in both KS and schizophrenia [42,43]. Crespi *et al.* described that Klinefelter syndrome (usually 47,XXY) should involve high rates of psychotic spectrum disorders, whereas Turner syndrome (usually 45,X) should involve a higher incidence of autism [25,26]. In our earlier psychiatric survey of 51 boys with KS, we did find high frequencies of both ASDs and psychosis [18]. It is possible that particular social-cognitive deficits in KS present differently at different ages. It could be that the developmental window in which they are assessed influences diagnoses [26]. Of the current younger sample, 11 subjects had been diagnosed with an ASD in the original psychiatric survey. Six of these were in the maternal group and five in the paternal group. Three of these ASD subjects had also suffered from at least one psychotic episode, all of which had a maternal origin of the extra X chromosome. This might be in accordance with the maternal effect on schizotypal traits that we observed in the older sample. The number of subjects in our study that were assessed for both schizotypal and autistic traits was underpowered for further comparisons. Future studies should aim to investigate whether a possible overlap in KS of autistic and psychotic spectrum psychopathology can be (partly) dissected through the parent-of-origin.

Not much is known about X-linked imprinting effects in humans as opposed to effects of imprinting through autosomal genes. It has been suggested that X-linked imprinting may serve as a mechanism for the evolution of sexual dimorphism in humans, given that gene dosages of X-linked imprinted genes are expected to differ between the sexes [26,29]. Traits encoded by paternally expressed X-linked genes can exhibit more qualitatively or bidirectionally distinct expression in male (XmY) and female progeny (either XmXp or XmXm), that is, absent in males, present in females. In contrast, the phenotypic difference between male and female offspring for maternally encoded traits may be quantitative, that is, a unidirectional effect [41]. It could be interesting to compare our results to the parent-of-origin effects described in Turner syndrome, monosomy X. By Skuse's hypothesis, that is, in analogy to, XmO (maternal origin) and XpO (paternal origin) individuals in TS, XmXmY (maternal origin) Klinefelter patients might be expected to show more male-typical traits, compared with XmXpY (paternal origin) patients [29,44].To our knowledge, no studies have found autistic trait differences between autistic males and females. Two recent studies that measured schizotypal traits in large samples of normal individuals showed that negative symptoms are more pronounced in males, whereas positive scales tend to be higher in females [45,46]. These findings and our maternal imprinting association with positive schizotypy scales do not suggest more malelike traits in XmXmY individuals.

In different mammalian species, including mice, paternal X chromosomal alleles are silenced in the extraembryonic tissue that constitutes the later placenta during development [47-49]. Interestingly, mouse embryos carrying supernumerary X chromosomes of maternal origin have shown reduced placental growth [50,51]. It is as yet unclear whether and to what extent preferential paternal X chromosomal inactivation takes place in the human placenta [52,53]. In the unusual human situation of XmXmY KS individuals, it might be possible that gene dosage effects are more pronounced in the fetal placenta through preferential paternal × chromosomal silencing. Maternal origin in KS could perhaps also result in reduced placental growth with more reduced brain growth and an increased susceptibility for psychosis analogous to the theories postulated by Badcock and Crespi for autosomal imprinting effects on psychosis and autism [27]. This would contrast the situation in XmXpY men in which normal Xp silencing should occur in the fetal component of the placenta. Perhaps of interest in this respect, Lahti *et al.* described lower placental weight, lower birth weight, and smaller head circumference at 12 months predicted elevated positive schizotypal traits in women after adjusting for several confounders ($p < .02$) [54]. Further gene dosage effects in the (developing) brain should explain the enhanced susceptibility for psychosis and/or ASD in KS also present among XmXpY individuals. Recent studies on (sex-specific) parent-of-origin allelic gene expression in the mouse brain in mice underscore the possibility that X chromosomal gene dosage differences of different parental origins are likely to cause differential effects in brain function [55,56].

In conclusion, it seems that the parent-of-origin of the extra X chromosome in KS may have a differential effect on autistic and schizotypal profiles in KS. KS could be a promising condition to further research X-linked imprinting effects on different psychopathologies. Such epigenetic knowledge could help to find the missing heritability in complex psychiatric disease.

SUPPLEMENTARY DATA

DETERMINATION OF PARENT OF ORIGIN AND CAG REPEAT LENGTH The parent-of-origin was determined parallel to the behavioral assessments. The researchers conducting the clinical interviews were blind to the parent-of-origin status of the subjects. Parent-origin of the X chromosome was determined through analysis of the polymorphic CAG tandem repeat of the androgen receptor (1). A blood sample was taken from each individual with KS and both his parents, if available, to determine the parental origin of the extra X chromosome. We used the human androgen receptor gene (HUMARA) as a polymorphic marker, which allowed us to investigate any skewing of X inactivation as well. The HUMARA gene contains a highly polymorphic trinucleotide CAG repeat in the first exon. The number of repeats varies from 10 to 35 and of all women with a normal karyotype 90% show heterozygosity for this repeat. Together with the analysis of the methylationsensitive restriction site, CfoI, that is located within 100 bp of the repeat, we can distinguish between the paternal and maternal alleles and determine their methylation status. For each DNA sample, two PCR reactions were carried out; one with an enzyme digestion of the genomic DNA prior to the PCR and one without. For the enzyme digestion reaction, CfoI enzyme was used to digest the active, unmethylated X chromosome. In the following PCR, a product will only be produced of the inactive X chromosome. The digestion was carried out using 2 µg DNA, 2 µl CfoI, 2 µl 10× enzyme buffer, and 6 µl Aqua dest, overnight at 37°C. Both the PCR reactions were performed in the same manner; per reaction 200 ng DNA, 100 ng forward (5'-TCCAGAATCTGTTCCAGAGCGTGC-3') and reverse (5'-GCTGTGAAGGTTGCTGTTCCTCAT-3') primers, 2.5 µl POL buffer, 2.5 µl DMSO, 1.5 µl dNTPs, 0.375 µl BSA, 0.1 µl Taq polymerase and 16.525 µl Aqua Dest were used. The samples were denatured for 2 min at 94°C followed by an amplification of 30 cycles of denaturation (1 min at 94°C), annealing (1.5 min at 58°C) and elongation (2 min at 72°C). PCR reactions were checked by electrophoresis on two percent agarose gels. The DNA samples of the fathers were used as internal controls for the restriction digest, since in males the X chromosome is always active and therefore there will be no PCR-product after the digestion reaction. Subsequently, all PCR products were run on an ABI 3100 capillary sequencer and analyzed using Genescan software (Applied Biosystems, Foster City, CA). The CAG repeat length was calculated from the size of the PCR products. A reference allele was sequenced to determine the relationship between the number of base pairs and the number of CAG repeats. First the genescans without the digestion step were compared

to determine the parental origin of the extra X chromosome for each of the Klinefelter patients. Next, we analyzed the results of the methylation assay to study the X chromosome inactivation pattern and assess any skewing in the Klinefelter patient. We defined skewing as a preferential inactivation of one of the two X chromosomes of more than 80%. Because analysis of the CAG repeat length did not allow identification of the parental origin in all cases, additional polymorphic X chromosomal DNA markers were analyzed in 16 cases.
For eight cases, we analyzed DXS1003, DXS6789 and DXS6797, and for another eight cases, we extended this set with DXS8049, DXS7132, DXS1001, DXS1193, DXS6807, DXS987 and DXS8090. The PCR reaction for these marker analyses contained: 50 ng DNA, 0.25 µl marker, 0.08 µl Taq gold, 1 µl buffer II, 25 mM MgCl2, 2 mM dNTPs and 5.67 µl Aqua Dest. The amplification was carried out by 10 min denaturation at 95°C followed by 33 cycles of denaturation (30 s at 95°C), annealing (30 s at 72°C) and elongation (30 s at 72°C). Again the PCR products were checked on agarose gels, run on an ABI3100 and analyzed using Genescan software.

Table S1. *ADI-R algorithm items sorted by labels and domains.*

	S: Qualitative Abnormalities in Reciprocal Social Interaction
	S1: Failure to use nonverbal behaviors to regulate social interaction
50	Direct Gaze
51	Social Smiling
57	Range of Facial Expressions Used to Communicate
	S2: Failure to develop peer relationships
49	Imaginative Play With Peers
62	Interest in Children
63	Response to Approaches of Other Children
64	Group Play with Peers (age < 10.0)
65	Friendships (age $\geq$ 10.0)
	S3: Lack of shared enjoyment
52	Showing and Directing Attention
53	Offering to Share
54	Seeking to Share Enjoyment With Others
	S4: Lack of socioemotional reciprocity
31	Use of Other's Body to Communicate
55	Offering Comfort
56	Quality of Social Overtures
58	Inappropriate Facial Expressions
59	Appropriateness of Social Responses
	C: Qualitative Abnormalities in Communication
	C1: Lack of, or delay in, spoken language and failure to compensate through gesture
42	Pointing to Express Interest
43	Nodding
44	Head Shaking
45	Conventional/Instrumental Gestures
	C4: Lack of varied spontaneous make-believe or social imitative play
47	Spontaneous Imitation of Actions
48	Imaginative Play
61	Imitative Social Play
	C2(V): Relative failure to initiate or sustain conversational interchange
34	Social Verbalization/Chat
35	Reciprocal Conversation
	C3(V): Stereotyped, repetitive or idiosyncratic speech
33	Stereotyped Utterances and Delayed Echolalia
36	Inappropriate Questions or Statements
37	Pronominal Reversal
38	Neologisms/Idiosyncratic Language
	R: Restricted, Repetitive, and Stereotyped Patterns of Behavior
	R1: Encompassing preoccupation or circumscribed pattern of interest
67	Unusual Preoccupations
68	Circumscribed Interests
	R2: Apparently compulsive adherence to nonfunctional routines or rituals
39	Verbal Rituals
70	Compulsions/Rituals
	R3: Stereotyped and repetitive motor mannerisms
77	Hand and Finger Mannerisms (score highest of 77/78)
78	Other Complex Mannerisms or Stereotyped Body Movements
	R4: Preoccupations with part of objects or non-functional elements of material
69	Repetitive Use of Objects or Interest in Parts of Objects
71	Unusual Sensory Interests (score highest of 69/71)

Table S2. *ADI-R algorithm items sorted by number.*

Item no	Item description	Domain label
31	Use of Other's Body to Communicate	S4
33	Stereotyped Utterances and Delayed Echolalia	R3
34	Social Verbalization/Chat	B2
35	Reciprocal Conversation	B2
36	Inappropriate Questions or Statements	B3
37	Pronominal Reversal	B3
38	Neologisms/Idiosyncratic Language	B3
39	Verbal Rituals	R2
42	Pointing to Express Interest	B1
43	Nodding	B1
44	Head Shaking	B1
45	Conventional/Instrumental Gestures	B1
47	Spontaneous Imitation of Actions	B4
48	Imaginative Play	B4
49	Imaginative Play With Peers	S2
50	Direct Gaze	S1
51	Social Smiling	S1
52	Showing and Directing Attention	S3
53	Offering to Share	S3
54	Seeking to Share Enjoyment With Others	S3
55	Offering Comfort	S4
56	Quality of Social Overtures	S4
57	Range of Facial Expressions Used to Communicate	S1
58	Inappropriate Facial Expressions	S4
59	Appropriateness of Social Responses	S4
61	Imitative Social Play	B4
62	Interest in Children	S2
63	Response to Approaches of Other Children	S2
64	Group Play with Peers (age < 10.0)	S2
65	Friendships (age > 10.0)	S2
67	Unusual Preoccupations	R1
68	Circumscribed Interests	R1
69	Repetitive Use of Objects or Interest in Parts of Objects	R4
70	Compulsions/Rituals	R2
71	Unusual Sensory Interests (score highest of 69/71)	R4
77	Hand and Finger Mannerisms (score highest of 77/78)	R3
78	Other Complex Mannerisms or Stereotyped Body Movements	R3

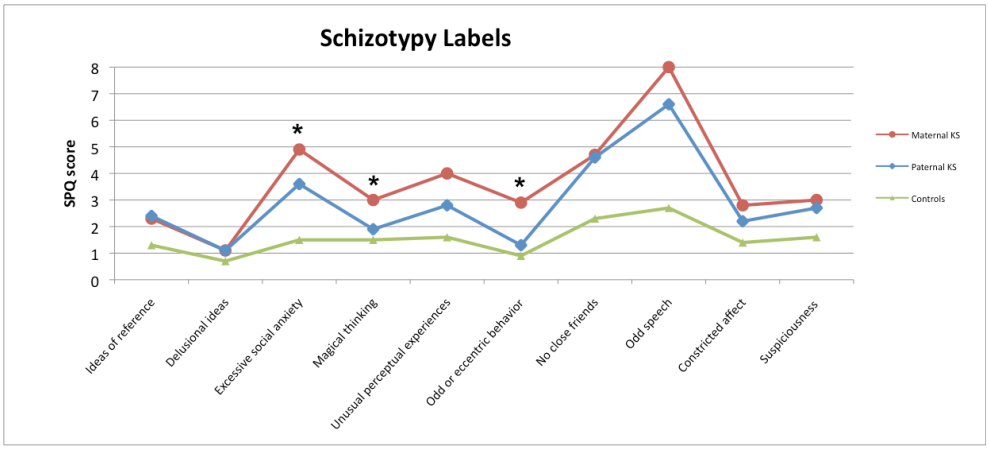


Figure S1. *Schizotypal Personality Questionnaire (SPQ) labels scores for the parent-of-origin groups in the older KS sample. * = p < 0.05, Helmert contrast analyses of maternal vs. paternal group scores.*

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CHAPTER 6

IN SEARCH FOR SIGNIFICANT COGNITIVE FEATURES IN KLINEFELTER SYNDROME THROUGH CROSS-SPECIES COMPARISON OF A SUPERNUMERARY X CHROMOSOME.

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ABSTRACT

The behavioral characterization of animals that carry genetic disorder abnormalities in a controlled genetic and environmental background may be used to identify human deficits that are significant to understand underlying neurobiological mechanisms.

Here we studied whether previously reported object recognition impairments in mice with a supernumerary X chromosome relate to specific cognitive deficits in Klinefelter syndrome (47, XXY). We aimed to optimize face validity by studying temporal object recognition in human cognitive assays.

Thirty-four boys with Klinefelter syndrome (mean age 12.01) were compared with 90 age-matched normal controls, on a broad range of visual object memory tasks, including tests for pattern and temporal order discrimination.

The results indicate that subjects with Klinefelter syndrome have difficulty in the processing of visual object and pattern information. Visual object patterns seem difficult to discriminate especially when temporal information needs to be processed and reproduced. Based on cross-species comparison, we propose that impaired temporal processing of object pattern information is an important deficit in Klinefelter syndrome.

The current study shows how cross-species behavioral characterization may be used as a starting point to understand the neurobiology of syndromal phenotypic expression. The features of this study may serve as markers for interventions in Klinefelter syndrome. Similar cross-species evaluations of standard mouse behavioral paradigms in different genetic contexts may be powerful tools to optimize genotype-phenotype relationships.

INTRODUCTION

Various naturally occurring human genetic disorders are associated with a broad mixture of cognitive and behavioral deficits. A challenge is to identify neurobehavioral features that are proximal to syndrome specific molecular dysfunctions to target treatment. Behavioral characterization of animals that carry syndromal genetic abnormalities in a controlled genetic background and living environment may be used to delineate significant deficits that eventually lead to the endpoint syndromal phenotypic expression. Recent studies have shown that mutant mice for Tuberous Sclerosis Complex (TSC) and Fragile X (FXS) capture cognitive deficits as observed in human patients [1-5]. Vice versa, a tempting hypothesis is that human analysis of features that resemble behavioral deficits observed in the animal model can facilitate the identification of close causal genotype-phenotype relationships. Furthermore, cross-species comparisons of well-validated mouse behavioral paradigms are important in order to estimate their face validity across different genetic contexts.

Here, we present an example of such a strategy in Klinefelter Syndrome [6]. KS is due to one (47, XXY) or more supernumerary X chromosomes. It is the most frequent aneuploidy in males with 1:700 affected [7,8]. The variable neurobehavioral phenotype in KS is probably caused by an interaction of hypoandrogenism and X chromosomal gene dosage effects. Subjects with KS often present with language-based learning problems, cognitive dysfunction and social difficulties with generally only mild intellectual disability [9-11]. These problems are accompanied by an increased vulnerability to a range of psychiatric disorders, in particular ADHD, autism spectrum disorders and psychosis [6,12-16].

Two mouse models for KS (male 41, XXY and the 41, XX^{Y*}) have been analyzed in depth [17-19]. Both models have been tested in behavioral tasks. The 41, XXY mice did not show impairments in sociability [18], which may have been expected by the frequently described social impairments in human KS [11]. 41, XX^{Y*} did also not differ from their wild type littermates in locomotion, exploration and anxiety related behaviors. Impairments were found in delayed conditional learning in a Pavlovian association, in which 41, XXY mice did learn the task but it took them significantly longer [19]. 41, XX^{Y*} mice were clearly impaired in a non-conditional Novel Object Task testing for memory recognition. Recognition of familiar objects failed when 41, XX^{Y*} mice were re-exposed to a familiar and two novel objects after a one-hour inter-exposure interval [17].

To translate these findings from KS mice models into human perspective, we compared 34 boys with KS with 70 age-matched normal controls, on various visual memory tasks with additional manipulations in order to mimic the components of the object recognition task in the 41, XX^{Y*} mice.

METHOD

SUBJECTS Thirty-four Boys with Klinefelter syndrome participated in the study (mean age 12.01, SD=3.26). The present study was part of a large study on cognition and psychopathology in Klinefelter syndrome at the two cooperating departments of the University of Leiden and the University of Utrecht. Written information was available at several centers for clinical genetics and pediatrics as well as on the webpage of the Klinefelter patients association to invite parents and children to participate in the study regardless of the presence of any psychological or behavioral problems. KS was confirmed by standard karyotyping, all boys had a 47, XXY karyotype none were mosaics. Seven boys were being treated with androgen supplements at the time of investigations. Three boys were on intramuscular testosterone injections (250mg/3wks regime), two others were taking testosterone undecanoate capsules (2 x 40 mg testosterone equivalent capsules), while two were on daily testosterone gel (50- 100 mg testosterone equivalent). Average treatment duration was 39.4 months, range 4-72 months, and average age at start of treatment was 13.6 yrs. All boys displayed marked androgen deficiency when treatment was started amongst other problems such as delayed puberty, chronic fatigue or accelerated growth. Age-matched normal control boys were included, that were recruited from regular schools in the region of Amsterdam and Utrecht, resulting in a control group of 90 boys. All children in the control group attended regular schools and represent a normal population sample, with average distribution of IQs. In the XXY group, the average total IQ for the KS was in the low average range (mean= 84.3, SD= 13.3) [20], with a range from 65-121.
The research was approved by the ethical committee of the University Medical Centre Utrecht and was in accordance with the declaration of Helsinki.

PROCEDURE AND MEASUREMENTS Assessment of cognitive functions in Klinefelter boys was performed at the department of Child and Adolescent Psychiatry of the University Medical Centre of Utrecht. KS subjects had visited the department prior to testing and had been instructed to minimize the influence of an unfamiliar testing environment versus the normal controls, who performed the tests at their school. All children were tested during morning hours in a quit room to avoid distraction.
Several visual recognition tasks of the Amsterdam Neuropsychological Tasks (ANT) program [21] were used. Test-retest reliability, construct-, criterion-, and discriminant validity of the computerized ANT-tasks are satisfactory and have extensively been described and illustrated elsewhere [21-23].

VISUO-SPATIAL PATTERN RECOGNITION After memorization of a predefined target pattern subjects have to detect this target pattern in a signal consisting of four patterns. Half the signals contain this target pattern (target signals) requiring the subject to press the 'yes'-key, the other half do not (non-target signals) in which case the 'no'-key should be pressed.

For 50% of the signals the distracter patterns look very similar to the target pattern ('similar' condition), while in the other 50% the distracter patterns are very dissimilar to the target ('dissimilar' condition). Because pattern similarity increases working memory demands, it is expected that the processing of 'similar' signals is slower and less accurate than 'dissimilar' signals. The task consists of 4×20=80 trials [24].

VISUO-SPATIAL SEQUENCING This visuospatial and spatial-temporal working memory task requires participants to remember a varying number of locations (minimally 3 out of 9 possible locations in a matrix presented on the screen). Participants are required to indicate the locations probed on the screen by mouse clicks on those locations, in the order they appeared on the screen (i.e., the correct temporal order). The difference between number of locations identified correctly (visuospatial WM) and number of (correctly identified) locations in the right order (visuospatial WM + visuotemporal WM) may be considered a measure of cognitive control [22].

SYMBOL RECOGNITION The memory search task employs a display load of four symbols and consists of three parts in which target set size (memory load) is increased from one to three symbols. Signals that contain the complete target set require a 'yes'-response. All other signals, also those containing an incomplete target set, require a 'no'-response. Reaction time (RT) to target signals is predicted to increase linearly with memory load, reflecting the prolongation of the memory search stage, with the slope of RT denoting the rate of memory search. In part 3, the presence of an insufficient number (0, 1 or 2) of target symbols ('distracters') in non-target signal affects performance in that RT and error rate will increase with the number of these 'distracters'. The redundancy of the information necessary to respond correctly to non-target signals that contain *zero* distracters increases with memory load. For example, when two of the four symbols in the signal do not belong to the target set, the subject knows it is a non-target signal and processing the remaining two symbols provides only redundant information [24].

Performance results were analyzed by repeated measures analysis of variance (ANOVA) with group (Klinefelters, controls) as between-subjects factor and task manipulations (e.g. pattern similarity, memory load) as within-subject factors, and age as a covariate. Similar ANOVAs were performed for the boys with KS only with Androgen treatment (yes, no) as between-subjects factor. Significance levels were set at p= 0.05

RESULTS

VISUO-SPATIAL PATTERN RECOGNITION Boys with KS made more errors than normal controls ($F(1,120)=33.72$, $p<.0001$, $\eta_p^2=.219$) (**Figure 1**, left). Group interacted significantly with pattern similarity ($F(1,120)= 6.47$, $p=.012$, $\eta_p^2=.051$), i.e. error rate increased

disproportionally for KS boys when pattern similarity was high. There was no effect of androgen treatment on errors made (p=.46))

VISUO-SPATIAL SEQUENCING Boys with KS identified less targets than normal controls ($F(1,101)=19.02$, $p<.0001$, $\eta_p^2=.158$). Group interacted significantly with scoring criterion ($F(1,101)=19.17$, $p<.0001$, $\eta_p^2=.160$), i.e. the number of correctly identified targets decreased more in boys with KS compared to normal controls when temporal order was (taken as) relevant (**Figure 1**, center). No effect of androgen treatment effect on performance was found (p=.26).

SYMBOL RECOGNITION Boys with KS made more errors on target signals ($F(1,122)=30.59$, $p<.0001$, $\eta_p^2=.200$), but differences in error rate between groups were not affected by memory load. Likewise, boys with KS made more errors on non-target signals ($F(1,122)=19.27$, $p<.0001$, $\eta_p^2=.136$), but differences in error rate between groups were not affected by the presence of distracter symbols. Signal redundancy led to a decrease in errors in the control group and an increase in errors for the boys with KS ($F(1,122)=17.55$, $p<.0001$, $\eta_p^2=.126$) (**Figure 1**, right). These results show that the central cognitive ability to maintain increasing informational load 'active' in working memory is equally developed in both groups, but boys with KS appeared to use a less efficient processing strategy. No effect of androgen treatment on task performance was observed (p=.83).

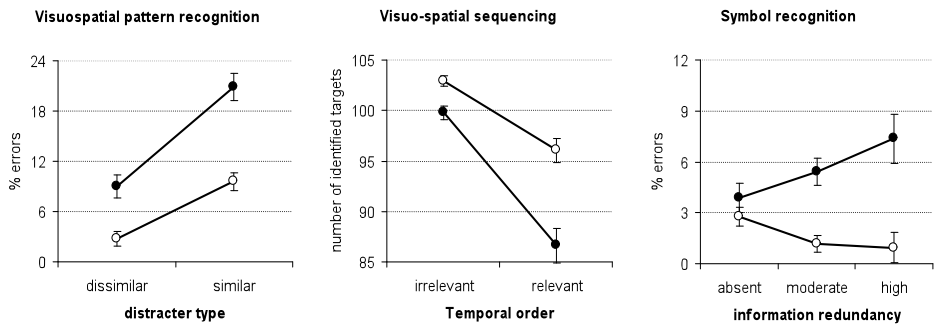

Figure 1. *Performance of 34 subjects with Klinefelter Syndrome (closed circles) and 90 controls (open circles) in different recognition subtasks: visuo-spatial pattern recognition, visuo-spatial sequencing, and symbol recognition. Error bars denote standard error of mean (SEM). Significance when p<0.05.*

DISCUSSION

The results indicate that subjects with KS have difficulty in the processing of visual object and pattern information, in particular when visual patterns are hard to discriminate. An additional cognitive weakness in boys with KS shows when working memory capacity is

challenged by the requirement to process and reproduce temporal information as well. These cross-species comparisons suggest that the processing of visual pattern information is a significant deficit in KS. Earlier studies have reported memory problems as important features in human KS but mostly in the context of language problems [9,10,16,25]. Verbal and auditory memory impairments are not easily feasible for cross species comparisons with mice. To provide face validity for cognitive processes downstream of sensory input, careful modeling of the mouse temporal object recognition task in humans is required. Olfactory and tactile information is a predominant source of non-verbal sensory input in mice, while humans mostly rely on vision for object recognition. Therefore, the current human tasks focused on visual discrimination capacity of familiar from unfamiliar object information and additionally challenged this capacity by temporal order.

These findings may help to provide further insights into the pathophysiology in KS. Another indication of importance of these traits would be their response to interventions in KS. There is no standard treatment in KS but testosterone treatment is frequently indicated in KS. Interestingly, the recognition performance in euploidic XY mice was shown to was shown to be associated with androgen levels [17]. The non-conditional Novel Object task showed a clear positive correlation between serum testosterone levels and learning performance in normal males but not in the 41, XX^{Y*} littermates. This could suggest that the human recognition deficits are also associated with a disturbance of androgenization. Cognitive changes after testosterone administration could be important to evaluate especially since the recognition deficits are likely to be related to behavioral problems like difficulty in decision-making and goal directed behavior. We did not find treatment effects on performance on any of the three recognition tasks. The sample size of seven androgen treated versus 27 non-androgen treated boys is underpowered to draw any conclusions about androgen treatment effects on these measures. Follow-up studies that combine endocrine monitoring to cognitive performance in KS are required to disentangle the complex interaction between cognition, androgen deficiency and androgen replacement effects. Visual pattern recognition performance may be associated with androgen deficiency, which could lead to an earlier or increased need for androgen replacement therapy. This could influence the evaluation of pure treatment effects on cognitive performance. Object pattern recognition performance may also be related to the developmental window, which would also influence the evaluation of treatment effects. Androgen treatment regimes may be explored in the context of cognitive enhancement although very careful monitoring of adverse metabolic effects would be required especially in younger individuals. The visual recognition deficits may deserve special attention in education and development and early specific training of visual cognitive skills may also improve outcome. Further cross-species experiments could explore underlying mechanisms such as low testosterone, inadequate hormone reception via the cerebral androgen receptor (AR) or X-linked gene dosage effects.

The current cognitive impairments may also be investigated from a neuroanatomical viewpoint as neural correlates may also be explored across species. Dere et al. have extensively reviewed the one-trial object recognition test in rodents [26]. It seems that in rodents the hippocampus and parahippocampal regions are not critical for one-trial object recognition per se. The medial prefrontal cortex seems to be important for the judgment of the sequence or order of object presentations. Several imaging studies in Klinefelter syndrome have found neuroanatomical gray and white matter abnormalities that relate to areas generally important for visual information processing, such as temporal lobe reductions [27-30]. It is not yet known which areas are important for visual information processing in KS. This might be a topic for further research. For instance, functional imaging studies in subjects with KS may indicate specific areas that can be further explored in the XXY mouse models. No studies have yet reported anatomical measurements in any of the XXY mouse models to be able to compare them to MRI findings in KS.

We have to address several weaknesses of the current study. The current study was part of larger study into the psychological consequences of Klinefelter syndrome. Our protocol did not include endocrine or bodily measurements; therefore, no further analysis of the relationship between cognitive and endocrine measures could be performed. To our knowledge, no studies have yet been able to study such a relationship in KS. Our sample may be prone to self-selection bias and may not be representative for average cognitive performance in KS. We chose not to control for general levels of intelligence in the present study, as this would likely have resulted in underestimation of problems with specific cognitive functions within this group [31]. Many studies have shown different areas of intact cognitive functioning in KS, which encourages the search for specific cognitive defects in the cloud of dysfunctions that often accompany genetic disorders such as KS [9]. We do realize that differences in the genetic background of mice and humans modify genotype–phenotype relationships across species and lead to the differential expression of certain KS-related phenotypes in mice and humans.

For now, the recognition of earlier encoded object and pattern information seems impaired and additionally challenged by temporal order both in humans and in mice with a supernumerary X chromosome. Based on recent mouse studies [17], the current results may provide translational parameters to relate endocrine but also non-endocrine factors to cognitive functioning. Earlier cross-species behavioral comparisons of learning and memory phenotypes have been crucial to put molecular findings into perspective and have led to development of treatments in TSC and FXS [2,5,32,33]. In addition to these examples, we aimed to show that careful cross-species translation of behavior itself could be a valuable starting point for further insights into neurobiological mechanisms. This study is an example how to make use of the increasing amount of behavioral findings of genetic

animal models and how to try to translate them to clinically important parameters. The amount of genomic signatures in behavioral disorders is rapidly increasing with a great demand of more specific behavioral readouts to refine genotype-behavioral phenotype relationships.

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CHAPTER 7

GENETIC MAPPING OF SOCIAL MEMORY COMPONENTS IN MICE SPECIFIES THE ROLE OF PCDH9 VARIANTS IN HUMAN PSYCHOPATHOLOGY.

Submitted.

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ABSTRACT

Diagnosing clinically relevant mouse behaviors is a critical next step in unraveling genetic and phenotypic heterogeneity of human psychiatric disorders, such as autism and schizophrenia. Here we aimed at the genetic mapping of quantitative measures of evolutionary conserved behaviors using advanced mouse strategies and hypothesized that specific genetic contributions to social memory in mice may be disrupted in human disorders of social behavior. Social discrimination assessment in chromosome substitution strains of mice revealed a different chromosomal origin underlying short-term and long-term memory. A quantitative trait locus (QTL) for long-term social discrimination was genetically mapped to a segment of mouse chromosome 14 harboring *Pchd9* (Protocadherin 9), a human autism candidate gene. *PCDH9* (human)/*Pcdh9* (mouse) belongs to the subfamily of non-clustered δ-protocadherins, which are expressed predominantly in the nervous system along spatiotemporally diverse expression patterns and are implicated in cell-cell adhesion processes. We generated *Pchd9*-deficient mice that showed consolidation deficits impairing long-term but not short-term social memory. These social memory impairments in *Pchd9*-deficient mice were associated with a brain-region specific reduction in cortical thickness in the higher order processing areas including secondary somatosensory cortices. These findings indicate that genetic mapping of essential behavioral components in mice can elucidate subphenotypes for genetic disruptions associated with psychiatric disorders, and suggest a conserved role of *PCDH9*/*Pcdh9* in cortical morphogenesis and long-term social memory.

INTRODUCTION

Unraveling the heterogeneity within human psychiatric disorders is hampered by individual variability in disease-related gene by environmental interactions at critical developmental stages in life. Functional studies in mice raised in controlled genetic and environmental conditions have revealed that a large number of candidate genes in psychiatric disorders are involved in different neurobiological processes. For example, mice deficient for a human candidate gene for these disorders, such as *Shank3*, *Disc1* or *Nrxn1,* showed neurobehavioral impairments [1-3]. Although mouse studies offer the possibility to disentangle the separate contributions of genetic and environmental factors to defined phenotypes, the question remains, which mouse phenotypes are clinically relevant to psychiatric complex diseases. This may be overcome through the controlled genetic mapping of behavioral components in mice that are crucial for adaptation and whose genetic regulation is likely to be conserved across species. For instance, most animals rely on the capacity to recognize and remember conspecifics to discriminate them from unknown conspecifics [4-6]. The ability to discriminate between familiar and unfamiliar individuals is critical for the stability of social groups [7]. Studies on the related nonapeptides oxytocin and arginine vasopressin have shown that social recognition and social memory formation are central to the modulation of complex social behaviors also in humans[8-10]. High-resolution QTL mapping of complex phenotypes such as social memory requires selective quantitative measures to infer the genetic contributions to detailed components of behavior. Temporal components of memory formation may be useful in this respect, as multiple temporal waves of protein synthesis are described in long-term social memory formation in mice, indicating that distinct memory formation processes occur in mammalian species[11]. Pharmacological studies have further demonstrated that drug treatments can modulate either short-term memory or long-term memory[12]. Genetic contributions to differential regulation of STM and LTM have been described in insects [13,14] but not yet in mammals.

We used mouse chromosome substitution strains (CSSs) for the genetic mapping of specific temporal components of social memory in mice by means of social discrimination. In each CSS, one chromosome of the donor strain A/J substitutes the corresponding chromosome of the background strain C57BL/6J (e.g., CSS1 has chromosome 1 of the A/J strain in a C57BL/6J genetic background)[15,16]. Phenotypic differences between a CSS and the C57BL/6J host strain indicate involvement of the substituted chromosome to the phenotype. The CSS panel has been designed as a simple yet powerful tool to identify small effect size QTLs affecting developmental, physiological and behavioral processes[17-20].

RESULTS

CHROMOSOMAL DISSECTION OF SOCIAL DISCRIMINATION COMPONENTS

A full panel of 21 CSSs, as well as the host and donor strain, (n = 8 per strain) were tested for temporal components of social memory in a two-day version of a social discrimination paradigm [21,22]. Mice had to discriminate a familiar from an unfamiliar conspecific following a 5-min training-test interval (TTI) (STM test) and a subsequent 24-hr TTI (LTM test). The average ratio of SD, the exploration of the novel intruder divided by the total time of exploration (r = Dn / (Dn+ Df), per strain was used as the outcome measure for discrimination performance (**Fig. 1a, b**).

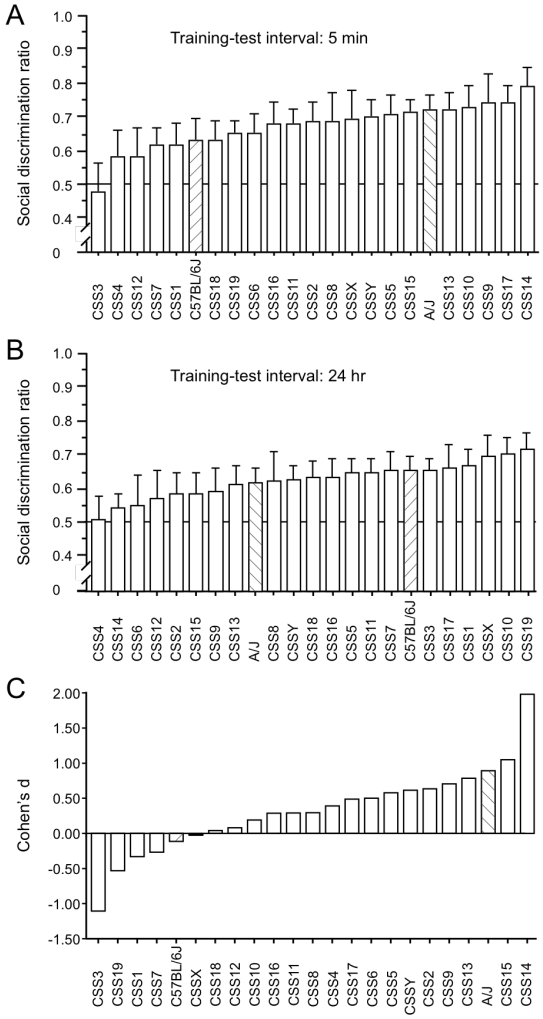

Figure 1. *Genetic dissection of social discrimination in a panel of mouse chromosome substitution strains (CSSs) and their parental strains following a 5-min (A) and 24-hr training test interval (TTI) (B), (A,B). Average ratios of social discrimination calculated as the exploration of the novel intruder dived by the total time of exploration (r = Dn / (Dn+ Df)) (n=8 per strain); cut-off ratio = 0.5. Indices of dissociated memory performance (C) calculated as Cohen's d [Mean_5 min -Mean_24-hr]/[(Pooled standard deviation]), the effect size of the difference between 5-min and 24-hr TTI SD performance.*

To select CSS strains with selective temporal memory impairments, indices of dissociated discrimination performance were used that were analyzed as the effect size of the difference between 5-min and 24-hr TTI SD performance (Cohen's *d* = [Mean_5 min -Mean_24 hr]/[(Pooled standard deviation]) (**Fig. 1c**) (**Table 1**).

Table 1: Genetic dissection of social discrimination in a panel of mouse chromosome substitution strains (CSS) and their parental strains (C57BL/6J and A/J) following the 5-min and the 24-hr training test interval.

	5-min Training-Test Interval				**24-hr Training-Test Interval**				**5-min/24-hr Comparison**	
Strain	**ratio**	**STD**	**SEM**	***p***	**ratio**	**STD**	**SEM**	***p***	**Δ ratio**	***Cohen's d***
CSS3	0.48	0.23	0.08	0.40	0.66	0.09	0.03	0.00	-0.18	-1.10
CSS19	0.66	0.10	0.03	0.00	0.72	0.13	0.05	0.00	-0.06	-0.53
CSS1	0.62	0.17	0.06	0.04	0.67	0.14	0.05	0.00	-0.05	-0.33
CSS7	0.62	0.12	0.05	0.03	0.65	0.14	0.06	0.02	-0.03	-0.27
C57BL6/J	0.63	0.18	0.06	0.04	0.65	0.13	0.05	0.01	-0.02	-0.11
CSSX	0.69	0.22	0.09	0.04	0.70	0.12	0.06	0.01	0.00	-0.01
CSS18	0.64	0.15	0.05	0.02	0.63	0.15	0.05	0.02	0.01	0.04
CSS12	0.59	0.22	0.08	0.15	0.57	0.24	0.08	0.22	0.02	0.08
CSS10	0.73	0.16	0.06	0.00	0.70	0.13	0.04	0.00	0.03	0.19
CSS16	0.68	0.19	0.07	0.02	0.64	0.11	0.05	0.02	0.04	0.29
CSS11	0.68	0.12	0.04	0.00	0.65	0.12	0.04	0.01	0.04	0.29
CSS8	0.69	0.24	0.08	0.03	0.62	0.25	0.09	0.12	0.07	0.30
CSS4	0.59	0.21	0.07	0.14	0.51	0.18	0.07	0.44	0.08	0.39
CSS17	0.74	0.15	0.05	0.00	0.66	0.18	0.06	0.02	0.08	0.49
CSS6	0.66	0.16	0.06	0.01	0.55	0.26	0.09	0.29	0.10	0.50
CSS5	0.71	0.12	0.06	0.01	0.64	0.09	0.05	0.01	0.06	0.58
CSSY	0.70	0.13	0.05	0.00	0.63	0.10	0.04	0.00	0.07	0.61
CSS2	0.69	0.15	0.05	0.00	0.59	0.18	0.06	0.10	0.10	0.64
CSS9	0.74	0.23	0.08	0.01	0.59	0.21	0.07	0.13	0.16	0.70
CSS13	0.72	0.13	0.05	0.00	0.61	0.15	0.05	0.04	0.11	0.79
A/J	0.72	0.11	0.04	0.00	0.61	0.13	0.05	0.02	0.11	0.89
CSS15	0.72	0.10	0.04	0.00	0.59	0.14	0.06	0.10	0.13	1.05
CSS14	0.79	0.15	0.05	0.00	0.55	0.10	0.04	0.13	0.25	1.98

Average ratios (av ratio) of social discrimination calculated as the exploration of the novel intruder dived by the total time of exploration (r = Dn / (Dn+ Df)) (n=8 per strain). One-sample t-test, cut-off ratio = 0.5 (=equal amount of time of social investigation towards familiar and novel intruder); p-values are based on 1-sample-t-test. CSS strains are ranked by Cohen's d [Mean_5 min -Mean_24 hr]/[(pooled standard deviation]), which expresses the effect size of the difference between the 5-min and the 24-hr TTI SD performance. Δ av ratio = Av ratio (5-min – 24-hr). SEM = standard error of the mean, STD = standard deviation.

CSS3 and CSS14 displayed the strongest temporal dissociation in social discrimination performance in opposite directions, with relatively impaired STM and intact LTM performance in CSS3 (Cohen's *d* = -1.10, lowest value of the panel) and the inverse result in CSS14 with intact STM and impaired LTM (Cohen's *d* =1.98, highest value of the panel).

No significant differences were found in locomotor activity, aggressive behaviors and non-social behaviors between CSS3, CSS14 strains and the C57BL/6J controls, indicating that the impaired social discrimination was not caused by the overrepresentation of non-social behaviors and/or by the lack of social investigation (data not shown). The dissociation of temporal social memory components in CCS 3 and CSS14 is also not likely explained by primary sensory deficits, which would be expected to lead to non-specific discrimination impairments affecting both long-term and short-term interval. The observed chromosomal dissociation of temporal social discrimination capacity (**Fig. 2A**) also segregated with temporal object discrimination capacity in CSS3 and CSS14. CSS3 did not show significant object OD after 1-hr TTI (t =1.488 p = 0.148, df = 7) but demonstrated successful OD after 24-hr OD (t = 3.804, p = 0.0067, df = 7). In contrast, CSS14 performed successful OD at 1-hr TTI (t= 3.939, p = 0.0056, df = 7) but lacked significant OD after 24 hr TTI (t= 1.176, p = 0.277, df = 7) while C57BL/6J showed successful OD after both TTIs (**Fig. 2B**).

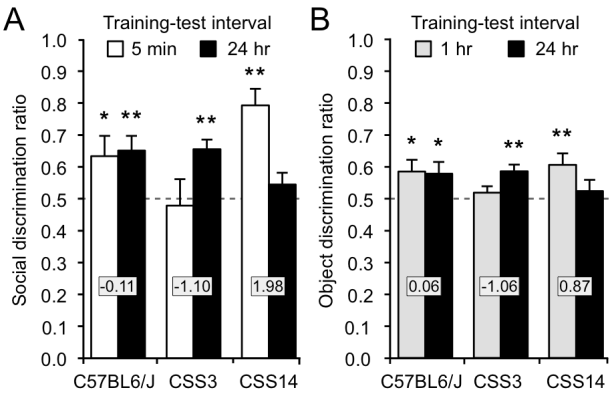

Figure 2. *Social (A) and Object (B) Discrimination in CSS3, CSS14 and the parental C57BL6/J strains as a function of training-test intervals One-sample t-test, cut-off ratio = 0.5; *p < 0.05,**p < 0.01. Values on top of bars indicate values for Cohen's d [Mean_STM - Mean_LTM]/[(Pooled standard deviation]), i.e. the effect size of the difference between 5-min and 24-hr TTI for social and 1-hr and 24-hr TTI for object discrimination performance.*

Spatial memory, measured in the Morris mater maze (MWM)[23], was intact in these strains and, therefore, this type of memory seems not associated with the distinct temporal discrimination impairments in CSS3 and CSS14 (**Fig. 3**).

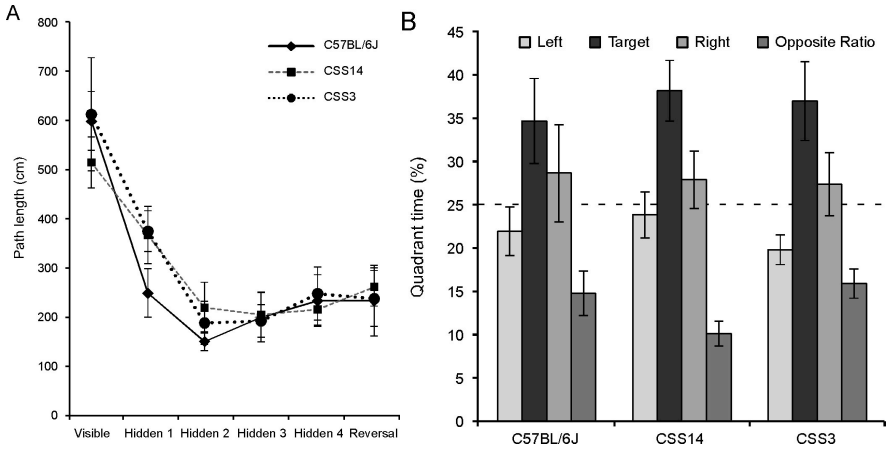

Figure 3. *Morris water maze results for selected CSS strains and the parental C57BL6/J strain. Path length, i.e. distance traveled from the trial start location to the platform in cm (A) and % of total time spent in each quadrant (B). Hidden_1 through Hidden_4 (in panel A) refers to the consecutive testing days that the mice had to search for the hidden platform.*

FINE MAPPING OF SOCIAL DISCRIMINATION COMPONENTS

CSS14 had the highest Cohen's D for temporal memory dissociation and was chosen for genetic mapping of the long-term discrimination locus on mouse chromosome 14 for. An F_2-progeny of 192 male (derived from an F_1 intercross of C57BL/6J-Chr 14^A/NaJ X C57BL/6J hybrids) were tested consecutively for social and object discrimination according to the paradigms used for CSS3 and CSS14 (fixed order). Following genotyping the F_2 individuals, QTL analysis using 18 markers covering chromosome 14 on the CSS14-F_2 population revealed several significant logarithms of odds (LOD) scores for both LTM SD and LTM OD. Three chromosomal segments were identified for LTM SD and 2 segments were identified for LTM OD (**Fig. 4**).

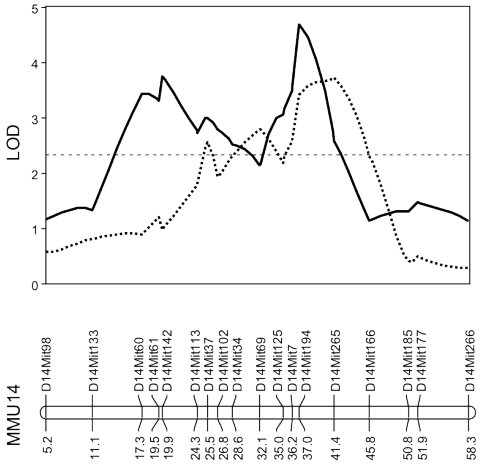

Figure 4: *Genetic map (down; mouse chromosome 14; the male revised Shifman map and LOD score plot (upper) for social discrimination ratio of novel intruder (doted line) and object discrimination ratio of novel object (solid line) following a 24-hr training-test interval in the F_2 population (n = 192 males). The dashed horizontal line represents the threshold value (2.32) of the LOD score considered significant for linkage.*

The highest peak for LTM SD had a LOD score of 3.72 with a 1.0 LOD support interval of 91.328.224 - 106.238.492 bp that showed partial overlap with a QTL segment for LTM OD. In this region we searched for all known genes containing known SNPs (with http://www.jax.org/phenome/snp.htm) between the progenitor strains of the CSS panel (A/J and C57BL/6J). This put forward 2 genes with a non-synonymous SNP, namely Kelch-like 1 (*Klhl1*) and protocadherin 9 (*Pcdh9*). *Klhl1* has been predominantly associated to motor neuron disease mainly affecting specific cerebellar motor function without report of cognitive or other behavioral impairments [24,25]. *Pcdh9* belongs to the family of non-clustered delta-protocadherins (δ-*Pcdh*s) which are predominantly expressed in the CNS along specific spatial and temporal patterns and are known to regulate synaptic function and cortical morphology[26,27]. Disruption of δ-*Pcdh* functioning has been suggested to lead to cognitive and behavioral impairments [27]. Genetic associations of several δ-*PCDH* family members have been reported with autism, schizophrenia, Alzheimer's disease and mental retardation have been reported [28-32]. With regards to *PCDH9*, de novo deletions of *PCDH9* loci have been identified in autism patients, a finding that has been confirmed in different samples [33,34]. Genetic mapping of behavior in dogs has also implicated *PCDH9* as candidate gene involved in canine social development[35].

BEHAVIORAL CHARACTERIZATION OF *PCDH9*-KO MICE

To confirm a role for *Pcdh9* in LTM SD, homozygous *Pcdh9* gene knockout mice (*Pcdh9* $^{-/-}$) were generated through a targeting vector that was designed to produce a null mutation of *Pcdh9* by replacing the whole first exon (**Fig. 5**).

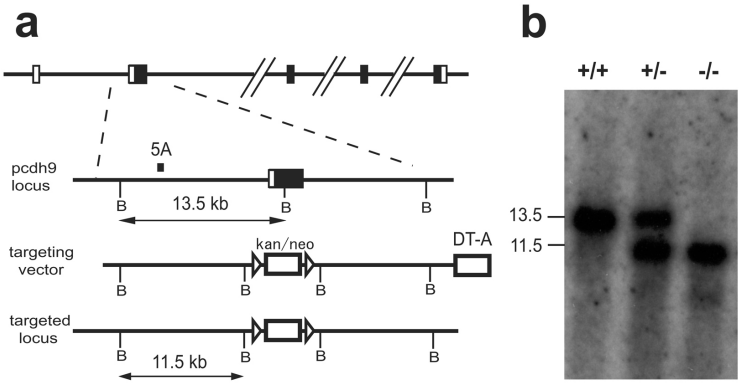

Figure 5. *Targeting strategy and phenotype of pcdh9 mutant mice. (A) The second exon including the start codon is deleted. The locus is indicated by restriction sites. Exons are depicted by boxes. Positions of probes (5A) for genotyping are also shown. B, BamHI. (B) Southern blot analysis with 5A probe defines the deletion of the wild type allele (13.5kb) and mutant allele (11.5kb).*

Pcdh9$^{-/-}$ mice and wild type controls were tested in the OD and SD paradigms used in the CSS14 strain and CSS14-F_2 population. Similar to the CSS14 phenotype, *Pcdh9*$^{-/-}$ mice showed impaired SD at 24-hr TTI (t = 1.11, p = 0,311, df = 6 one sample t-test) while SD was intact at 5-min TTI (t= 2.92, p= 0.027, df = 6,) (**Fig. 6A**), whereas SD performance was intact in WT mice at both, the 5-min and 24-hr TTI (t = 2.55, p = 0.038, df = 7,; t = 3.43p = 0.011, df = 7) (**Fig. 6A**). In contrast to the CSS14 phenotype, object discrimination performance following 1-hr and 24-hr TTI was unimpaired in *Pcdh9*$^{-/-}$ and WT controls (t = 2.72, p = 0.035, df = 6; t = 2.33, p = 0.04, df = 7,), indicating that *Pcdh9* affects social but not non-social LTM (**Fig. 6B**).

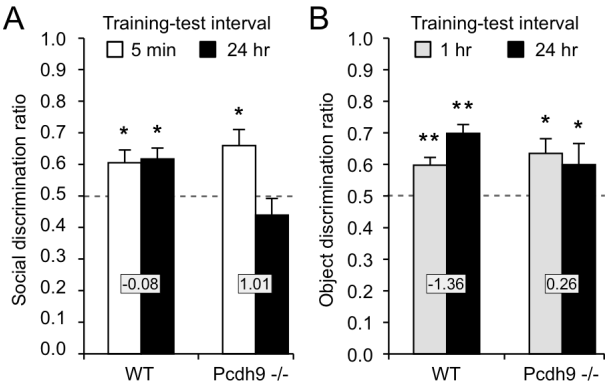

Figure 6. *Social (A) and Object (B) Discrimination in Pcdh9-knockout (-/-) versus wild-type controls as a function of training-test intervals (one-sample t-test, cut-off ratio = 0.5; *p < 0.05, **p < 0.01). Values on top of bars indicate values for Cohen's d [Mean_STM - Mean_LTM]/[(Pooled standard deviation]), i.e. the effect size of the difference between 5-min and 24-hr TTI for social and 1-hr and 24-hr TTI for object discrimination performance.*

This context specific dissociation (social versus non-social) of long-term discrimination capacity is consistent with the missing correlation in the CSS14 F_2 animals between their individual LTM SD and OD readouts (r = 0.034, p = 0.695) (**Fig. 7**). Partitioning of A/J chromosome 14 seems to further dissociate genetic contributions to long-term social and non-social discrimination capacity at the level of memory-context specificity, i.e. social versus non-social cues.

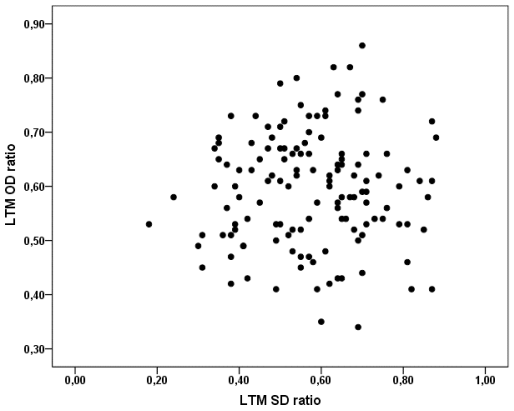

Figure 7. *Lack of association between social and object discrimination (OD) LTM (24-hr TTI) ratio readouts.*

FUNCTIONAL CHARACTERIZATION OF *PCDH9*-KO MICE

The suggested role of *Pcdh9* in cortical morphogenesis and synaptic function [27,36,37] was further analyzed from an anatomical point of view. The overall brain size in the adult was not different between the wild type and *Pcdh9*$^{-/-}$ mice, but Nissl staining showed a regional difference in cortical thickness in the neocortex. It was slightly but significantly decreased in a part of cortical association areas of the *Pcdh9*-deficient cortex, including the secondary somatosensory and visual areas (**Fig. 8**).

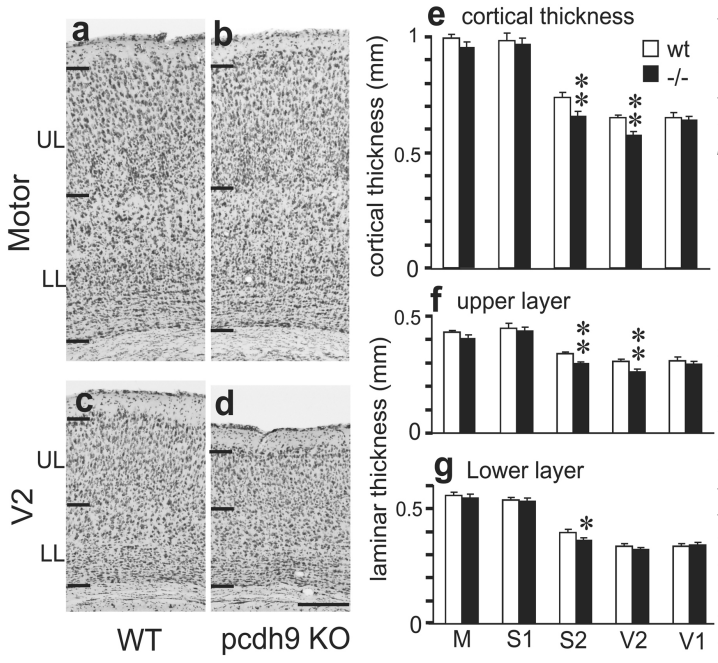

Figure 8. *The cortical structures in 6-week-old wild type and homozygous Pcdh9-knockout (-/-) mice. Nissl staining sections of the neocortex in WT (a, c) and pcfh9 KO (b, d). These sections were obtained from the motor cortex (a, b) and secondary visual cortex (c, d). Quantitative analysis of thickness of the cortex (e), upper (f) and lower layers (g) of each cortical region. Open and filled columns represent WT and pcdh9 KO, respectively. UL; upper layer, LL; lower layer, M; motor cortex, S1; primary somatosensory cortex, S2; secondary somatosensory cortex, V1; primary visual cortex, V2; secondary visual cortex. Bar in (d) represents 0.3 mm and is applied to (a-d). *p<0.05, **p<0.01.*

A quantitative analysis of cell number further demonstrated that this decrease was attributable to the increased cell density rather than neuronal number. Some of the non-clustered protocadherins have been shown to be expressed in specific cortical layers [38,39]. Similarly, *Pcdh9* was also expressed strongly in layer 4 of the developing brain, although weakly expressed in layer 6 (**Fig. 9**).

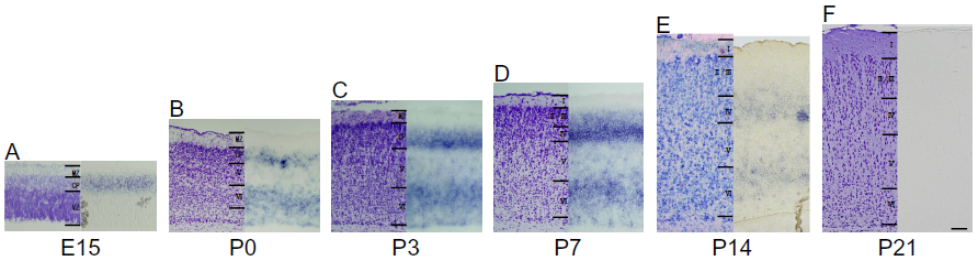

Figure 9. *Expression patterns of pcdh9 during development in the neocortex (A-F). The pcdh9 mRNA was analyzed in the occipital neocortex of mice. Nissl staining is shown on the left, in situ signals are shown on the right of each panel. Pcdh9 expression is restricted to layers 4 and 6 in the developing cortex. Pcdh9 signal increased after birth and peaked at P7, thereafter declined and was absent at P21 (F). In the later stage, the expression of pcdh9 is decreased. MZ, marginal zone; PPL, preplate; IZ, intermediate zone; VZ, ventricular zone; CP, cortical plate; I, II, III, IV, V, VI, cortical layers. Bar represents 100 µm.*

Accordingly, the reduction of cortical thickness in the *Pcdh9*$^{-/-}$ mice was more apparent in the upper layers. We also conducted an aggregation assay using *Pcdh9*-expressing transfectants and confirmed that *Pcdh9* has an adhesive property (**Fig. 10**) as suggested in other protocadherins[27]. Considering the fact that PCDH9 is accumulating in synapses in cortical dissociated cell culture (**Fig. 11**), the increased cell density and the reduction of cortical thickness may probably due to alterations in neuropil development.

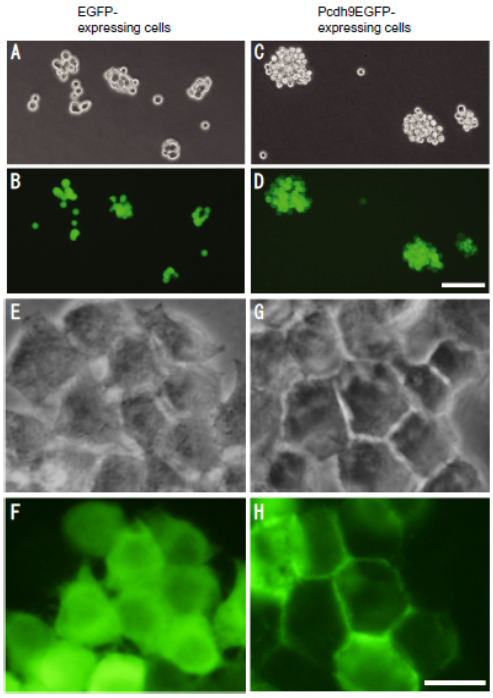

Figure 10 *Adhesive activity of pcdh9-expressing cells. Panels A and B show EGFP-expressing cells, and panels C and D show pcdh9-EGFP-expressing cells. Pcdh9-EGFP-expressing cells adhere to each other and form larger aggregates than EGFP-expressing cells. Panel G shows localization of pcdh9-EGFP in mouse Neuro2A cells. Pcdh9-EGFP signals are localized to the boundary of cells, whereas EGFP signals distribute throughout the cells. Scale bar indicates 50 µm (A-D), 20 µm.*

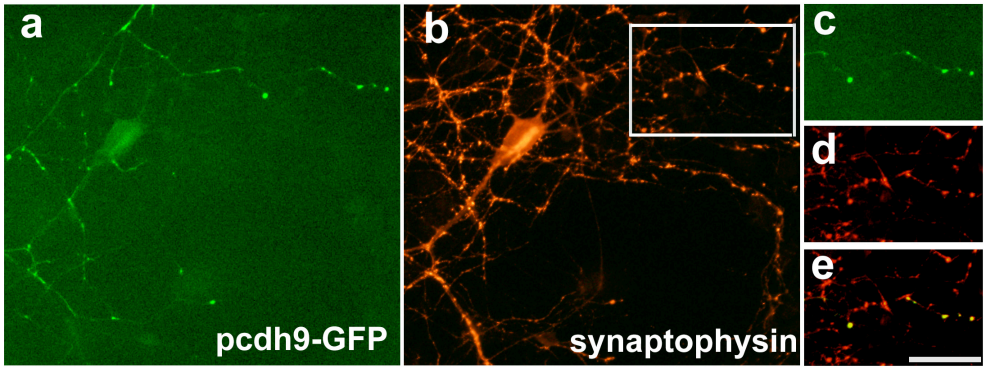

Figure 11. *Localization of PCDH9 into synapses. Cortical cells are dissociated from embryonic brains and transfected with a pcdh9-egfp expression vector. After a few weeks in vitro, the cultures were fixed and subjected to immunocytochemistry with an antibody against synaptophysin. The signal was visualized by cy3-conjugated secondary antibody (b, d) and compared to EGFP labeling (a, c). (d) shows a merged image. Bar represent 20 um.*

DISCUSSION

These findings indicate a genetic basis for the temporal behavioral expression of social discrimination in mice. *Pcdh9*, a positional and functional candidate gene within a mapped QTL interval for LTM social discrimination, seems to have early developmental effects on the cortical integrity of higher order processing areas in mice which may be conserved in humans. This interpretation is supported by the observed deficits in *Pcdh9*$^{-/-}$ mice in consolidation of earlier acquired social information downstream from encoding of information. Reduction of cortical thickness is a common anatomical finding in human psychiatric disorders with disturbed social behavior[40-42] and has been found specifically in the secondary processing areas of the cortex[43]. Cortical thinning due to increased cell density as seen in the Pcdh9$^{-/-}$ cortices has also been described in post-mortem studies of psychiatric disorders, such as schizophrenia[44-47]. Our findings indicate that loss of *Pcdh9* function results in a localized loss of cortical integrity that reduces sensory processing of information relevant for social adaptation. This loss of integrity probably relates to the observed loss of adhesive property of *Pcdh9*, as earlier suggested for other *Pcdhs*[27]. Overall, complementary genetic mapping of clinically relevant mouse phenotypes may bridge the gap between genotypes and phenotypes in human psychiatric disorders, leading to greater understanding of the underlying biology.

MATERIALS AND METHODS

ANIMALS

Breeding pairs for C57BL/6J, A/J and all 21 CSSs (autosomal, X and Y) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Colonies of each strain were subsequently bred in-house. Animals were kept in a controlled 12-hr light-dark cycle (light on 07:00–19:00; light intensity of 60 lx) at a room temperature of 22 ± 1°C and a humidity of 60%. Food and water were available ad libitum (2111 RMH-TM diet; Hope Farms/Arie Blok BV, Woerden, The Netherlands). All animals were housed in standard cages (Macrolon type II) with sawdust bedding and a paper tissue for nest building. All experiments were performed according to the institutional guidelines of the University Medical Center (UMC) Utrecht. Four weeks after birth, mice were weaned and socially housed (2–4 same sex littermates per cage). Litters used were derived from multiple breeding pairs. The animals were subsequently maintained in a 12-hr light/12-hr dark cycle (lights off at 13:00). The ethical committee for animal experimentation of the UMC Utrecht approved all experimental procedures that were in full compliance with the European Council Directive (86/609/EEC). All mice were between 3-5 months old at the start of the tests and were ear-marked for identification purposes. Only male mice were used in the

current study, as earlier pilot studies in our laboratory revealed SD impairments in the parental C57BL/6J strain in female mice (unpublished data).

SOCIAL DISCRIMINATION TEST

We tested 8 animals per parental (C57BL/6J and A/J) and CSS. Animals from each CSS and parental strains were tested across the 9 months screening period to control for potential environmental or seasonal variation. All experiments were performed in the second half of the light phase at a light level of 30-35 lx. A lid with air holes was placed on top of the experimental cages to minimize external olfactory stimuli that potentially could interfere with the experiment. A modified 2-day version of the SD test was used[22]. Test animals were initially exposed to an A/J intruder for 2 min and then, after a training-test interval (TTI) of 5 min and of 24 hr, exposed for 2 min to the familiar intruder and a novel A/J intruder. A/J mouse intruders were matched for age and gender. Clean cages (Macrolon type II) were used after each exposure. Cages were filled with 400 ml wood chips. To avoid any sensory contact prior to and between testing, intruders and test animals were housed in separate rooms for at least 2 weeks before the start of the experiment. After completion of each social interaction trial, each mouse was returned to its home cage. Behavior was recorded by a camera positioned overhead. Social investigation was defined as the total time mice engaged in social sniffing, anogenital sniffing and allogrooming. These behaviors were manually analyzed from the video recordings using The Observer software version 1.1.123 (Noldus Information Technology, Wageningen, The Netherlands). Other non-social behaviors were also measured during the social interactions: absence of motor activity, rearing and autogrooming. Any aggressive act between animals during the social interaction sessions resulted in the immediate cessation of the experiment and the data from this session were excluded from further analysis. Data from mice with a social investigation time of less than 5-sec during the first or second exposure were excluded from the analysis. Less than 2% of the mice tested had to be excluded based on this criterion. The ratio of social discrimination was calculated as the duration of exploration of the novel intruder divided by the sum of the time spent exploring both the novel and familiar (D_f) conspecifics: $r = D_n / (D_n + D_f)$. A value of this ratio of 0.5 indicates that the mouse did not distinguish the novel from the familiar intruder, i.e. did not display social discrimination. To estimate the relative magnitude of the difference in ratio after 5 min and 24 hr training test interval (TTI), Cohen's *d* effect size coefficients was used. The Cohen's *d* score was here defined as the difference between the means for 5-min TTI and 24-hr TTI divided by the pooled standard deviation. The familiar versus novel intruder novel intruder ratios of discrimination were analyzed statistically per interval with two-tailed one-sample *t*-test (i.e. testing for deviation from 0.5). Ratios significantly larger than 0.5 indicated successful SD, where alfa was set at p= 0.05 for 2 strain comparisons. SPSS 12.0 for Windows statistical package were used for all behavioral analyses.

OBJECT DISCRIMINATION TEST

OD testing took place in empty transparent plastic cages (42.5 x 26.5 x 18 cm; length x width x height) covered with a plastic transparent lid. The light level in the test cage was 30-35 lx. Eight animals were tested per strain, including the parental C57BL/6J strain. Test animals were initially exposed to 2 familiar objects for 5 min and then, after a training-test interval exposed for 5 min to the familiar object and a novel object.

The objects used were made of glass, metal or plastic and were of sufficient height to prevent mice climbing on the objects. The test cages and objects were cleaned with a mild detergent (10% Trigene solution) between each trial. Preference for the objects used had been tested prior to the study and was not observed (data not shown). Novel objects were counterbalanced to avoid effects of spatial preference. Behavior was recorded by a camera positioned overhead. After each trial, the mouse was returned to its home cage. Object exploration was defined as approaching the object and having physical contact with the object; sniffing the object, turning the head towards it, touching it with the forepaws. This was visually scored using the Observer software. 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 during the first or second exposure were excluded from the analysis.

As with the SD test, the ratio of object discrimination was calculated as the duration of exploration of the novel object divided by the sum of the time spent exploring both the novel and familiar (D_f) objects: $r = D_n / (D_n + D_f)$. Equivalent to the SD paradigm, a value of this ratio of 0.5 indicates that the mouse did not distinguish the novel from the familiar object, i.e. did not display Object Discrimination.

Given the different cues and motivations of the object discrimination paradigm versus the social discrimination paradigm, we investigated the most informative TTI for OD in pilot studies of C57BL/6J mice with 3 groups of different TTI all defined as STM intervals 1-3 between the first and second exposure (5, 30 or 60 min); all groups subsequently had a 24 hr interval. C57BL/6J mice could significantly discriminate the novel object from the familiar at each time point in their second 24hr exposure. Optimal discrimination in the 24-hr TTI was shown after 60 min TTI, therefore 1-hr and 24-hr timepoints were selected for testing.

To estimate the relative magnitude of the difference in ratio after 1-hr and 24 hr training test interval (TTI), Cohen's *d* effect size coefficients was used. The Cohen's *d* score was here defined as the difference between the means for 1-hr TTI and 24-hr TTI divided by the pooled standard deviation. The familiar versus novel object ratio of discrimination were analyzed statistically with two-tailed one-sample *t*-test (i.e. testing for deviation from 0.5). Ratios significantly larger than 0.5 indicated successful OD, where alfa was set at $p = 0.05$ for 2 strain comparisons. SPSS 12.0 for Windows statistical package were used for behavioral analyses.

MORRIS WATER MAZE (MWM)

This standard and commonly used spatial navigation task requires mice to locate a submerged target platform by means of triangulation using visual extramaze cues. Over a series of daily trials, each mouse had to locate a submerged platform in the pool. To assess retention of spatial memory, the platform was subsequently removed in the probe trial (after a series of trials with the hidden platform) and the time spent in platform location (target quadrant) was measured). A pool (made of grey PVC, diameter 77 x 75.5 cm and depth of 35.5 cm) was placed in the center of the room and was filled with tap water (21.5 ºC). The light intensity was 21-24 lx and approximately 28 lx at the walls of the maze. A transparent plastic square platform, 6 x 6 x 21 cm, was placed in the pool (1 cm above the water level for visible training and 1 cm below the water level for all hidden and reversal trials). A set of visual extramaze cues was placed on the walls surrounding the pool. The trials were recorded by a camera positioned overhead with a CCTV feed through to the adjacent room to minimize disturbance of the mouse during the test. The activity of each mouse (distance swam and swim speed) was tracked using Ethovision v.3 (Noldus Information Technology, Wageningen, The Netherlands). Water-soluble, non-toxic white paint was used to color the water to hide the platform from sight below the water level and to aid tracking. In Ethovision, the pool was virtually divided equally into 4 quadrants. Each quadrant was used to determine the placement of the mouse into the maze, which was run in a pseudorandom order for successive trials.

The test animals of selected strains (CSS3, CSS14, n = 10) and control strain, (C57BL/6J, n = 10) were individually placed in the pool in either of 4 equidistant quadrants according to a balanced (randomized) schedule. Trials started at the moment the animals entered the water and each mouse was given a maximum of 60 s to find Thereafter, the mouse was guided to the platform and returned to its home cage. Once mice placed all 4 paws on the platform, the latency was recorded. Mice with a latency of 60 s for 2 or more trials a day were excluded from the analysis. Morris water maze experiments were carried out over 6 consecutive days, and per day each mouse underwent 2 sessions, each consisting of 4 trials per session except on day 1 with one afternoon session only. The inter-trial interval was approximately 6 min. Latency to reach the platform was recorded for each mouse when all four paws were on the platform. On day 1 the visible platform training was performed. On days 2 and 3 two sessions of hidden platform training occurred. Day 4 started out with a hidden platform training followed by a probe trial without escape platform in the 6th session. On day 5 hidden platform re-training occurred (after probe trial disruption) in two sessions with the same escape platform position as used before. On day 6 a reversal test was performed in two sessions in which the platform was located in the opposite quadrant to test for cognitive flexibility as used before.

The mean latency of each animal was calculated from the trials within one session. A significant decrease of the mean latency to enter the escape platform indicated a positive

learning effect of mice across sessions. The time spent in each quadrant and the ratio of each quadrant (time spent in each quadrant divided by total time [60 s] given in %) was analyzed with two-tailed paired Student's t-test. SPSS 12.0 for Windows was used for this analysis. One-way analysis of variance was used to analyze inter trial differences.

GENERATION OF CSS14-F_2 POPULATION

The F_1 generation was derived by reciprocal matings of C57BL/6J and C57BL/6J-Chr 14^A/NaJ animals. The F_1 hybrids were intercrossed (brother x sister mating), producing 192 male F_2 segregants. A separate control group of C57BL/6J mice was used. The animals were tested at the same age range as the CSS mice for SD and OD consecutively in this order, with a minimum of one and a maximum of two weeks between tests.

QTL MAPPING

DNA SAMPLES

Spleens were dissected after decapitation and kept in -80°C until used for DNA isolation. Total genome DNA was isolated using standard DNA isolation procedures for tissue. Isolated DNA was re-suspended in TE buffer (10 mM Tris, 0.2 mM EDTA, pH 8.0) at a concentration of 40 ng/µl. The DNA concentrations were determined by measuring the A260 with a PU8700 UV/Visible spectrophotometer (Eppendorf BioPhotometer). DNA samples were stored at 4°C.

PCR AMPLIFICATION OF MOUSE CHROMOSOME 14 MICROSATELLITE LOCI

For generating a genetic map of chromosome 14, eighteen microsatellites were chosen from the mouse genome database (Mouse Genome Informatics, http://www.informatics.jax.org/). These markers were dispersed throughout mouse chromosome 14. Primers flanking these microsatellites (MIT mouse MapPair primers) were purchased from Sigma-Aldrich. Thermal cycling was performed in a DNA Engine (Peltier Thermal Cycler-200, MJ Research). For the following fifteen microsatellites: D14Mit98, D14Mit133, D14Mit60, D14Mit61, D14Mit142, D14Mit113, D14Mit37, D14Mit102, D14Mit34, D14Mit69, D14Mit125, D14Mit7, D14Mit194, D14Mit265, D14Mit166, D14Mit177, D14Mit185, D14Mit266 the genomic DNA samples were amplified according to the supplied protocol accompanying the microsatellite primers using Taq polymerase (GE-Healthcare)). PCR products were analyzed by electrophoresis on 3% (w/v) agarose gels (Agarose MP, Roche)) and visualized by ethidium bromide staining (0.5 µg/ml PCR products). From the gel a digital image was created and alleles were assigned by sight.

MAP CONSTRUCTION

Segregation ratio of the genotypes of individual microsatellite markers was checked with the Chi-squared goodness-of-fit test. None of the markers showed significant segregation distortion ($P > 0.05$). Cox et al. (2009) constructed a revised genetic map of the mouse genome and demonstrated that utilization of the revised map improves QTL mapping [48]. Therefore, marker positions were taken from this map by using the 'mouse map converter' (http://cgd.jax.org/mousemapconverter/).

QTL ANALYSIS

The location of the QTLs affecting the measured quantitative traits was determined by using the scanone function in the R/qtl package (Broman et al. 2003) and using cross as an additive and interactive covariate. Because the traits were normally distributed the interval mapping module was used. Results were expressed as LOD scores. LOD score threshold (5% significance) was determined using 1000 random permutations. An association was assumed significant when the LOD score was ≥ 2.32.

GENERATION OF *PCDH9* GENE KNOCKOUT MICE

GENE TARGETING

A targeting vector was designated to delete the second exon of the mouse pcdh9 gene, which encodes extracellular cellular, transmembrane and part of cytoplasmic domains (**Fig.** 5). The targeting vector was constructed by using RPCI-23BAC library (Genetycs, Tsukuba). A 17.2kb genomic DNA fragment was cloned into pBRSDT. The 5' homology arm was a HindIII/SacI-digested 8.7-kb fragment, and a floxed PGK-neo positive selection marker was placed at its downstream. The 3' homology arm was a SacI/NheI-digested 8.8-kb fragment, and a diphtheria toxin A (DT-A) fragment containing a poly-A signal was added as a negative selection marker [49]. We obtained homologous recombinants using TT-2 embryonic stem cells. Mice possessing the neo cassette were produced using standard procedures for chimeric mouse production [49]. Successful gene targeting was confirmed by Southern blotting analysis, as shown in Fig. 5.

BEHAVIORAL CHARACTERIZATION OF *PCDH9* GENE KNOCKOUT MICE

Chimaeric mice were crossed to C57Bl/6J females. Initially, F1 hybrids from heterozygous × heterozygous matings were generated. Repeated back-crossing with C57BL/6J mice (> 10 generations) was conducted to ensure an isogenic C57BL6/J background, These isogenic animals were then used to generate time-mated homozygous×homozygous breeding pairs to obtain wild-type and mutant animals from at least three different litters to mimic the background of the parental CSS14 and C57BL/6J controls. *Pcdh9*$^{-/-}$ and WT controls (n=7

per group) were subjected to the social and object discrimination tests similar to those performed for CSS14 and C57BL/6J controls.

ANALYSIS OF CORTICAL THICKNESS

To measure the cortical thickness, serial sections at about 100μm intervals were prepared from WT and *Pcdh9*$^{-/-}$ mice, and Nissl staining was performed. For Nissl staining, sections were immersed in 0.5% cresyl violet for several minutes and then subjected to the ethanol series and emmedding. Analysis of the cortical thickness was performed within the parietal (0.46~2.06mm proximal to bregma), and occipital region (2.18mm~ proximal to bregma). Barrel field (Area 3), the primary somatosensory area, lateral parietal association area (Area 1), secondary visual area (Area 18), and primary visual area (Area 17) were identified in the same section of each region using standard stereotaxic coordinates [50]. It assumes that the brain structure of P7 and adult mice are similar. According to the stereotaxic coordinates, it is determined that Area 1 existes in the medial part to Area 3, and then, Area 18 is in the medial part to Area 17, which is in the posterior to Area 3. The measurement was performed on at least three sections in each area. In each section, cortical thickness from layer2/3 to 6 was measured at two points at about 100μm interval. In addition, to measure the thickness of the upper layers and deep layers, the measurement was done from layer 2/3 to 4, and from layer 5 to 6, respectively. The boundary between layers 4 and 5 was identified based on morphological distinctions. The data was obtained from WT and *Pcdh9*$^{-/-}$ mice (adult mice; n=5, 1-week-old; n=3). Groups were compared using a Student's t-test.

IN SITU HYBRIDIZATION PROFILING OF *PCDH9* EXPRESSION

DIG-labeled RNA probes were used for hybridization. The DNA fragments obtained from the subtraction library were used as templates for RNA probes. To produce linearized templates for the synthesis of riboprobes, inserts in pGEM-T vectors were amplified by PCR using oligonucleotides that contain T7 and SP6 promotor sequences. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and in vitro transcription was carried out (DIG-RNA Synthesis Kit, Roche, Tokyo). Finally, these probes were purified with gel filtration columns (mini Quick Spin RNA Columns, Roche, Tokyo) and kept at -80℃. In this study, pcdh9 RNA probe included the common region of 5 splicing variants, 5'UTR and the start of ORF.

Animals were decapitated after anesthesia to obtain whole brains from postnatal animals (P0, 3, 7, 14 for rats and P7 and P42 for mice). E15 and 18 mouse brains were taken from fetuses under deep anesthesia. The brains were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffers [51] 30min at room temperature and then 2-3 hours at 4℃. After overnight incubation with a sequential step of 15% and 30% sucrose in PBS, the brains were frozen and then cut into 10 or 20μm sections (coronal or sagittal section) with a cryostat.

Sections were post-fixed in 4% PFA in 0.1M PB, washed with distilled water and 0.1M triethanolamine, then acetylated in 0.25% acetic acid in 0.1M TEA, followed by a final wash in PBS. Prehybridization was carried out for 1hour in hybridization buffer (50% formamide, 5% SDS, 5×SSPE, 0.2mg/ml tRNA), followed by hybridization for 12 hours at 60℃ in hybridization buffer containing 1μg/ml DIG-labeled RNA probe. After three washes in 50% formamide and 2×SSC at 60℃, these sections were subjected to blocking (blocking reagent Roche, Tokyo, Japan) for 1hours at room temperature, and then incubated overnight with alkaline phosphatase-conjugated anti-DIG antibody (1:2000, Roche, Tokyo, Japan). After washing five times at room temperature, the color reaction was carried out at room temperature in BM purple (Roche, Tokyo, Japan). The reaction was terminated by immersing the sections in TE buffer (100mM Tris-HCl, 1mM EDTA, pH8.0), and then the sections were fixed with 4% PFA in 0.1M PB for 15minutes. Then sections were treated in 50%,70%, 90%, 95% and 100% ethanol, and xylene, and then embedded.
For Nissl staining, adjacent or proximal sections were used. These sections were immersed in 0.5% cresyl violet for several minutes and then subjected to the ethanol series and embedding.

PCDH9 CELLULAR AGGREGATION ASSAY

To express full-length protocadherin9 (PCDH9) protein together with the enhanced green fluorescent protein (EGFP), the expression plasmid pCAGGS-PCDH9-EGFP was constructed. The human- pcdh9, transcript variant 2 cDNA was pharchased from ORI GENE (Ori Gene Technologies, Inc Rockville, MD) and subcloned into mammalian expression vector (pCAGGS) that harbors a powerful and ubiquitous CAG promotor [52]. For visualization of pcdh9, GFP was tagged with C-terminal side.
L cells were transfected with the pCAG-EGFP and pCMV-DsRed or pCAG-pcdh9EGFP and pCMV-DsRed by lipofectamine 2000, and then were passaged on the next day. After 2days, the medium was exchanged with D-MEM containing 4mM glutamine, 10% FBS and Geneticin (250μg/ml or 1,000μg/ml). Medium exchange was performed every 3 days. After 10 days in culture, several clones were randomly picked up. To confirm the expression of pcdh9-EGFP, Western blot analysis and immunocytochemical analysis were performed. We established two clones for L cell line.
Cell aggregation assay was performed basically according to the method previously described [53,54]. Briefly, for dissociation of cells, which express EGFP or pcdh9EGFP stably, 0.125% trypsin-containing Dulbecco's PBS was used, and then we performed shaking culture at 80rpm with 1.7ml of the culture medium for 3.5hours. The observation of aggregates was performed at several time points (after 1hr, 2hr and 3.5hr).

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CHAPTER 8

GENERAL DISCUSSION

GENE FINDING IS NOT ENOUGH

The most important challenge in psychiatry is to connect molecular science to clinically oriented research. This will not be possible when broad diagnostic constructs such as autism and schizophrenia remain leading principles. Then, we will remain lost in translation. A more explicit acknowledgement of the phenotypic heterogeneity of psychiatric disorders is still lacking. Genomic technologies have announced a new era in the genetic analysis of psychiatric diseases. This may help to further advance psychiatry into biology-based medicine, since the primary goal of human genetics is to transform the treatment of disease through an understanding of underlying molecular pathways. Rapid advancements are made to uncover which genes are involved and how they interact in spectra of neurodevelopmental disorders. Unfortunately, none of these discoveries has led to better treatment options so far [1]. Perhaps too much focus has been put on population genetics in psychiatric research and too few efforts have been made to put genetic findings into functional perspective. Notably, the only two examples where new psychiatric medication has been derived from genetic insights emerged from Fragile X and Tuberous Sclerosis Complex, both long-known mono-genetic disorders associated with neurodevelopmental disease [2-5]. In these disorders, careful behavioral monitoring of learning and memory phenotypes were crucial to put molecular genetic findings into perspective and led to development of novel treatments. The first part of this thesis further shows that the exploration of rare genetic behavioral disorders such as Klinefelter syndrome (KS) can pave the way for understanding the causes and mechanisms of more common psychiatric disorders [6]. The studies on KS were focused on behavioral phenotypes and delivered some insights that are relevant for developmental disorders in general.

TAXONOMY OF GENETIC AUTISTIC PHENOTYPES

Suppose that such a 3-dimensional space, like an aquarium, delineates the total spectrum of human autism spectrum disorders (ASDs). The heterogeneity of ASDs may then be visualized as a wide but bordered space that contains all possible constellations of autistic phenotypes. Every position in the aquarium would signify a different ASD phenotype, yet all possible positions belong to the same family (spectrum) of ASDs. The genetic autistic phenotypes of disorders such as KS and 22q11DS seem to cluster around specific ‘coordinates’ in the aquarium of ASDs. The ‘scatterness’ of this clustering may be an indicator of specificity of genetic phenotypes and may be an estimate of the penetrance or phenotypic impact of the variants under investigation. Such an estimate will always be an average estimate and based upon the sample in which it is evaluated. In fact, selection in this respect should be ‘encouraged’ in order to ‘purify’ samples and to enhance the chance of finding specific phenotypes. These strategies should not be hampered by illusions to find phenotypes that represent all carriers of a genetic disorder. They merely attempt to dissect the ASD population into subphenotypes of enhanced biological validity: subjects that share

'coordinates' in the ASD aquarium may share factors that lead to the identification of underlying biological mechanisms. For instance, subjects with Klinefelter syndrome may share coordinates with ASD subjects that are also affected by X-linked pathways. A preliminary partitioning of the 'ASD aquarium' might become feasible when similar assessments are repeated in samples of other well-known genetic disorder variants like mutations in the MECP2 gene (Rett syndrome) or TSC1 and 2 gene (tuberous sclerosis complex), deletions of the 15q11 region (Angelman and Prader-Willi syndrome) or the 22q11 region (22q11 deletion syndrome) that we already included in this study. Collaborative efforts from different sites could aim to gather the standardized autism phenotype data (ADI-R) of larger genetic subsamples to achieve this goal. This may lead to a genetic behavioral refinement of ASDs and to start evaluating the 'ethological' impact of particular genotypes [7]. Perhaps this can result in the assembly of a map of the 'autistic landscape'. Also individuals of undetermined genetic make-up may then be allocated on the basis of their clinical presentation.

X-LINKED PARENTAL IMPRINTS ON BEHAVIOR

Like in euploidic (46, XX) females, one X-chromosome in Klinefelter Syndrome is probably inactivated in each cell to compensate for gene dosage. In euploidic females, it is assumed that this X-inactivation is random in each cell, but about 15% of X-chromosomal genes stably escape inactivation and 10% of genes have a heterogeneous inactivation [8]. Those genes that escape inactivation as well as the genes in the pseudoautosomal region are likely to cause gene dosage differences in 47, XXY subjects. The results from our parent-of-origin study indicate that gene dosage effects in KS may be pronounced when non-inactivated or pseudoautosomal genes are imprinted. Thus, gene dosage effects in KS may result from three factors:

1.Is the gene inactivated?
2.Is the gene expressed preferentially from either a maternal or paternal allele?
3.Is there a homologue present on the Y-chromosome (pseudoautosomal genes)?

A further question is how or when preferential inactivation of parental X-alleles may take place in human development. Preferential silencing of paternal X chromosomal alleles may take place in the human placenta as has been shown in other mammalian species like mice and cows[9-11]. In the unusual human situation of XmXmY KS individuals (maternal origin), gene dosage effects could become pronounced if this preferential paternal X chromosomal silencing also occurs in the human fetal placenta. In 'maternal' KS, both X chromosomal alleles will then be active which can not occur in euploidic (XmXy) females or (XmY) males. In analogy to Crespi's theory on autosomal imprinting effects, maternal origin in KS could likewise result in reduced placental growth with more reduced brain growth and an

increased susceptibility for psychosis via X-linked imprinting in the extraembryonic placenta [12]. Highly interesting in this respect is the reference to the study of Lahti et al., who described lower placental weight, lower birth weight, and smaller head circumference at 12 months predicted elevated positive schizotypal traits only in women (n=1200) after adjusting for several confounders ($p < .02$) [13]. Recent studies on (sex-specific) parent-of-origin allelic gene expression in the mouse brain in mice underscore the possibility that X chromosomal gene dosage differences of different parental origins are likely to cause differential effects in brain function [14,15].

The parent-of-origin effects in KS confirm earlier notions that X-linked imprinting may be an important mechanism in behavioral regulation. Apart from one study in Turner syndrome, no earlier X-linked imprinting effects have been described in humans [16,17]. The prospect, that parental X-chromosomal alleles exert influences via imprinting early in development, opens the door for new experiments. These could involve X-chromosomal gene expression assays and the coupling of brain growth and placental function to psychiatric outcome.

ANIMAL MODELS ARE ESSENTIAL FOR FURTHER DISSECTION OF BEHAVIORAL PHENOTYPES

The Klinefelter studies elucidated clear effects of supernumerary X chromosomes on behavior and on the risk for psychopathology. They could not identify more precise behavioral genetic phenotypes. Such efforts in human genetic samples are seriously hampered by heterogenic backgrounds and environmental factors. Mechanistic insights into the molecular causation of disturbed behaviors require control over these effects, which can be achieved by the complementary use of animal models. Mouse studies offer the possibility to disentangle the separate contributions of genetic and environmental factors to defined phenotypes [18,19]. Functional studies in mice raised in controlled genetic and environment conditions have shown disorder-like phenotypes for human psychiatric candidate genes, such as Shank3, Disc1 or Nrxn1 [20-22]. Vice versa, we add that the behavioral characterization of animals that carry genetic disorder abnormalities in a controlled genetic and environmental background may be used to identify human behavioral deficits. These deficits may be significant to understand underlying neurobiological mechanisms. We used available XXY mouse data to give an example of such a strategy. This study is an example to make use of the increasing amount of behavioral findings of genetic animal models by trying to translate animal traits to clinically important parameters.

A drawback of studying genetically modified animals is that it addresses gene function in a single genetic background. We know that differences in the genetic background of mice and humans modify genotype–phenotype relationships and that differences in genetic backgrounds will also lead to the differential expression of phenotypes in mice and humans.

If we pursue a one-by-one evaluation of candidate genes in animal models we will have the advantage of controlled experiments but we will not further our understanding of their 'live' actions in mixed genetic architectures.

FROM GENETICS OF DISORDERS TO GENETICS OF BEHAVIORS

The large mouse mapping study in this thesis describes a strategy to extract individual genetic contributions to behavior out of complex backgrounds using novel mouse genetic mapping panels. The results show that quantitative trait mapping in mice can be used to put data from human population (GWAS) genetic studies into functional perspective. The novelty of this approach is to switch from complex disorder-like behaviors to components of naturally occurring behaviors. The aim hereby is to find genetic variation affecting behavioral adaptation across species. In the genetic mapping study of this thesis, we hypothesized that social memory in mice is an essential behavior homologous across species. Our results imply that genes that disrupt social cognition in mice can be associated to social maladaptation in humans.

Quantitative trait mapping in genetic reference panels of mice may be essential to strengthen research into psychiatric etiologies. A prerequisite will be to start translational research programs focusing on a particular group of behaviors or symptoms (instead of disorders) that have homologous components between mice and men. For instance, behavioral flexibility entails components such as rigidity, inflexibility and disinhibition. All of these have measurable counteracts in humans and mice and are observed across many different psychiatric disorders such as anorexia nervosa, autism, schizophrenia and obsessive compulsive disorder. The genetic mapping of these components in mice can deliver QTLs with candidate genes that can be tested in human samples across these disorders. This will give further insights in the true extent of functional gene pleiotropy and variability. Mouse models of 'mapped' genes can provide functional targets for human studies as was shown in the cortical deficit associated to PCDH9.

The prospect of complementary quantitative genetic analysis is especially intriguing as mice 'line up for success' in the so-called Collaborative Cross (CC) [23-25]. The Collaborative Cross (CC) is a large panel of recently established multiparental recombinant inbred mouse lines specifically designed to overcome the limitations of existing mouse genetic resources for analysis of phenotypes caused by combinatorial allele effects. The CC models the complexity of the human genome and supports analyses of common human diseases with complex etiologies originating through interactions between allele combinations and the environment. The CC constitutes a unique mammalian resource because of its high and uniform genome-wide genetic variation effectively randomized across a large, heterogeneous, and infinitely reproducible population. The CC supports data integration

across environmental and biological perturbations and across space (different labs!) and time. The CC might be an important link to overcome the extremely complex genetic and phenotypic architecture of human behaviors.

PCDH9: A PROTO- GENETIC PHENOTYPE?

The PCDH9 (Protocadherin9) findings described in the present thesis highlight the possibility to identify genetic alterations that can impact behavior through localized cortical disruptions. PCDH9 belongs to the superfamily of cadherin genes, which are known to play a role a wide variety of developmental processes and mature functions of the vertebrate brain. The cadherin family is classified into classical cadherins, desmosomal cadherins and protocadherins (PCDHs). Genomic structures distinguish between PCDHs and other cadherins, and between clustered and non-clustered PCDHs. PCDHs are further classified by genomic sequence into three subgroups: δ1 (PCDH1, PCDH7, PCDH9, PCDH11 and PCDH20), δ2 (PCDH8, PCDH10, PCDH12, PCDH17, PCDH18 and PCDH19) and ε (PCDH15, PCDH16, PCDH21 and MUCDHL) [26-28]. Non-clustered PCDHs are expressed predominantly in the nervous systems. They have spatiotemporally diverse expression patterns [26,27]. Especially, the region-specific expressions of non-clustered PCDHs have been observed in cortical areas during early postnatal stages (as we also confirmed for PCDH9), suggesting that non-clustered PCDHs play roles in the circuit formation during development. The non-clustered PCDHs appear to have homophilic/heterophilc cell-cell adhesion properties, and each member has diverse but distinct cell signaling partnership. Non-clustered PCDHs seem involved in neuronal disorders such as autism-spectrum disorders, schizophrenia, and female-limited epilepsy and cognitive impairment. This suggests that they play several, tightly regulated roles in normal brain function. Interestingly, most single neurons express more than one (proto)cadherin in a combinatorial fashion in all layers of cerebral cortex. This combinatorial code is comprehensive. The pairwise expression of cadherins can give any type of combinations (complementary, partial or complete overlap, subset-specific expression, cell-size specific expression, etc.) [29] and may well contribute to the molecular specification of the complexity of neurons in the cerebral cortex. This may also explain why a full functional deletion of PCDH9 results in relatively subtle change.

No earlier examples have been noted, in which a single psychiatric candidate gene has been coupled to discrete cortical changes via the quantitative assessment of behavioral differences (e.g., social discrimination capacity). Importantly, regional reduction of cortical thickness is a central anatomical finding in human schizophrenia [30-32], also found with specific reference to the secondary somatosensory cortex [33] and secondary somatsensory function[34]. Moreover cortical thinning due to increased cell density, as we found in Pcdh9 mutant cortices, has also been described in post-mortem studies in

'general' schizophrenia (SZ) [35-38] and has been posed as a central 'pathogenic' mechanism in schizophrenia [36]. This led us to speculate on a role for PCDH9 across different human developmental disorders other than already shown for autism. Obviously, we would be very keen to analyse the observed anatomical (MRI) and cognitive effects of this gene in the human situation. A first survey across a large genetic schizophrenia sample in our institution showed that possibly 10 affected subjects carry PCDH9 deletions, which was not observed in any of the controls subjects (without schizophrenia). Such findings would be timely as evidence is accumulating from novel techniques such as transcriptome analysis that early developmental events lead to changes in cortical circuit patterning that leaves certain cell types or regions vulnerable to later neurodegeneration [39,40].

"CAUSE" VERSUS "OUTCOME" IN PSYCHIATRIC DISEASE

Perhaps psychiatric research is in the stages of the discovery of 'Langerhans islets' in 1869 (by Paul Langerhans) in the sense that we are beginning to identify 'cortical circuitry' as the primary substrate of disorders such as autism and schizophrenia. Obviously, all sorts of secondary influences, mechanisms and insults are required to further develop disorders. But how genes relate to the long road of cortical construction and these how genes impact its later (cognitive) function are fundamental questions. The answer will perhaps be more complicated than the human genome project in itself.

Ross and Geschwind write in a recent article*: "Once such pathogenic mechanisms are better understood, cognitive classifications based upon etiology "cause" might become feasible, such as distinct disorders of neural migration, or cell fate, which could presumably share molecular guided therapies more successfully than therapies targeting current disease classifications based upon observed disorder "outcome"* [39].

Ideally, an integration of high-resolution genetic mapping of relevant mouse behaviors and brain morphology might tackle the heterogeneity of psychiatric disease from both ends. As this thesis is mainly on behavioral phenotypes, I should conclude with a remark on the prospect of behavioral assessments in psychiatric research. Perhaps, genetic variations are easier found that lead to changes in 'innate' rather than 'learned' behaviors [41]. In my opinion, it seems promising to conduct genetic studies of automated behaviors and to focus on the loss of adaptational capacity as a primary explanation for involuntary madness or short-circuited brain.

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CHAPTER 9

SUMMARY

SUMMARY

The main aim of this thesis was to identify behavioral consequences of specific genetic variants. This should lead to enhanced genotype – phenotype descriptions that can provide more understanding of psychiatric pathogenesis.

PART 1: THE CONSEQUENCES OF AN EXTRA X-CHROMOSOMES ON BEHAVIOR

In the first part of the thesis, Klinefelter syndrome (KS; 47, XXY) was used as a model to study a specific human genetic alteration. The studies on Klinefelter syndrome captured signatures of supernumerary X-chromosomes on different levels of phenotype expression:

1. On the prevalence of DSM-IV psychiatric classifications
2. On the specificity of autism trait profiles
3. On the specific relationship between executive functions and autism traits
4. On the impact of the parent-of-origin of the supernumerary X chromosome on autistic and schizotypal traits
5. On the alignment of mouse XXY behaviors to human KS cognitive traits

The starting point of the Klinefelter studies was to confirm that an extra X-chromosome is substantially associated with developmental psychopathology. In **CHAPTER 2** fifty-one boys with KS were assessed for the presence of psychiatric symptoms and disorders through semi-structured and standardized interviews. A wide range of DSM-IV diagnoses could be applied. The majority of the boys with KS (>70%) had formal attentional and/or language disorders, which is in accordance with earlier studies. Autism spectrum disorders (ASD) (26 %) appeared to be highly prevalent and autistic symptoms were abundant in XXY boys without a formal ASD. This was a finding that had been not been reported before. Affective disorders (12 %) and psychotic disorders (12 %) were also highly prevalent, next to frequent hallucinations and paranoid ideations in those XXY boys without psychotic disease. Considerable overlap was observed between ADHD, autism spectrum disorders and language disorders in this sample. Overall, the survey clearly confirmed that XXY boys are at risk for developmental psychopathology. The manifestations of psychopathology seemed variable from the DSM-IV point of view.

In **CHAPTER 3**, we hypothesized that autistic trait measurements could be used to show social behavioral similarities in subjects with KS. The overall aim of the study was to provide proof of concept of genetic autistic phenotypes. A new method was developed to specify genetic behavioral phenotypes by entering autistic trait variables in different statistical analyses. Not only boys with KS but also children with 22q11deletion syndrome (22q11DS) were incorporated in this study as a second genetic disorder sample. A large group of children with ASD without KS or 22q11DS was used as a 'genetically

heterogeneous' control sample. It was hypothesized that the autistic profile of subjects with KS or 22q11DS *with* a diagnosed autism spectrum disorder (KS-ASD / 22q11DS-ASD):

1. could be delineated from the genetically heterogeneous control ASD sample
2. was more homogeneous than the genetically heterogeneous control ASD sample

A robust discrimination between 22q11-ASD and KS-ASD and genetically heterogeneous ASD phenotypes appeared to be feasible on the basis of a reduced number of autistic traits. This suggested that combinations of certain behaviors were significantly more prevalent in both the genetic disorder ASD samples than in the genetically heterogeneous ASD sample. Different symptoms were discriminating in KS-ASD versus 22q11DS-ASD. Thus, the analyses indicate that the ASD profiles were also different *between* KS-ASD and 22q11DS-ASD. Increased autistic symptom homogeneity was also found in comparison in both genetic disorder ASD groups compared to ASD controls. This was expressed by a reduced average number of different symptoms contributing to the ASD diagnosis. The results of this study may contribute to a clinical refinement of autism spectrum disorders into more biologically more valid phenotypes [1].

In **CHAPTER 4** we further studied genotype-phenotype relationships in KS by investigating the relation between cognition and autistic morbidity. Such relationships may be more exclusive in human genetic disorders such as KS than in the general population. In a subset of the original sample of boys with KS (n=44), we measured cognitive executive functioning (EF) profiles as these have been implicated as important deficits in KS [2,3]. EF is also referred to as the higher brain functions necessary for planning and organizing behavior. First we wanted to add to the empirical studies of EF in KS that are still fairly sparse and have addressed different aspects of EF in different age groups [2]. We found clear deficits in major EF domains. Boys with KS demonstrate poor attention regulation, inhibition problems and poor mental flexibility. Second, we wanted to assess if deficits in cognitive control lead to social maladaptation by correlating EF performance to empirically validated autism trait scales. Deficits in attention regulation and inhibition coincided with social communication abnormalities and stereotypical behaviors. We concluded that EF problems in KS are associated to social behavioral problems and are probably risk markers for developmental psychopathology in KS. This study showed that important relationships between cognitive control and behavioral outcome can be specified in genetic disorders, which is less feasible in genetically undefined populations.

The variability of KS behavioral phenotypes will partly be due to genetic mechanisms influencing supernumerary X chromosome gene expression. Of these mechanisms, parent-of–origin effects of the supernumerary X chromosome are particularly interesting to investigate, as these can indicate X-linked imprinting effects on behavior. In **CHAPTER 5** we assessed parent-of-origin effects of the supernumerary X chromosome on autistic and

schizotypal traits in a sample of boys and in a mixed sample of boys and adults with KS respectively. In these samples, we found remarkable parent-of-origin effects on behavioral trait expression. The parent-of-origin was reflected in the level of autistic symptoms and schizotypal traits by multivariate effect of group (maternal vs. paternal vs. controls) and also by individual differences in trait scores between maternal and paternal groups. Interestingly, higher scores on schizotypal traits were consistently associated with maternal origin, while the significant differences in autistic traits were not consistent in the direction of "imprinting". In further analyses, the parent-of-origin of the extra X chromosome could be discriminated through specific autistic and schizotypal trait profiles. More than 80% correct classification of parental groups was feasible on the basis of the autistic and schizotypal trait profiles. Overall, these results were unexpectedly robust and were more pronounced than the earlier described parent-of-origin effects on somatic features in KS [4]. They raised several new thoughts not only on pathophysiological mechanisms in KS but also on X-linked imprinting in general.
A further question is how parent-of-origin effects may influence X chromosomal gene dosage effects and thereby alter behavioral outcome in KS. This seems possible when preferential expression of either paternal or maternal alleles somehow interact with X chromosomal inactivation patterns in KS. This may occur in early embryogenesis. It has been shown for some mammals that paternal X chromosomes are temporally preferentially inactivated in the early stages of the fetal placenta.

A further challenge was to find those cognitive traits that directly relate to supernumerary X-chromosomal gene expression. **CHAPTER 6** proposes the complementary use of animal models of genetic disorders to identify 'significant human cognitive defects'. We hypothesized that analysis of human features that resemble behavioral deficits observed in the animal model can facilitate the identification of close causal genotype-phenotype relationships. The rationale for this approach was that behavioral deficits elucidated under controlled genetic and environmental circumstances can be attributed to the genetic defect engineered in the animal model. The challenge for this study was to find human traits that represent the same cognitive dysfunctions found in animal models for KS. Two mouse models for KS (male 41, XXY and the 41, XX^{Y}*) have been developed, which have been behaviorally characterized in different behavioral mouse paradigms [5,6]. Locomotion, exploration, sociability and anxiety related behaviors seemed unaffected in the KS mouse models. Object recognition had been shown to be clearly impaired in the 41, XX^{Y}*mice. Object recognition in rodents has been shown to involve sensory recognition and object perception [7]. It may therefore be measured in humans via visual object and visual pattern recognition. We carefully selected human paradigms in which these capacities were challenged by temporal order as in the 41, XX^{Y}* mice paradigm. We could confirm that these tasks were clearly impaired in a subset of the original sample of boys with KS (n=34)

in comparison to an age matched population control sample. Thus, these cross-species comparisons suggested that the processing of visual pattern information is a significant deficit in KS. There may be other core features with close relation to X-chromosomal imbalances, but here we focused on those traits that have yet been validated in animals model of KS. This study provided proof-of-concept that animal models may be used to extract relevant traits out of the cloud of cognitive dysfunctions that usually accompany genetic disorders such as KS. Also, cross-species comparisons of standard mouse behavioral paradigms such as object recognition are important to evaluate their face validity in different genetic contexts.

PART 2: GENETIC MAPPING OF MEMORY COMPONENTS IN MICE

The KS studies further show that particular cognitive defects can be central to problems in social adaptation. A further challenge was to find genes regulating cognitive functions that influence social outcome. Therefore, a genetic mapping study in mice was started to find loci regulating 'social cognition' and to evaluate their function in humans. Thus, behavioral components were now used as a 'forward' starting point to find regulatory genetic loci instead of the 'reversed' behavioral phenotyping of genetic alterations as in the KS studies. In **CHAPTER 7**, it was hypothesized that temporal components of social memory (short-term or long-term) could be genetically dissected and that this could lead to finding of genes regulating particular aspects of social cognition and behavior.

We used mouse chromosome substitution strains (CSSs) as a genetic reference panel (GRP – see introduction of the thesis) [8,9]. A CSSs panel was screened for temporal components of social memory in a modified social discrimination paradigm after a first short-term (STM) memory 5-minute training test interval (TTI) and a long-term [10] memory 24-hour TTI [11]. We selected the 2 strains, CSS3 and CSS14 that displayed the strongest dissociation of social discrimination performance. We defined that dissociation was indicated by discrimination impairments either in STM (as was shown in CSS3) or LTM (as was shown in CSS14), with an intact discrimination performance in the counteract STM or LTM interval. CSS3 and CSS14 strains were also screened in other memory paradigms to test for context specificity of the social memory impairments. We found that the dissociation of STM versus LTM performance in CSS3 and CSS14 was also apparent in (non-social) object discrimination, while spatial memory in the Morris Water Maze was intact.

An F_2-generation of CSS14 (n = 192) was generated and tested in the social and object discrimination paradigms for further 'fine' mapping of the CSS14 LTM locus. Several genetic loci were found to be associated to LTM impairments in both social and object discrimination. The locus with the highest score of positive genetic linkage for LTM social discrimination impairment contained only 2 candidate genes with differences in coding

DNA between the original parental CSS strains (based upon recent mouse genome databases). One of these 2 genes was *Pcdh9*, a gene that has been very well characterized and is assumed to have a role in cortical morphogenesis [12-17]. Interestingly, *Pcdh9* has already been implicated in human autism. De novo deletions of PCDH9 have been found in at least 2 independent human autism samples [18,19]. *PCDH9* has also been implicated as a candidate gene to affect social behavior in dogs, which further indicates a regulatory role for *Pcdh9* (*PCDH9*/PCDH9) across species [20].

The laboratory of Nobuhiko Yamamoto in Osaka (Japan) had generated *Pcdh9* deficient mice (conventional knock-outs) to study the role of *Pcdh9* in cortical development. The homozygous *Pcdh9* gene knockout mice (*Pcdh9*$^{-/-}$) were tested on the CSS14-F_2 behavioral readouts on site in Japan. An LTM social discrimination defect was clearly confirmed in the *Pcdh9*$^{-/-}$ mutant mice while, like in CSS14, STM social discrimination was intact. In contrast, no impairments in object discrimination were found, suggesting role for Pcdh9 in LTM formation that is dependent on the context in which the memory capacity is challenged.
A role of *Pcdh9* in cortical morphogenesis was confirmed as Nissl staining showed a regional difference in cortical thickness in higher cortical association areas of *Pcdh9*$^{-/-}$ mice, including the secondary somatosensory area. This decrease was attributable to the increased cell density rather than neuronal number. An aggregation assay confirmed that *Pcdh9* has an adhesive property as suggested for other non-clustered protocadherins[16]. We further showed an accumulation of *Pcdh9* gene product in cortical synapses. These results suggest alterations in neuropil development, as has been observed in human psychiatric disorders such as schizophrenia [21]. The association of PCDH9 with human autism might indicate that PCDH9 deletions are a further example of how genetic events can cause subtle morphological disruptions that can impact brain function [22]. Alterations in cell-cell adhesion may converge with the genetic basis of synaptic and neuronal signaling dysfunction that is strongly suggested in neurodevelopmental disorders[23].

PCDH9 seems to have early developmental effects on the cortical integrity of higher order processing areas. This interpretation was supported by the observed deficits in *Pcdh9* mutant mice in the consolidation of earlier acquired social information. Overall, this study demonstrated that the genetic mapping of essential behavioral components in mice can provide important functional context to genetic disruptions associated to human psychiatric disorders. As proof-of-principle, the role of *Pcdh9*/PCDH9 in human autism was specified through a genetic and morphological link with context specific long-term memory in mice.

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SAMENVATTING

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Dit proefschrift gaat over de identificatie van gedragsveranderingen die door specfieke genetische afwijkingen zijn veroorzaakt. Het doel hierbij is relaties tussen genetische aanleg en waarneembare kenmerken (genotype-fenotype relaties) nauwkeuriger te kunnen beschrijven.

DEEL 1: DE GEVOLGEN VAN EEN EXTRA X-CHROMOSOOM OP GEDRAG

In het eerste deel is het Klinefelter syndroom (KS) gebruikt als model om een specifieke humane genetische afwijking te bestuderen. Het Klinefelter syndroom is een genetische stoornis die wordt gekenmerkt door een of meer extra X chromosomen (meestal 47, XXY), die bij ongeveer 1 op de 700 mannen voorkomt. Het XXY chromosomaal patroon wordt al decennia bestudeerd en is al frequent geassocieerd met beperkingen in gedrag en verminderde cognitieve vaardigheden. De Klinefelter studies in dit proefschrift studies onderzochten specifieke effecten van een extra X-chromosoom op verschillende niveaus van gedragsexpressie:

1. op de prevalentie van psychiatrische DSM-IV classificaties
2. op de specificiteit van autistische gedragsprofielen
3. op de relatie tussen cognitieve controle functies en symptomen van autisme
4. op de impact van de ouderlijke herkomst van het extra X-chromosoom op autisme en psychotische symptomatologie
5. op de vergelijking van gedrag in XXY muizen met cognitie in humaan XXY

Eerst wilden we bevestigen dat een extra X-chromosoom substantieel geassocieerd is met psychiatrische ontwikkelingsstoornissen. Ondanks de vele eerdere studies naar gedrag in KS is dit een onderbelicht aspect gebleven. In **HOOFDSTUK 2** werden 51 jongens met het KS beoordeeld op de aanwezigheid van psychiatrische stoornissen en symptomen door middel van semigestructureerde en gestandaardiseerde interviews. Een opvallende verscheidenheid aan DSM-IV diagnoses (het handboek voor diagnose en statistiek van psychische aandoeningen) kon worden vastgesteld. De meerderheid van de jongens met KS (>70%) bleek een taalstoornis en/of een aandachtstoornis te hebben, zoals verwacht op basis van eerdere literatuur. Autisme spectrum stoornissen (ASS) (26%) bleken ook prevalent en autistische symptomen waren overvloedig aanwezig bij de meeste XXY jongens zonder een formele ASS. In deze jonge groep waren ook al veel psychotische stoornissen (12 %) opgetreden, naast veel voorkomende psychotische symptomen zoals hallucinaties en paranoïde ideaties. Er was verder een aanzienlijke overlap tussen ADHD, ASS, en taalstoornissen in deze groep. In conclusie, dit overzicht bevestigde dat jongens met KS een hoog risico hebben op psychiatrische ontwikkelingsbeelden, waarbij de manifestatie van de psychopathologie variabel was vanuit DSM-IV oogpunt.

De hypothese van **HOOFDSTUK 3** was dat autisme symptoom profielen gebruikt kunnen worden om overeenkomsten in sociaal gedrag bij jongens met KS aan te tonen. Een nieuwe methode werd ontwikkeld om deze genetische gedragsfenotypen te specificeren door middel van statistische analyse van autisme kenmerken. Naast jongens met KS werden ook kinderen met het 22q11 deletie syndroom (22q11DS) meegenomen in deze studie, als een 2de groep kinderen met een gedeelde genetische afwijking (een deletie van een deel van chromosoom 22). Deze groepen werden vergeleken met een grote groep kinderen met een autisme spectrum stoornis (ASS), waarbij geen bekende genetische afwijking was vastgesteld en die werd beschouwd als een genetische heterogene controle groep met autistische subjecten. Als een indicatie voor het bestaan van genetische autisme fenotypes werd verondersteld dat autisme symptoom profielen van autistische subjecten met *ook* KS of 22q11DS:

1. kunnen worden onderscheiden van het genetisch heterogeen controle ASS sample
2. meer homogeen zijn dan het genetisch heterogene controle ASS sample

Een robuuste discriminatie tussen 22q11DS-ASS en KS-ASS en genetische heterogeen ASS was mogelijk op basis van een beperkt aantal autistische eigenschappen. Dit betekent dat bepaalde combinaties van gedragingen zijn significant meer prevalent voorkomen bij de twee genetische stoornissen dan bij de genetische heterogene populatie. Het viel verder op dat verschillende autistische symptomen onderscheidend waren in KS-ASS versus 22q11DS-ASS, dus dat de profielen van de genetische stoornissen onderling ook verschillen. Toegenomen homogeniteit van autisme symptomen werd ook gevonden in beide genetische stoornissen in vergelijking met het genetisch heterogene ASS controle sample. Dit werd uitgedrukt als het gemiddeld aantal symptomen dat had bijgedragen aan de ASS diagnose, dus hoe minder verschillende symptomen hoe homogener het autistische fenotype van de groep.

In **HOOFDSTUK 4** werden genotype-fenotype relaties in KS verder onderzocht door de verhouding tussen cognitie functies en autisme morbiditeit te onderzoeken. Zulke relaties zijn mogelijk meer 'exclusief' in humane genetische condities zoals KS dan in de algemene (genetisch heterogene) populatie . In een gedeelte van het originele KS sample (n=44) werden zogeheten executieve functie (EF) profielen gemeten aangezien deze eerder als belangrijke defecten in KS zijn aangemerkt [1,2]. Onder EF worden de hogere controlefuncties van de hersenen verstaan, nodig voor het plannen, organiseren en controleren van gedrag. We vonden duidelijke uitval in belangrijke EF domeinen. Jongens met KS demonstreerden zwakke aandachtsregulatie, problemen met inhibitie en beperkte mentale flexibiliteit. Ten tweede wilden we bepalen of de beperkte cognitieve controle zou kunnen leiden tot verminderde sociale adaptatie. De EF prestatie in de KS groep werd gecorreleerd aan empirisch gevalideerde autisme symptoom schalen, om zo te kijken of

relatieve uitval in controle leidt tot uitval in sociaal gedrag. Defecten in aandachtsregulatie en inhibitie van gedrag vielen inderdaad samen met afwijkingen in sociale communicatie en stereotyp gedrag (het herhalen van steeds dezelfde handelingen). We concludeerden dat EF problemen in KS geassocieerd zijn met sociale gedragsproblemen en dat ze waarschijnlijk risico markers zijn voor psychiatrische ontwikkelingsstoornissen bij KS. Deze studie laat zien dat belangrijke relaties tussen cognitieve controle en latere uitkomst in gedrag gespecificeerd kunnen worden in genetische stoornissen, wat minder goed mogelijk is in de algemene populatie.

De variabiliteit van het KS gedragsfenotype wordt mogelijk veroorzaakt door genetische mechanismen die overexpressie van X-chromosomale genen beïnvloeden. Van deze mechanismen was het effect van de parentale (ouderlijke) herkomst van het extra X-chromosoom interessant om te onderzoeken. Het is namelijk bekend dat ongeveer in de helft van de mannen met KS het extra X chromosoom van de moeder afkomstig is en in de andere helft van de vader. Het is al aangetoond dat in niet geslachtsgebonden chromosomen (niet X of Y) bepaalde genen alleen tot expressie komen als ze worden afgelezen als ze van een bepaalde ouder afkomstig zijn en daardoor het fenotype beïnvloeden. Dit fenomeen heet imprinting. In **HOOFDSTUK 5** werden parentale herkomst effecten onderzocht op autisme en schizotypie symptoom profielen in twee verschillende KS samples. Ter verduidelijking, schizoptypie schalen zijn hier ingezet om symptomen te meten, die gerelateerd zijn aan psychose en schizofrenie. In beide samples vonden we opmerkelijke parentale herkomst effecten op de expressie van gedragskenmerken. De parentale herkomst van het extra X-chromosoom was niet alleen gereflecteerd in de globale mate van autisme en schizotypie kenmerken, maar ook bleken er bepaalde specifieke psychiatrische kenmerken met name aanwezig als het extra X-chromosoom van moeder of van vader afkomstig was. Opvallend hierbij was dat hogere schizotypie scores consistent geassocieerd waren met maternale herkomst, terwijl de significante verschillen in autisme scores niet consistent gebaseerd waren op een type parentale herkomst parentale herkomst.
In een andere analyse kon de herkomst van het extra X-chromosoom gediscrimineerd worden door specifieke combinaties van autisme en schizotypie kenmerken. Meer dan 80% succesvolle classificatie van parentale herkomst groepen was mogelijk op basis van deze profielen. Dus, op basis van autistische en psychotische symptoom profielen kan de origine van het extra X chromosoom in meer dan 80% van de gevallen correct voorspeld worden.

Deze resultaten waren onverwacht robuust en waren meer uitgesproken dan eerder beschreven parentale herkomst effecten op somatische kenmerken van KS [3]. Ze geven mogelijk nieuwe inzichten over pathofysiologische mechanismen in KS en over X-linked imprinting in het algemeen. Als deze parentale herkomst effecten kunnen worden gerepliceert, dan rijst de belangrijke vraag hoe ze X-chromosomale genexpressie en

daarmee gedrag beïnvloeden. Dit zou mogelijk kunnen zijn wanneer de preferentiële expressie van maternale of paternale allelen op een of andere wijze interacteert met X-chromosomale inactivatie patronen (zie introductie). Mogelijk gebeurd dit in de vroege embryologische ontwikkeling. In bepaalde diersoorten worden namelijk de paternale X chromosomen tijdelijk geïnactiveerd in de vroege stadia van de foetale placenta.

Een verdere uitdaging was om die cognitieve kenmerken te vinden die in direct verband staan met extra X-chromosomale genexpressie. **HOOFDSTUK 6** propageert het complementaire gebruik van diermodellen van genetische stoornissen om 'significante cognitieve defecten' te identificeren. Het idee was dat de analyse van humane gedragskenmerken die overeenkomen met defecten in een diermodel kan leiden tot de identificatie van etiologisch belangrijke genotype-fenotype relaties. De rationale voor deze benadering was dat gedragsdefecten die gevonden zijn onder gecontroleerde genetische en omgevings-omstandigheden, werkelijk toegeschreven kunnen worden aan het 'gemanipuleerde' genetisch defect van het diermodel. De uitdaging voor deze studie was om humane eigenschappen te vinden die de cognitieve dysfunctie van het diermodel representeren. Twee muizen modellen voor Klinefelter syndroom (41, XXY and 41, XX^{Y*}) zijn ontwikkeld van wie het gedrag in eerder studies is gekarakteriseerd [4,5]. Deze muizen lieten ongestoorde locomotie, basale exploratie, sociabiliteit en angst gerelateerde gedragingen zien, terwijl herkenning van objecten wel gestoord bleek in 41, XX^{Y*} muizen. Aangezien de herkenning van objecten in knaagdieren zowel sensore (zintuiglijke) recognitie en object herkenning vereist [6], zou objectherkenning mogelijk in mensen relateren aan visuele object en patroon herkenning. Voor deze studie hadden we zorgvuldig humane paradigma's gekozen waarin deze capaciteiten op temporele basis worden beproefd zoals in de 41, XX^{Y*} muizen studie. We konden bevestigen dat deze taken duidelijk gestoord waren in een deel van het originele sample van jongens met KS (n=34) in vergelijking met controles van dezelfde leeftijd. Dus, deze vergelijking tussen verschillende (dier)soorten suggereert dat het verwerken van visuele patroon herkenning een etiologisch belangrijk defect in KS kan zijn. Er zijn waarschijnlijk nog meer 'core' defecten met een nauwe relatie tot X-chromosomale verstoringen, maar hier hebben we ons gericht op die eigenschappen die reeds gevalideerd zijn in muizen modellen van KS. Deze studie bewijst dat diermodellen gebruikt kunnen worden om relevante gedragskenmerken te extraheren uit de brei van cognitieve dysfuncties, die een genetische ontwikkelingsstoornis zoals KS vaak omgeven. Ook is het belangrijk de symptoom validiteit (face validity) van standaard muizen gedragsparadigma's, zoals object herkenning, in verschillende genetische contexten te beoordelen.

DEEL 2: GENETISCHE MAPPING VAN GEHEUGEN COMPONENTEN IN MUIZEN

De Klinefelter studies laten zien dat cognitieve defecten centraal kunnen staan aan problemen in sociale adaptatie. Een volgende uitdaging was om genen te vinden die bijdragen aan de cognitieve regulatie van sociaal gedrag. Met behulp van speciaal ontwikkelde panels van muizenstammen is het mogelijk om zogeheten genetische mapping van gedragskenmerken uit te voeren. Hierbij kan de fysieke locatie van genen (loci) op een bepaald chromosoom in relatie tot verandering van een bepaald gedrag worden bepaald. Hoofdstuk 7 beschrijft een grote genetische mapping studie om genetische loci of genen te vinden die sociaal gedrag reguleren. Dus, gedragscomponenten worden hier gebruikt als een "forward" startpunt om genetische invloeden te vinden, in plaats van de "reversed" gedragsfenotypering van genetische varianten zoals in de KS studies. In **HOOFDSTUK 7** was het uitgangspunt dat temporele componenten van sociaal geheugen (i.e., korte termijn (STM) of lange termijn (LTM) [7]) genetisch kunnen worden ontleedt om zo genen te vinden die specifieke aspecten van sociale cognitie en gedrag reguleren.

We gebruikten muizen chromosoom substitutiestammen (CSS) als een genetisch referentie panel (GRP- zie introductie (Hoofdstuk 1) voor details) [8,9]. Een compleet CSS panel werd gescreend op temporele componenten van sociaal geheugen in een sociaal discriminatie paradigma [10]. Bij sociale discriminatie wordt gemeten of dieren meer sociale exploratie van nieuwe versus bekende dieren laten zien, nadat ze op een eerder tijdstip al aan de bekende muis waren blootgesteld. Dit werd getest na respectievelijk een 5 minuten korte termijn training test interval (TTI) en een 24 uur lange termijn TTI [11-13]. We selecteerden de 2 CSS stammen, CSS3 en CSS 14, die de sterkste dissociatie van sociale discriminatie lieten zien. Dissociatie was hierbij gedefinieerd als gestoorde discriminatie in het korte termijn (CSS3) of lange termijn (CSS14) interval, met intacte discriminatie in het tegenovergestelde interval. De CSS3 and CSS 14 stammen werden verder gescreened in andere geheugen testen om de context specificiteit van de gevonden sociaal geheugen defecten te testen. We vonden dat dezelfde temporele dissociatie effecten in object discriminatie bij CSS3 en CSS14, terwijl spatieel (ruimtelijk) geheugen intact was.

Een F_2-generatie van CSS14 (n = 192) werd gegenereerd om binnen chromosoom 14 genetische loci te kunnen detecteren. Deze generatie werd getest in dezelfde sociale and object discriminatie paradigma's en werden verschillende loci voor lange termijn sociale en object discriminatie gevonden. De locus met de hoogste genetische associatie score voor LTM sociale discriminatie bevatte slechts 2 kandidaat genen die bekende verschillen in coderend DNA tussen de oorspronkelijke parentale CSS stammen droegen. Een van deze 2 genen was *Pcdh9,* een gen dat eerder uitgebreid is gekarakteriseerd en verondersteld wordt een rol te hebben in de aanleg van de cerebrale cortex [14-19]. Hoogst interessant was dat *Pcdh9* eerder was geassocieerd met humaan autisme. De novo (ie., niet bij ouders

aanwezige) deleties en dus mogelijk causale deleties zijn aangetroffen in ieder geval 2 onafhankelijke humane autisme samples [20,21]. *PCDH9* is verder ook geïmpliceerd als een kandidaat gen voor sociaal gedrag in honden, wat een verdere indicatie is voor een rol voor *Pcdh9* (*PCDH9*/PCDH9)* in het reguleren van sociaal gedrag [22].

Het laboratorium van Nobuhiko Yamamoto in Osaka (Japan) had *Pcdh9* mutante muizen (conventionele knock-outs, $Pcdh9^{-/-}$) gegenereerd om de rol van Pcdh9 in de ontwikkeling van de cortex te bestuderen. We hebben ter plekke in Japan deze $Pcdh9^{-/-}$ muizen getest op dezelfde uitkomsten als de CSS14-$F_{2,}$. Het LTM sociale discriminatie defect werd duidelijk bevestigd, terwijl STM sociale discriminatie weer intact bleek. Er werden geen verstoringen in object discriminatie gevonden in de $Pcdh9^{-/-}$. Dit suggereert een rol voor Pcdh9 in LTM geheugen formatie die sociale context afhankelijk is.

Een rol voor *Pcdh9* in cerebrale corticale morfogenese werd bevestigd doordat Nissl kleuring een regionale verdunning van corticale dikte liet zien ter hoogte van de corticale associatie gebieden, inclusief de secundaire somatosensore en visuele cortex gebieden. Deze afname konden worden toegeschreven aan verhoogde cel densiteit en niet aan een afname van het aantal neuronen. Een cellulair aggregratie assay bevestigde dat *Pcdh9* neuronale celadhesie bevorderende eigenschappen bezit, zoals al gesuggereerd is voor andere non-clustered protocadherinen [16]. Het is dus wellicht mogelijk dat een verlies van corticale integriteit door verminderde celadhesie zich kan openbaren als corticale verdunning. Dit kan belangrijk zijn aangezien corticale verdunning en verhoogde neuronale celdensiteit eerder is beschreven in studies van schizofrenie patiënten [23-27]. De defecten in het consolideren van sensorische informatie via deze hersengebieden zouden dus ook relevant kunnen zijn voor de pathogenese van schizofrenie en aanverwante humane stoornissen.

Concluderend, deze studie laat zien dat de genetische mapping van essentiële gedragscomponenten in muizen belangrijke functionele context kan blootleggen van genen die geassocieerd zijn met humane psychopathologie. Als 'proof-of-principle', de rol van *Pcdh9*/PCDH9 in humaan autisme werd hier gespecificeerd door een genetische en morfologische link met context specifieke lange termijn geheugen defecten in muizen.

*aanduiding voor een gen (*Pcdh9*/*PCDH9*/PCDH9) is species-afhankelijk

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DANKWOORD

In de oudste lagen
van mijn ziel
waar hij van stenen is gemaakt
bloeit als een gaaf
ontkleurd fossiel
de stenen bloem van uw gelaat

Ik kan mij niet van u bevrijden
er bloeit niets in mijn ziel
dan gij
De oude werelden zijn voorbij
maar niets kan mij van
u meer scheiden

Vasalis

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CURRICULUM VITAE

Hilgo Bruining werd geboren op 1 april 1972 in Leiden. Na een verblijf in de Verenigde Staten, bracht hij zijn verdere jeugd door in Leiden, alwaar hij in 1990 aan het Stedelijk Gymnasium zijn schooldiploma haalde. Eerst heeft hij een propedeuse biochemie aan de University of Surrey (UK) gehaald om vervolgens in Rotterdam aan de studie geneeskunde te beginnen. In 1997 werd de doctoraalfase afgerond en in 1999 werd het artsexamen cum laude volbracht. Vervolgens is hij begonnen met de opleiding kindergeneeskunde in het AMC Amsterdam en later in het AZM Maastricht. Tussentijds heeft hij gewerkt als kinderarts in opleiding in Great Ormond Street children's hospital in Londen en in het children's hospital at Westmead in Sydney. In 2005 is hij overgestapt naar de psychiatrie en is hij de opleiding tot kinder- en jeugdpsychiater begonnen met als opleider prof. dr. RS Kahn. Tegelijkertijd is hij begonnen aan een onderzoekstraject dat tot de huidige dissertatie heeft geleid onder leiding van prof. dr. H van Engeland en prof. dr. H Swaab.