Universität Trier

Genetics of common psychiatric disorders: A Mendelian perspective based on genetic analyses of large pedigrees

Dissertation zur
Erlangung des Doktorgrades der Naturwissenschaften
aus dem
Fachbereich I – Psychobiologie

vorgelegt von

Michelle Kaiqi Lin

Betreuer:
Prof. Dr. Jobst Meyer
Prof. Dr. Claude Muller

Trier, 2011

Chapter 1

Introduction: The search for causal genes or genetic loci in psychiatric disorders and behavioral traits through multi-generational pedigrees

In psychiatric and behavioral sciences, many scientists face complex problems in trying to unveil the underlying mechanisms that brought about psychiatric and behavioral phenotypes. Mendelian disorders or traits are phenotypes, which are caused by one genotype at one genetic locus (i.e. monogenic) and can be found to occur with high frequencies in large families. They are contradictory to the non-Mendelian multifactorial disorders/traits, otherwise known as complex disorders/traits, which are brought about by the effect of multiple genotypes located at multiple genetic loci and the environment (i.e. polygenic). Psychiatric and behavioral disorders/traits are generally categorized as complex disorders/traits, similarly to that of many other physiological disorders/traits [1]. However, until recently, efforts to understand their genetic mechanisms have been lagging behind that of their more physio-physical clinical counterparts, which may be due to the later acknowledgement that psychiatric and behavioral disorders/traits should be treated as any other genetic disorders/traits. Despite that, the field of psychiatric and neurobehavioral genetics has progressed rapidly in recent years with the extensive collaborations within various international consortia that encompass research perspectives ranging from endophenotyping to the classical linkage and association studies [2,3]. In this chapter, we will contemplate the progress of molecular genetics, with special emphasis on the pattern of Mendelian inheritance, in the general field of psychiatric and behavioral disorders/traits.

From family, twin and adoption studies, many psychiatric and behavioral disorders/traits are known to be heritable and are influenced by genetic factors, albeit not totally (i.e. environmental factors and non-familial genetic factors, which we will not be going into details here). Francis Galton (cousin of Charles Darwin, founder of the evolution theory), who devoted much of his

research passion on quantifying human traits variation (e.g. height, weight, eye colour, arm length), recognized the value of twins for studying human genetics and is the documented first to inquire into the concept of heritability and environment [4]. Monozygotic (MZ) twins are genetically identical as compared to dizygotic twins (DZ), who only share half of their genes. Hence, if a trait has a large genetic component driving its expression, MZ twins will display higher instances of the trait than DZ twins or other unrelated individuals. If a trait has a larger genetic component than environmental component, the MZ twins who were brought up apart (i.e. adopted into different families, living in different environment) will nevertheless have the same chances of expressing the trait. However, if the trait in question is largely under environmental influences, the adopted MZ twins will have different probabilities of expressing the trait. The earliest modern studies showing heritability in psychiatric disorders through such family, twin and adoption study designs were largely made with schizophrenic samples in the 1960s and 1970s, propelled by Gottesman and Shields, who went on to establish the yardstick for many modern researches into the genetics of behavior and psychopathy [5,6].

There are different camps of thought with regards to the genetic model of most psychiatric/behavioral disorders/traits. The two main views by which the phenotypes arise are via: 1) multiple common genetic variants, each of small effect (i.e. polygenic), otherwise known as the common variant – common disorder (CVCD) hypothesis [7]; or 2) a single rare genetic variant of major effect (rare variant occurs in different genes for different families or individuals, who display the same phenotype), also known as the common disease/rare variant (CD/RV) hypothesis [8]. Genome-wide association studies (GWAS) are carried out in the hypothesis-free (with no candidate genes apparent in mind) search for genetic loci associated with a particular psychiatric disorder or behavioral trait. The main assumption for GWAS is the accumulative small effects of multiple genes, belonging to the CVCD camp, which cause the phenotypic disorder/trait observed [9]. In spite of the ever-increasing sample sizes, which has recently reached over tens of thousands

of subjects, replication of results across independent studies for any particular trait/disorder proved difficult in many cases [10]. On the other spectrum, rare genetic variants of major effects are considered in linkage studies using extended pedigrees, with the CD/RV hypothesis in mind.

Under normal circumstances, psychiatric disorders or behavioral traits are considered to be polygenic, involving several to many genes of both small and large effect sizes, ultimately contributing to the phenotype. This is unlike monogenic disorders/traits, which are due to the effect of a single gene. However, the existence of multiplex families with numerous individuals carrying a particular psychiatric disorder or behavioral trait, steer us towards a Mendelian genetic exception in these families. The existence of large multi-generational pedigrees, comprising of multiple affected members, are present for quite a number of psychiatric disorders or behavioral traits such as bipolar disorder in the Amish extended pedigree [11], autism in the Utah autistic pedigrees [12] and schizophrenia in the Palau schizophrenic pedigrees [13], just to name a few. The revelation of singular rare variants in the respective families cumulatively adds to the genetic databases for the disorder/trait, which will in turn allow a larger picture of the genetic mechanism driving the disorder/trait that affects others in the general population. Mitchell and Porteous have recently written a review on understanding the genetic architecture of psychiatric disorders through Mendelian cases occurring in large pedigrees [14]. Using schizophrenia as an example, they argued that despite only a small percentage of schizophrenic cases being explained by highly penetrant mutation in a single gene each time, if these exceptional cases (with their unique rare variant or mutation) appears frequently enough, then it makes logical sense that one can assume that a significant portion of the psychiatric disorder is accounted for by rare variants or mutations.

In the following paragraphs, we shall discuss how large families with many individuals affected by psychiatric disorders can 1) be useful in understanding genetic mechanisms of behavioral pathologies; 2) provide sample cases to dissect overlapping genetic causes in cross-disorders of two or more

psychiatric traits in comorbidity; 3) offer better insights in introducing clearer subtyping of psychiatric diagnoses; and 4) give clues as to which environmental factors may come into play in the disease manifestation.

(1) Mendelian patterns in large families affected by psychiatric disorders

In extended families with several generations, consisting of multiple affected individuals in each generation, one can follow the transmission of the disorder from parents to offspring. The first step is usually a genome-wide linkage analysis in these families in search of chromosomal regions, which is highly linked to the disorder being studied. After which, a segregation analysis ensues to more accurately fine-map the chromosomal regions, which co-segregate with the disease status in the family. This is can be done by analyzing genetic biomarkers, which are either the fragment size of microsatellite repeats or the single nucleotide polymorphisms (SNPs), in the genome of as many family members as possible. By doing so, it is possible to construct haplotypes, which are certain sets of microsatellite fragment sizes or SNPs in a region of the genome. Haplotypes can be traced from a 'founder' affected individual (usually the eldest in the pedigree with clinical diagnosis and genotype known) down to his or her descendants. In the event that the psychiatric trait has a Mendelian tendency in a particular family, a specific haplotype is seen in all affected members in the family but not in the unaffected members. In ideal situations as such, one has come across a fully penetrant dominant psychiatric trait in that family, with a known causative genetic locus.

Genetic mutations or variations in the genetic locus co-segregating with the psychiatric disorder in the family can be further explored in an attempt to better understand the genetic mechanisms underlying the disorder. Almost all genetic variants or mutations uncovered via such approach in large pedigrees are rare and private to the families being investigated. These rare and private mutations or variants are believed to exert the majority of the effect resulting

in the disorder. As they are rare, they will not likely show up in most association studies utilizing the general population as sample base. It follows that large-scale GWAS most probably has a very low/or no chance of picking up these genetic loci signals. Therefore, multiplex families can play a very important role to reveal genetic anomalies, which induce complex disorders or traits [15].

Ultimately, many researchers and clinicians are interested in what causes the disorder. Rare genetic mutations and variants give clues to which genetic pathways and thus, the bio-chemical pathways when affected, give rise to the disorder. In complex disorders, as most psychiatric disorders are believed to be, many genes interact with each other in extensive webs of communication and the malfunction of any one of the genes may result in the same disorder, known as genetic heterogeneity. In other words, rare and private genetic mutations/variants can provide insights into the pathophysiological pathways of the disorder, which are also affecting other patients from the general population. For many psychiatric disorders to date, their etiologies are still an enigma in many aspects but extended pedigree studies can serve to add more information to uncover the causation of the disorder.

More often than not, there are several exceptions, which one has to consider while looking for Mendelian inheritance of psychiatric disorders in large family-based studies and they are chiefly, i) incomplete penetrance; ii) sporadic cases; and iii) bilineality. i) Incomplete penetrance happens when an unaffected individual carries the haplotype that all other affected individuals in the family are carrying. Depending on the occurrence rate of such status in the family, the penetrance level for the disorder at a particular genetic locus can vary drastically. This is where one has to consider other non-familial genetic factors and/or environmental factors exerting their influences, and only having fulfilled the criteria of those factors, will the said haplotype co-segregating with the diseased trait lead to the expression of the phenotypic trait [16]. ii) On the other hand, there may be instances when an affected individual diagnosed with a psychiatric disorder, does not carry the same

haplotype, which all other affected family members carry. Sporadic cases, though rare in large pedigrees, can still be expected to appear at about the same percentage rate as in the general population for the disorder being investigated. iii) Apart from such problems, there is also bilineality in extensive pedigrees to consider. As with many disorders, the reproductive fitness in psychiatric individuals is lower than that of the general healthy population. Unless it is an early on-set disease like autism, many people afflicted with psychiatric disorders still manage to marry and start their own families [17-19]. Thus, there are two possible lines of Mendelian alleles (either from the paternal side or the maternal side of the family) being transmitted to their descendants. This complicates matters as further down the family tree, one will most probably come across several individuals who are affected but are carrying different haplotypes (some from the paternal and others from maternal line). Another usual occurrence is large pedigrees with multiple generations involving more than one affected spouse/s can be found in consanguine families in certain social cultures, which encourage close-ties (e.g. between first cousins) marriages [20,21]. In large inbred pedigrees, rare genetic variants of recessive psychiatric disorders can be detected very easily.

(2) Cross-disorders among psychiatric traits – how multiplex families may support the analyses

Shared heritability arises due to genetic pleiotropy, a situation where one gene gives rise to different phenotypes. A recent example of shared heritability was presented in a metal-analysis of genetic studies carried out on attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD)[22]. There is a high frequency of co-occurrence of ADHD and ASD in children, hence the hypothesis that the two disorders share similar genetic factors. In multiplex families consisting of individuals diagnosed with multiple psychiatric problems, tracking differences in genetic sequences (which are cost-realistic and do-able within an acceptable time-frame with the diving

costs and improvements in the next-generation sequencing technologies) and comparing among affected individuals with comorbidities, affected individuals without comorbidities and unaffected individuals in the same family may be easier to yield leads to the dissection of genetic components which give rise to genetic pleiotropy. As many of the psychiatric disorders have one or more criteria that may overlap, the differences in diagnosing these criteria may be subtle. Since large families are powerful sample bases to detect genetic anomalies of major effect sizes, it is more plausible to find hints to tease apart the overlapping genetic mechanisms between disorders via their gene-gene interaction network. This will in itself also allow better comprehension of the etiologies of each disorder.

A recent success on psychiatric cross-disorders of schizophrenia and bipolar disorder used information collected from families as a foundation for the study. Lichtenstein *et al.* utilized more than two million nuclear families in Sweden to assess the effects of genes and the environment in contributing towards the two disorders [23]. They managed to show evidence that schizophrenia and bipolar disorder share a common genetic cause to a certain extent. Another genome-wide study by Huang and colleagues attempted to investigate the cross-disorder of schizophrenia, bipolar disorder and depression using large number of unrelated affected individuals but they were not as successful [24].

(3) Subtyping of psychiatric disorders for improved diagnostic criteria with multiplex pedigrees

Psychiatric genetics is quite unlike the other branches of human genetics. A salient difference is the diagnostic tools used to determine the disorder – usually a series of questionnaire tests and structured interviews of the individuals in question, which may or may not include interviews of other non-affected individuals in close contact with the clinical proband. ‘Non-physical’ methods of examination and categorization of psychiatric disorders as such

are open to debate at times about their objectivity during diagnoses despite much effort of the clinicians to standardize and coordinate across different executers of the tests and interviews. Hence, psychiatric diagnoses have been termed as 'the weak component of modern research' [25]. Over the years, there are constant improvements in the psychiatric testing tools but contrasting to most other human disorders, which can be more easily concluded via a physical test like blood testing of hormones or x-rays and mammograms of body parts, such physical tests for use in psychiatry are still in early stages of development. Many of the psychiatric labels are broad and encompass several subtle phenotypes that can either be different categories (e.g. DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition; and ICD-10, International Statistical Classification of Diseases and Related Health Problems, 10th edition) [26,27] of the same disorder or a wholly different disorder as in the cases of co-mobidities/cross-disorders. Perhaps such broad definitions for psychiatric diagnoses are the main culprit for the increased toughness in delving their genetics compared to other complex diseases. Efforts to subtype each psychiatric disorder to provide a finer diagnostic report are underway to combat these problems in many laboratories and clinics presently. As discussed in the preceding paragraphs on dichotomizing the subtleties between cross-disorders, multiplex families offer a beautiful and effective sample set-up to discover genes of large effect size, which may be hidden in the different sub-groups of a particular disorder [28,29]. These major effect genes may offer more options in defining a cleaner cut between categories of a disorder through groupings by pathway-specific gene malfunction; and may also serve as predicament of other onset of psychiatric problems in the same patients in future via observations of the causative genes in their possible gene-gene interaction network.

An example, periodic catatonia (OMIM: %605419), which belongs to a sub-category of schizophrenia with a bipolar course, is presented in chapter 3. A polymorphism of the *MLC1* gene, which was found to co-segregate with all periodic catatonia-affected members in one of our large families, was associated only to the periodic catatonia sub-group of schizophrenia [30]. In the

chapter, we did further functional studies on this candidate gene of periodic catatonia to investigate its role in relation to this sub-group of schizophrenia.

(4) Clues in large family-based studies to possible environmental consequences in development of psychiatric symptoms or behavioral traits

Some may argue about certain behavioral phenotypes being the result of nurture (environmental factors) as oppose to nature (genetics factors). In eating disorder cases, there is a markedly increased recurrence risk in first-degree relatives [31,32]. Despite so, it has only been recently (in the past two decades) acknowledged that it is a psychiatric disorder with a genetic basis [33,34]. The genetic work on eating disorders is in the early stages but it is still considered by many clinicians as having a strong familial behavioral influence [35]. As eating disorders have early onset at adolescence [35], multiplex families offer a good experimental setting in studying the environmental influence on the genetic factors in such disorder with known high heritability rate. Assuming the family members are still staying close to each other during the discovery of onset of the disorder at each generation, the environmental factors for most in the family members for that generation can be considered uniform to a certain extent. By investigating large multi-generational pedigrees of such disorders, one can elucidate the effect on the environment on the genes by looking within each generation and also comparing them to that of across the different generations and branches of the main families (which have different environmental influences since one can assume that after setting up a new family with their spouses, each nuclear family then live separately). In other words, it is possible to compare several different environment before/during onset of the disorder for one familial set of genes with increased power to detect the disease heritability.

Guide to the subsequent chapters

The special theme of this thesis is to investigate large pedigrees showing Mendelian pattern for various psychiatric disorders (namely attention-deficit/hyperactivity disorder (ADHD), schizophrenia and bipolar disorder), in the bid to understand more about the genetic architecture of general psychiatric/behavioral disorders/traits, which have a late start as compared to many other physiological disorders/traits. Other problems mainly specific to the psychiatric and behavioral sciences, like frequent cross-disorders and sometimes quite subjective diagnostic tools used, further add to the difficulties of psychiatric/behavioral genetics research.

The following chapters explore a series of experiments using the classical genetic approach, as explained in the following figure (figure 3), on various psychiatric disorders and behavioral traits. This classical two-stage genetic approach is widely used by many human geneticists to study a wide range of human physiological disorders in the past decades but is only recently being applied to the field of psychiatric and behavioral sciences. One of the earlier successful efforts to execute a large-scale international two-stage genetic approach in psychiatry was carried out by Moises and *et al.* in schizophrenic samples using large multi-generational pedigrees from an Icelandic population isolate in the linkage step and they proceeded on to the association step with independent population (i.e. non-pedigreed) samples from eight different countries [36].

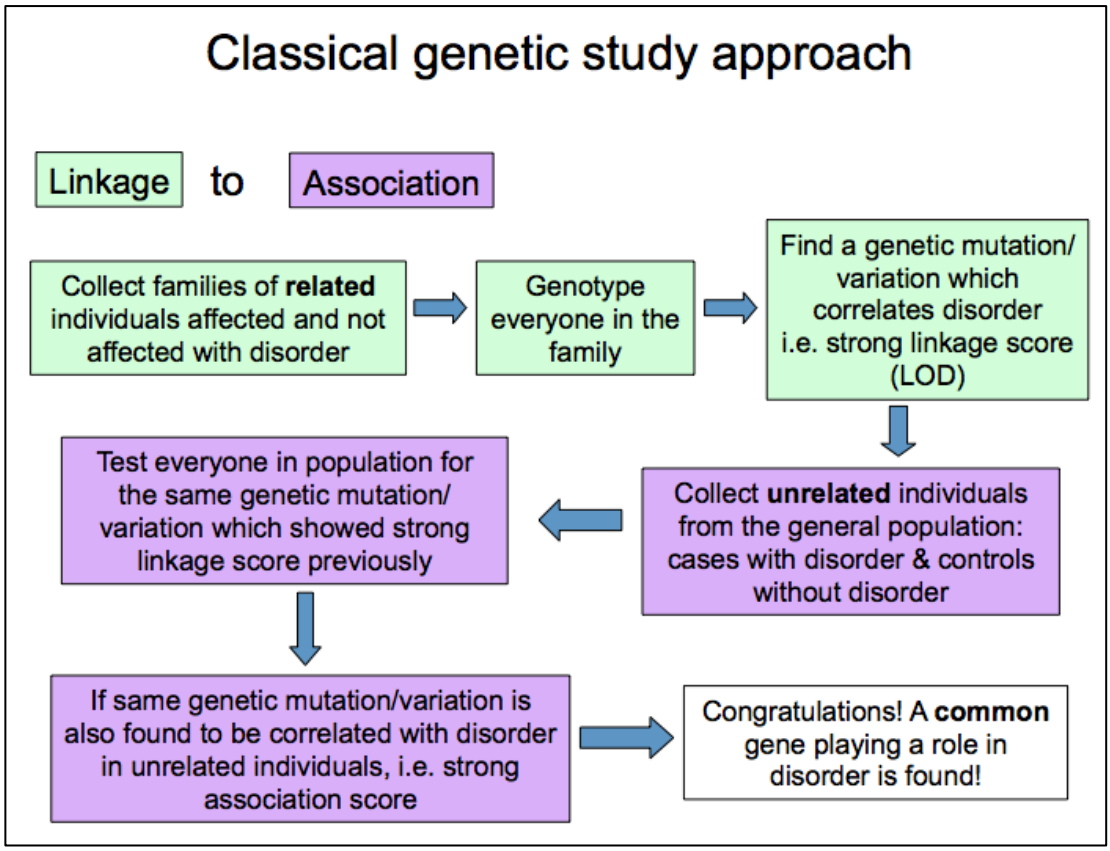


Figure 3: Diagram of a classical two-stage genetic approach. Linkage stage is in green and association stage is in purple.

The linkage study stage (in green) of the classical genetic approach is usually carried out in large families with multiple members affected with a particular disorder. Rare variants, and common variants as well, may be revealed at this stage, which is especially useful under circumstances where there is limited genetic knowledge about the disorder (as the case with many psychiatric and behavioral disorders/traits). The association study stage (in purple) is usually carried out in unrelated individuals of the general population when the first linkage step done is successful.

Generally, after a large family with multiple individuals affected with a psychiatric disorder or behavioral trait has been recruited, the first step is to carry out a linkage analysis followed by fine-mapping of chromosomal regions in all members of the family in search of genetic loci co-segregating among

family members with the disorder/trait. The hypothesis for this particular step is that causal genes or genes playing a major role in contributing to the susceptibility of the disorder/trait are present within the co-segregating chromosomal region. Chapter 2, 'Fishing for afflicting chromosomal regions in psychiatric/behavioral disorders/traits: Fine-mapping in ADHD large families', examines the fine-mapping of chromosomal regions from prior linkage analysis done to search for narrowed down genomic regions, which has a high probability in containing genes contributing to the said disorder.

The second step following a successful positional cloning effort (i.e. fine-mapping) is to identify interesting candidate genes within the narrowed down chromosomal regions. Functional studies on the candidate genes are later carried out to better understand if and how they may play a part in causing the disorder/trait. In chapter 3, 'Candidate genes within the afflicting chromosomal regions: Functional studies of a selected candidate gene, *MLC1*, in schizophrenic large families', shows an example of a candidate gene within a fine-mapped chromosomal region, which was selected for further functional study to learn if, and how, the gene may contribute to the phenotype of the disorder.

In parallel, the next step after successful positional cloning, is to investigate whether the particular mutations or variations of the candidate genes identified in the narrowed down chromosomal regions are also found in unrelated individuals in the general population who are similarly affected by the same disorder/trait. Chapter 4, 'Extrapolating genetic results from large families to the general population: Association study of SNPs in unrelated bipolar and schizophrenic individuals from results of prior large family studies', completes the whole process of the classical two-stage genetic approach, which brings the reader through linkage study in large families to association study in the general population of two well-known psychiatric disorders.

Chapter 5, 'Feasibility of replicating genetic results from large family studies in general population of unrelated individuals for complex psychiatric disorders

and behavioral traits: From eQTL linkage to association analyses in vervets', focuses on another genetic aspect, expression quantitative trait loci or eQTL (not only on genes, but the expression level of genes), but still following the classical two-stage genetic approach. This chapter also presents attempts to answer three main questions relevant to the summation of the current thesis and the possible future direction in psychiatric and behavioral genetics: 1) does the classical genetic two-stage approach works as well when applied to complex traits with a smaller but still suitably sized population of unrelated individuals as compared to many of the large sample-sized experiments in psychiatric and behavioral genetic association studies nowadays. This question is paramount to today's psychiatric genetics research since large samples, amounting to tens of thousands of subjects, are necessary to detect significant results in the association stage [37]; 2) aside from functional genes, which is the original focus of many psychiatric/behavioral genetic studies, is it possible for other genetic aspects to be involved, e.g. eQTL (genetic regions which regulate the expression of genes); and 3) is it possible to set up an accurate map of blood biomarkers to study the neuro-physical states of both psychiatric disorders and behavioral traits. This is a pilot-study to create a blood-brain correlated gene expression map that can used to study physical brain changes for psychiatric/behavioral disorders/traits with just blood samples alone. Future experimental successes may lead to plausible definitive genetic markers (similarly to many clinical physiological disorders) for psychiatric disorders or behavioral traits in the coming years and more importantly, deeper insights into the web of gene interactions resulting in the psychiatric/behavioral phenotypes.

Chapter 6, the concluding chapter of this thesis, will touch upon several popular ideas that are driving recent psychiatric and behavioral genetics studies, in which large pedigrees can be of good use.

Chapter 2

Fishing for afflicting chromosomal regions in psychiatric/behavioral disorders/traits: Fine-mapping in ADHD large families

This chapter is presented as a manuscript (under re-review by the European Journal of Human Genetics as of 5th September 2011) entitled:

Haplotype co-segregation with ADHD in unrelated German multi-generation families

Michelle K. Lin[1], Christine M. Freitag[2], Haukur Pálmason[1], Christiane Seitz[3], Tobias J. Renner[4], Marcel Romanos[4,8], Susanne Walitza[4,7], Christian P. Jacob[5], Andreas Reif[5], Andreas Warnke[4], Rita M. Cantor[6], Klaus-Peter Lesch[5] and Jobst Meyer[1]

[1] Department of Neurobehavioral Genetics, Institute of Psychobiology, University of Trier, Germany;

[2] Department of Child and Adolescent Psychiatry, Goethe University Frankfurt am Main, Germany;

[3] Department of Child and Adolescent Psychiatry, Saarland University Hospital, Homburg, Germany;

[4] Department of Child and Adolescent Psychiatry and Psychotherapy, University of Wuerzburg, Germany;

[5] Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Germany;

[6] Department of Human Genetics, School of Medicine, University of California Los Angeles, United States of America;

[7] Department of Child and Adolescent Psychiatry and Psychotherapy, University of Zurich, Switzerland;

[8] Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital of Munich, Germany.

Abstract

Complex disorders proved to be elusive in the search for underlying genetic causes. In the presence of large multi-generation pedigrees with multiple affected individuals, a Mendelian dominant inheritance model can be observed in those families by following the haplotypes being transmitted predominantly to all the affected members in the families. Particular shared haplotypes among all affected individuals can reveal the chromosomal regions where the disease-related genes may be located and the critical pathways involved can be exposed.

We have recruited eight large Attention-Deficit/Hyperactivity Disorder (ADHD) families of German descent. Densely spaced informative microsatellite markers with high heterozygosity rates were used to fine-map and haplotype chromosomal regions of interest in these families. In three subsets of the eight ADHD families, haplotypes co-segregating with ADHD-affected individuals were identified at chromosomes 5q11 – 5q13, 9q31 – 9q32 and 18q11 – 18q21, which are supported by positive LOD scores.

The existence of haplotypes co-segregating among all affected individuals in large ADHD pedigrees suggests the existence of Mendelian forms of the disorder and that ADHD-related genes are located within these haplotypes. New-generation sequencing tools will allow whole of these regions to be sequenced more easily to identify causal genetic mechanisms, providing further insights into the genetics of ADHD.

Introduction

Genetic architecture of complex disorders is generally difficult to elucidate and the search for causal gene or a set of causal genes, where multiple pathways may be involved, is an uphill work. Unlike monogenic disorders, complex disorders do not usually follow a Mendelian inheritance pattern. However, the existence of large pedigrees with many related individuals affected with the same complex illness, demonstrate the possibility of that complex disorder segregating in a Mendelian dominant fashion. Examples of past results from numerous large family-based studies of complex diseases for hypertension [38], type II diabetes and Maturity Onset Diabetes of the Young (MODY) [39], inflammatory bowel disease [40], Parkinson's disease [41], bipolar disorder [42], and schizophrenia [43], to name a few, support the Mendelian model of inheritance in these families. For a particular complex disorder, different families may exhibit different causal genes, alleles or genetic loci, pointing to private rare mutations in the different families. A probable explanation for such is that among the intricacies of complex disorders lies genetic heterogeneity, where different variants or mutations in the same gene, and/or different genes in the same or related pathways, may lead to the manifestation of the same disorder.

Large pedigrees with multiple individuals afflicted with a complex disease permit the tracking of haplotype transmission among the affected family members and the lack of transmission to those who are unaffected. The recurrence of a particular haplotype among all the affected family members, but not the unaffected, can provide valuable insights into the plausible candidate genes in those regions, which may cause the disorder running in the family. Despite the high probability of uncovering genetic variants or mutations that are rare and usually private to the respective families, the knowledge gleaned from this approach can ultimately be extrapolated into the general affected population by providing clues about the pathophysiological pathways causing the disease from these genes.

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neuro-psychiatric disorder, which affects approximately 1 in 20 children [44], and has about a 50% probability of persisting into adulthood [45]. This etiologically complex and clinically heterogeneous condition is characterized by an early onset (before 7 years) of age-inappropriate inattention, hyperactivity and increased impulsivity observed in different settings (e.g. at school, at home and/or at work), leading to impairment in social functioning. Previous twin and adoption studies showed a strong heritability (70 – 80 %) of childhood ADHD [46,47], which is comorbid with many other traits like anxiety disorder, affective disorders, depression, oppositional defiant or conduct disorder and substance abuse [48]. However, in a recent twin-family study, heritability of adult ADHD was estimated to be about 30 % [49].

We have recruited eight large ADHD pedigrees of German origin. These families comprised of a total of 191 individuals, of whom 95 are affected with ADHD. From the family trees, ADHD appears to segregate in a dominant manner. Hence, postulating a dominant Mendelian model of genetic inheritance of ADHD in these pedigrees, we investigated densely spaced (~ 0.5 Mb) microsatellite markers on all available family members in chromosomal regions derived from our previous 50 K SNP chip-based study on these families [50]. The previous 50 K SNP genome-wide linkage study was done using a few selected individuals to represent each family, which did not allow us to obtain enough information to examine the co-segregation of haplotypes with the disorder. However, it provided us with important leads to where we can apply our microsatellite markers in the vast genome. From our haplotype segregation analysis in the present study, we were also able to determine the borders of regions on the chromosomes that are shown to be co-segregating with ADHD, which was not possible in the earlier study. The present study allows us to identify chromosomal regions co-segregating with ADHD in the respective families by the observation that these haplotypes are predominant in the affected individuals in the families but not in the unaffected members.

We have investigated all reported linkage regions from our 50 K SNP study in all available family members, in accordance as to which significant linkage regions coincide with which large ADHD families [50]. Interestingly, most genetic loci with significant linkage peaks in the previous SNP study did not show co-segregation with ADHD but were shown to be present in a random manner among the members of the families, maintaining the need for this study. Through the present analysis, three chromosomal regions are revealed, at chromosomes 5q11 – 5q13, 9q31 – 9q32 and 18q11 – 18q21, to be co-segregating with the affected status in our different ADHD pedigrees. Genetic mutations, variants or structural variants within these regions found to co-segregate with ADHD may contribute to the better understanding of the molecular mechanisms leading to ADHD.

Materials and methods

Family ascertainment/assessment and sample description

Families were recruited through index children referred to the three outpatient clinics in Trier, Homburg and Wuerzburg and were of German origin. The index children were aged 6 or above and met the criteria for the ADHD combined type according to DSM-IV. Individuals exhibiting ADHD symptoms without a typical childhood history and individuals with unclear etiology of symptoms were considered as 'unknown' in the diagnosis. The families P1, P2, P3 and P4 were recruited in Wuerzburg, and families P5, P6, P7 and P8 in Homburg and Trier. The eight families included in this study comprised of 191 individuals, of whom 95 were affected with ADHD. These families are 2 - 4 generation multiplex pedigrees, which were depicted in more details in figure 1 of Romanos *et al*. [50]. All participants and/or their legal guardians have signed an informed written consent. Approval by the ethics committees at the Julius-Maximilians University (Wuerzburg) and the Saarland Doctors' Association (Saarbruecken) was granted.

For a full description of the diagnostic procedure, please refer to Romanos *et al*. [50]. In short, children and adolescents were assessed by semi-structured interview (Kiddie-SADS-PL German version [51] or Kinder-DIPS [52], the Child Behavior Checklist [53]), by an ADHD diagnostic checklist applying DSM-IV criteria (ADHD-DC) [54], and teacher ratings (TRF [53] or FBB-HKS [55]). All adults were assessed by SCID I [56] and SCID II [57], the ADHD diagnosis checklist (ADHD-DC) and the Wender Utah Rating Scale [58]. Measurement for intelligence was by the non-verbal CFT1/20 [59,60] in children, and MWT in adults [61]. None of the participants display an IQ below 80. All participants were assessed by at least two clinicians experienced in childhood and adult ADHD diagnosis. Under circumstances that a complete assessment was not possible, the individual's ADHD status is taken to be 'unknown'.

Genotyping and haplotype estimation

DNA was extracted from blood samples according to standard protocols. Fine-mapping was done with microsatellite markers over the regions that exhibited significant or suggestive linkage from the ~ 50 K SNP analysis done previously [50]. A total of thirteen regions were tested out of the fifteen previously reported, with each region having a MOD score of at least 2.56 to 4.17 in the ~ 50 K SNP study. Unlike the previous study, the present study focuses on assessing alleles, which co-segregate particularly with ADHD in a Mendelian model. All 155 individuals with available DNA, both affected and unaffected, were genotyped in the present study. The microsatellite markers were selected from the deCODE linkage maps and the genotyping was done with a resolution of ~ 0.5 Mb. A total of ninety-nine microsatellite markers were tested. Numbers of markers for each of the thirteen regions tested vary according to the size of the region. In the cases where no microsatellite markers in the region of interest were observed in the deCODE linkage map, self-designed microsatellite markers were used (self-designed marker primers can be made available on request). Linear extrapolation between the two closest flanking markers at each end with known positions was used to

calculate the genetic position of self-designed markers. Positions of markers are defined according to the UCSC genome bioinformatics browser, human genome assembly March 2006.

Polymerase chain reactions (PCRs) were done using primers specific for the deCODE markers as stated in the UniSTS platform. Primers were dye-labeled at the 5'-end with either BMN6 or Cy5 and were ordered from Biomers (Ulm, Germany). The PCR products were then processed in the Beckman Coulter CEQ 8000 Genetic Analyzer with the Beckman Coulter GenomeLab™ fragment analysis protocol. The different fragments were analyzed and recorded using the Beckman Coulter CEQ analysis system. The fragment information was imported into SimWalk2 [62] using the EasyLinkage bundle (version 5.02) [63] to obtain haplotype information. Following which, the Haplopainter software (version 1.043) [64] was used to draw the haplotypes for each of the genotyped members of the families.

Linkage analysis

Parametric multi-point linkage analysis was conducted using SimWalk2 [62] under a dominant model. Such a model was used in conjunction with our hypothesis of a Mendelian model of ADHD in some sub-branches of the multi-generational pedigrees. The disease allele frequency was set to 0.001 and it was assumed that there was no heterogeneity (alpha = 1.000) within an individual family. A series of penetrance level between 80 % to 100 % was used and those, which produced the best LOD scores, were recorded (fully penetrant for families P1 and P3, and 0.80 for family P2). Generally, only markers with above 0.70 heterogeneity according to the Marshfield genetic maps [65] were used, and marker allele frequencies were estimated using all individuals in the pedigrees. All runs were checked for non-Mendelian genotyping errors using Merlin (version 1.1.2) [66] and markers with genotyping errors were removed for those showing the genotyping error.

Results

Haplotype segregation and LOD scores

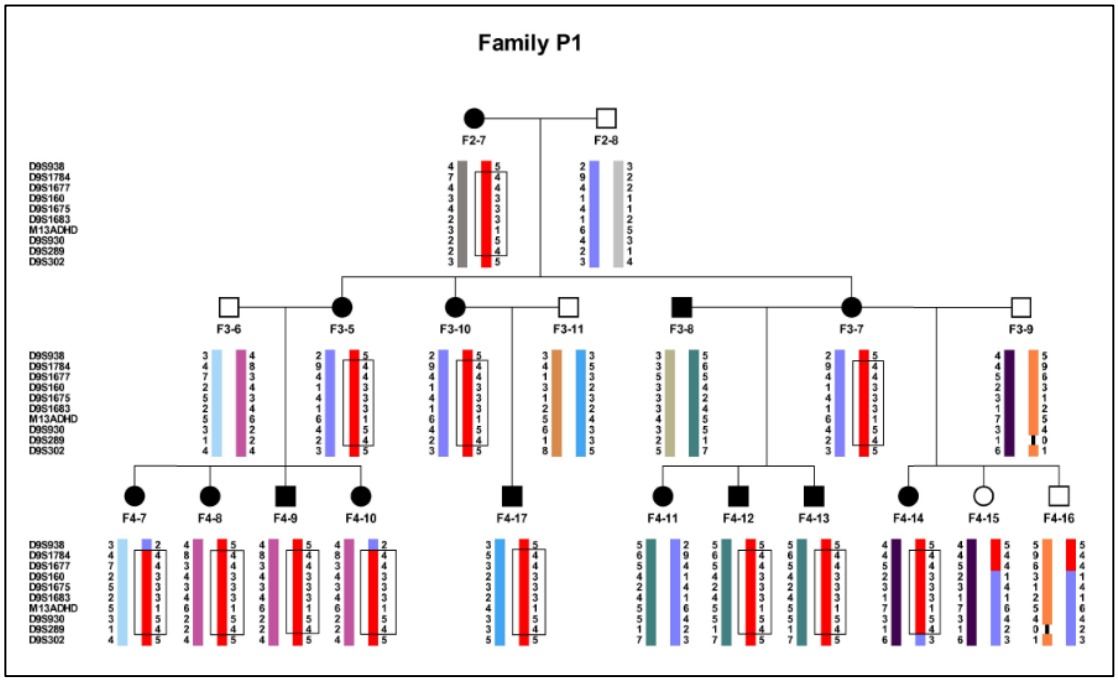


Figure 1: Haplotype segregation (in red and boxed) at chromosome region 9q31 – 9q32 in a subset of Family P1

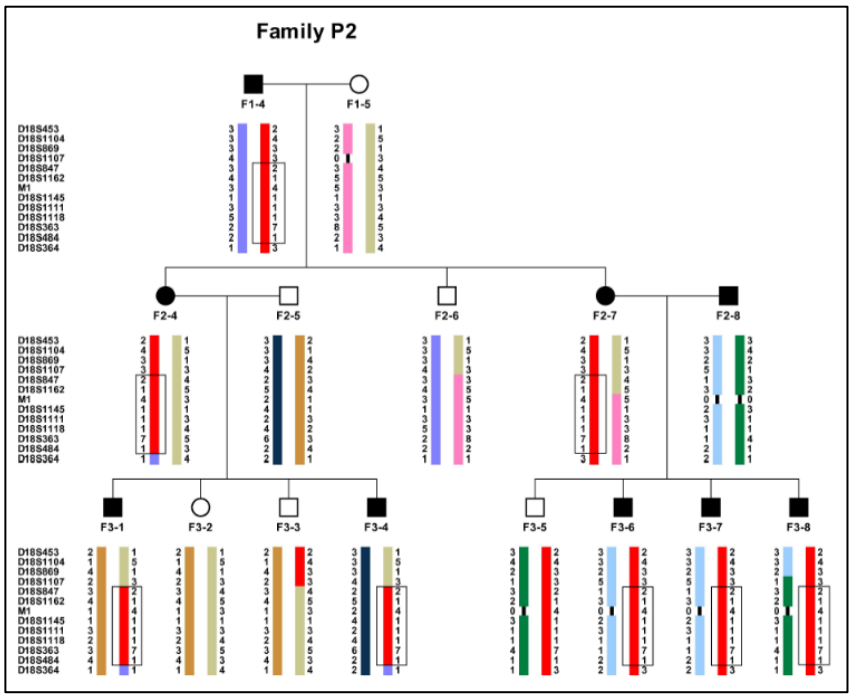


Figure 2: Haplotype segregation (in red and boxed) at chromosome region 18q11 – 18q21 in a subset of Family P2

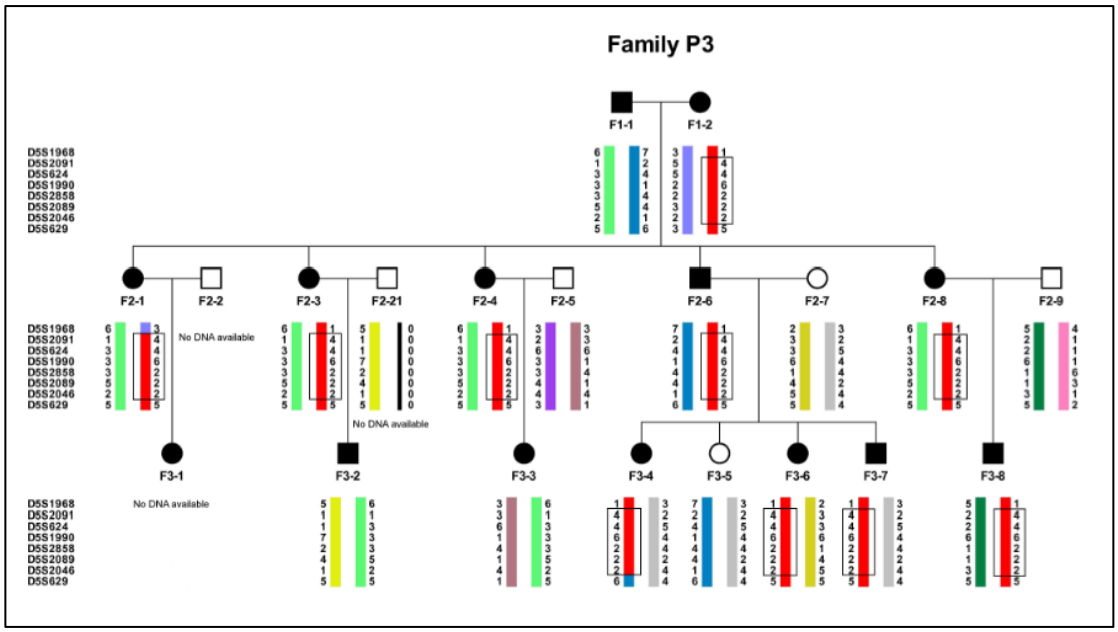


Figure 3: Haplotype segregation (in red and boxed) at chromosome region 5q11 – 5q13 in a subset of Family P3

Legend

Females are depicted as circles and males as squares in the pedigree trees. Individuals diagnosed with ADHD have their gender symbols shaded black and family members not diagnosed with ADHD are unshaded.

All three segregating chromosomal regions in their respective family subsets showed positive LOD scores greater than 1. Results are summarized in table 1.

Family	Chromosome	Upper border		Lower border		Region size (Mb)	LOD score
		Marker	Position (Mb)	Marker	Position (Mb)		
P1	9q31 – 9q32	D9S938	105.0	D9S302	116.1	11.1	3.08
P2	18q11 – 18q21	D18S1107	20.4	D18S364	51.5	31.1	1.64
P3	5q11 – 5q13	D5S1968	53.3	D5S629	68.3	14.8	2.11

Table 1: Summary of chromosome regions co-segregating with ADHD and their corresponding LOD scores.

For all of the above pedigrees, ADHD status is defined as either one of the following: inattentive, hyperactive/impulsive, combined type (of inattentive and hyperactivity/impulsive).

Out of the thirteen regions tested, only three showed haplotypes co-segregating with ADHD in the subsets of three different families. In a subset of the extended family P1 (figure 1), a haplotype, which has been narrowed to chromosome 9q31 – 9q32 (LOD score = 3.08), is found to be segregating in all ADHD-diagnosed individuals except for individual F4-11, who is a descendant of both parents (F3-7 and F3-8) affected by ADHD. This incident of bilineality arises when F3-8, who belongs to another family and thus, carrying a different line of ADHD genetic heritage from the rest of family P1, married into the family. F4-11 most probably carries another set of ADHD-related genes from her father's (F3-8) side of family (not recruited for the study), which is different from the rest of her ADHD-affected relatives in family P1. An ADHD-related region at 18q11 – 18q21 in a Dutch ADHD population isolate, previously reported by Amin and colleagues [67], was also found to segregate in all affected members of a branch of family P2 (figure 2) (LOD score = 1.64). Similarly, a recent Dutch adult ADHD heritability and linkage study carried out by Saviouk *et al.* showed significant evidence for inattention at 18q21 [68]. In another branch of family P3 (figure 3), a haplotype spanning 5q11 – 5q13 (LOD score = 2.11) segregates predominantly among the ADHD-diagnosed individuals. This family faced the same problem of bilineality as that of family P1, with more than one known lines of ADHD genetic transmission possible. Modest LOD scores between 1 to 2, similar to that from our subsets of these ADHD families, have been promising in identifying a causative variant through sequence analysis in several pedigree-based studies of dominant diseases (e.g. metachondromatosis [69]). These pedigrees usually appear to follow a dominant model with a certain degree of incomplete penetrance, which is also characteristic of our ADHD pedigrees. A fourth region co-segregating with ADHD is found at chromosome 1q25 in family P8 (LOD score = 1.79) (data not shown). In this particular case, the haplotype only co-segregates with ADHD in affected individuals with full clinical ADHD

status but not in those who are diagnosed with subclinical ADHD. Such difference between full clinical and subclinical groups in genetic susceptibility to the disorder show the importance of subtyping phenotypes at the diagnostic stage for genetic studies.

Discussion

ADHD is widely believed to be a multifactorial disorder, which arises from the overall contribution of many genes, each with a small phenotypic effect, as well as environmental influences. Association studies are usually applied to identify genes with small genetic influences [70] in cases like this. However, large-scale association analyses of many different complex disorders in the psychiatric field are difficult to replicate in independent samples. Up to now, genome-wide association studies (GWAS) in ADHD did not yield as many significant results across the different studies as expected [71]. Additionally, prior to association studies, various linkage analyses of relatively small sib pair families with ADHD did not replicate as consistently as hoped, saved for one region on chromosome 16 so far [72].

Given these inconsistencies, we have come to view the genetics of ADHD from a different perspective – that perhaps, in large multi-generation pedigrees, one may have a better chance to pick out single genes of large effect. This approach has been effective for some complex common disorders, with the classic example being the Maturity Onset Diabetes of the Young (MODY; OMIM #606391) where pedigrees were identified among individuals affected with type II diabetes [39]. It was discovered that although the causal genes for the different MODY families were different, they still led to similar phenotype. From such information, the pathways leading to the disorder was more efficiently dissected and the pathology of the disease better understood.

We have been fortunate to identify large pedigrees in which ADHD appears to be segregating in a Mendelian dominant manner. As has been done in many pedigree studies with traits believed to be the result of a single gene of large effect, a haplotype segregation analytical approach was applied to these ADHD families. This enabled us to uncover haplotypes that co-segregate with ADHD, resulting in specific chromosomal regions, which are shown to be jointly inherited by the affected members in the families. A recent copy number variation (CNV) screening carried out in a separate study, showing co-segregation of a duplication on chromosome 7p15 with the ADHD status in a branch of our multi-generational ADHD family (family P3), further supports monogenic dominant inheritance of ADHD in parts of our large pedigrees [73].

Here, we present three subsets of our initial eight large ADHD pedigrees, which displayed haplotypes that are co-segregating predominantly among the affected individuals. Several of these eight pedigrees contain episodes of bilineality through assortative matings (when two affected individuals marry and have descendants), which introduces more than one line of possible ADHD genetic heritage. Hence, a certain branch of a large pedigree may exhibit a particular haplotype co-segregating with ADHD but not another branch of the same pedigree due to multiple founder ancestors who are affected by the disorder in just one pedigree. The remaining branch of the said pedigree will most probably have another haplotype co-segregating with ADHD, which is not explored by the set of microsatellite markers in the present study. Only subsets, or branches, of the extended pedigrees with haplotype and ADHD status co-segregation were shown to avoid confusion even though we genotyped all available family members of the whole large pedigrees. The size of our subsets are comparable to that of a recent study of Dutch multi-generational ADHD family by Vegt and colleagues, which revealed similar co-segregation patterns of haplotypes with ADHD in chromosomal regions on 7p15 and 14q11 [74].

In another study, Arcos-Burgos and colleagues have also recruited ADHD families in a large population isolate of Colombian Paisa lineage and similarly,

carried out a fine-mapping study in an attempt to find chromosomal regions, which co-segregate with ADHD in those families [75]. They were successful with this approach, leading to the identification of the latrophilin 3 gene (*LPHN3*) as an ADHD susceptibility gene, which was statistically confirmed in an association study in a worldwide ADHD cohort of 2627 ADHD cases and 2531 controls [76]. Population isolates such as theirs have large advantages in mapping complex traits genes due to the larger effect of genetic and environmental homogeneity than outbred populations [77]. However, in our study, we collected ADHD families of German descent, which are not of a population isolate and are unrelated in terms of known recent common ancestors. Although this may give us a more representative genetic pool for ADHD in the German population, we are limited by the usually smaller size of the families, and more heterogeneity in the genetic make-up and environmental factors. ADHD is also known to develop under certain environmental conditions, and gene x environment interactions [78]. Furthermore, we cannot exclude the possibility that some of the ADHD cases, which are present may be due to chance, given that the prevalence for the disorder is about 5 % in the general population [44]. In line with this thought, our study design was not apt to control for possible comorbid disorders that may segregate within the respective families as well. Still, the data garnered from this study can bring us a little step towards comprehending the genetics of ADHD by directing our attention on several chromosomal regions of these families, which most probably contain ADHD-related genes, variants, mutations or even non-coding mutations and structural variants.

Our previous 50 K SNP study reported fifteen suggestive and significant linkage regions, most of which we were unable to replicate when we genotyped more family members of the respective families with more densely spaced microsatellite markers if we only accept a Mendelian co-segregation of the haplotype with ADHD status. As there is currently no sole reigning genetic model for ADHD, we also accept that there exist other possibilities, aside from the Mendelian model, as observed from the discrepancies between the non-Mendelian specified model of our previous 50 K SNP and

the present haplotype-based study. On the other hand, familial haplotype segregations, predominantly among the ADHD individuals in subsets of the presented large pedigrees, point to a probable Mendelian dominant model in these families. Using a Mendelian approach in large pedigrees for a complex disorder like ADHD may reveal rare familial forms that can provide insight into critical pathways that may not be previously known to be associated with ADHD. With the availability of the next-generation sequencing technology, whole-region sequencing is feasible in the narrowed chromosomal regions. Genes, as well as non-coding mutations, in these regions that may be contributing to ADHD in these families can thus be identified. Such a Mendelian strategy in the study of complex disorders remains one of the most powerful and successful methods to date for gaining valuable genetic and molecular information in complex diseases such as ADHD [15].

Chapter 3

Candidate genes within the afflicting chromosomal regions: Functional studies of a selected candidate gene, *MLC1*, in schizophrenic large families

This chapter is presented as a manuscript entitled (accepted by Journal of Nucleic Acids Investigation):

Unusual 5'-regulatory structure and regulation of the murine *Mlc1* gene: Lack of promoter-specific functional elements

Darja Henseler[1], Jonathan D. Turner[2], Matthias Eckhardt[3], Maaike van der Mark[4], Yanina Revsin[4], Michelle K. Lin[1], Thorsten Kranz[1], Claude Muller[2] and Jobst Meyer[1]

[1] Department of Neurobehavioral Genetics, Institute of Psychobiology, University of Trier, Germany;

[2] Institute of Immunology, Centre de Recherche Public-Santé, Laboratoire National de Santé, Luxembourg;

[3] Institute of Biochemistry and Molecular Biology, University of Bonn, Germany;

[4] Division of Medical Pharmacology, LACDR/LUMC, Leiden University, The Netherlands.

Abstract

The *MLC1* gene is involved in an autosomal recessive neurological disorder, megalencephalic leucoencephalopathy with subcortical cysts (MLC), which is characterized by macrocephaly during the first year of life and swollen white matter (leucoencephaly). Variants of *MLC1* have also been associated with psychiatric disorders such as schizophrenia, major depression and bipolar disorder. Currently, little is known about the encoded protein (MLC1). Judging from its similarity to other known proteins, it may serve as a trans-membrane transporter. However, the function of the encoded protein and its gene regulation has not been investigated successfully so far. We investigated the 5' region of the murine *Mlc1* with respect to regulatory elements for gene expression. A promoter search and an *in silico* analysis were conducted. Luciferase reporter gene constructs with potential promoter regions were created to study promoter activity *in vitro*. We found two alternative first exons for the murine *Mlc1* but were not able to detect any promoter activity for the investigated reporter gene constructs in different cell lines, thus pointing to the presence of essential *cis*-acting elements far outside of the region. *In silico* analysis indicated an uncommon promoter structure for *Mlc1*, with CCAAT-boxes representing the only noticeable elements.

N.B. *MLC1*/MLC1 refers to the human gene/protein and *Mlc1*/Mlc1 refers to the murine gene/protein.

Introduction

Autosomal recessive mutations, either homozygous or compound heterozygous, of *MLC1* cause megalencephalic leucoencephalopathy with subcortical cysts (MLC). This disease was first described in 1995 by van der Knaap and colleagues as a neurological disorder characterized by macrocephaly and leucoencephalopathy. Onset occurs during the first year of life and subsequently leads to deterioration of motor functions and mental decline. Magnetic resonance imaging (MRI) showed swollen white matter of the cerebral hemisphere and subcortical cysts in the anterior-temporal and frontoparietal regions [79]. Furthermore, several reports point to an association of *MLC1* variants with several psychiatric disorders. Meyer and co-workers described a Leu309Met mutation of *MLC1*, co-segregating with schizophrenia in a large family [80]. Similarly, a Leu308Gln mutation co-segregated with bipolar disorder in a single affected family, and single nucleotide polymorphisms of *MLC1* have been associated with schizophrenia and bipolar disorder [81]. Rubie and colleagues found the Leu309Met mutation in a single schizophrenic patient and his father [82], and Selch and co-workers confirmed association of *MLC1* polymorphisms with schizophrenia [30]. Additionally, Spijker and colleagues described a change in *MLC1* gene expression level in patients suffering from major depression [83]. The *MLC1* gene (OMIM *605908) is mainly expressed in astrocytes, as well as in ependymal cells, Bergman glia and leucocytes [84-86]. In mice, *Mlc1* expression has also been detected in neurons [87]. Immunostaining and electron microscopy demonstrated the localization of Mlc1 in perivascular astrocytic end-feet and astrocyte-astrocyte contact regions. Mlc1 is concentrated at brain barriers like pia mater and ependymal [84,86]. However, the actual function of Mlc1 is still unknown. It has been reported that Mlc1 is a protein with most probably eight trans-membrane domains and a marginal sequence identity to ABC2 transporters and the potassium channel Kv1.1 [80,84,88]. Since most of the proteins containing eight trans-membrane domains have transporter or channel function [84], the function of Mlc1 as trans-membrane transport protein seems to be probable, even if no ion transport activity could be demonstrated and no ligand of Mlc1 has been

identified so far [80,88]. The murine *Mlc1* is located on chromosome 15E3. It consists of 12 exons, whereby the translation start site (TLSS) is located in exon 2. The gene encodes a 382 amino acid protein, which shows 87 % sequence identity to the human protein. To date, nothing is known about the regulation of *Mlc1*, however, whilst determining the genomic structure, Steinke *et al.* suggested a putative promoter region. Several transcription start sites were found in a 5' region of approximately 70 bp, with the putative TSS being deposited in the GenBank database under accession no. BG297871 [89], in line with the transcriptional start site provided by Ensembl version 56. In this study, the 5' region of *Mlc1* was analyzed in more detail to achieve further information about the structure of the regulatory region and the regulation of *Mlc1*. Several putative promoter regions have been investigated by luciferase reporter gene assays. *Mlc1* was also studied with respect to four potential alternative first exons, and *in silico* analyses of the upstream region of *Mlc1* were conducted.

Design and methods

Expression studies

Whole brain, testis, amygdalae, hippocampi, hypothalami, cortex and cerebella of *Mus musculus domesticus* Black 6 (C57BL/6) were used for mRNA isolation. Reverse transcription into cDNA was performed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligodT-primers. Primers used for expression analysis of the alternative first *Mlc1* exons are shown in table 1. Primers were optimized on genomic DNA. The PCR for *Mlc1* exon1A was performed using 10 pmol of each primer, 200 µM dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM TrisHCl (pH 8.3), 0.0025 mg/ml BSA, 0.025 % Tween20 and 1 U Taq-Polymerase. PCR for *Mlc1* exon1B, exon1C and exon1E were performed using 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TrisHCl (pH 8.3), 0.0025 mg/ml BSA, 0.025 % Tween20 and 1 U Taq-Polymerase. The following thermocycler protocol was used: initial denaturation step (4 min, 94 °C), followed by 39

cycles of denaturation (30 sec, 94 °C), annealing (30 sec at appropriate temperature) and extension (30 - 60 sec, 72 °C), ending with a final extension (10 min, 72 °C) and cooling down to 4°C. PCR for *Mlc1* expression was conducted with an annealing temperature of 63 °C. Purity of cDNA was controlled by taking intron spanning *Beta-actin* (*ActB*) primers (for: 5'-AGGCTGTGCTGTCCCTGTAT-3'; rev: 5'-GTTTGCTCCAACCAACTGCT-3') using an annealing temperature of 60 °C. Primers for detection of *Pomp* pseudogene expression (for: 5'-GGCTGTGCTAGAGGTCCTTG-3'; rev: 5'-AGAGCTCCGCAACTGGAATA-3') worked at an annealing temperature of 57 °C.

Assembly of luciferase reporter gene constructs

Reporter gene constructs were assembled by cloning putative promoter sequences into the pGL3-Basic vector (Promega). The putative promoter sequences were originated from the 5' region of *Mlc1* (ENSMUSG 00000035805; Ensembl version 56) and amplified by PCR. High Fidelity Taq Polymerase from Fermentas was used for amplification. The primers used contained *Mlu*I- and *Xho*I-specific restriction sites for subsequent cloning into the pGL3-Basic vector. Primer sequences, sizes and positions of the investigated putative promoter sequences are shown in table 2. Restriction sites and nucleotide overhang of 3 bp, which were used for a better restriction efficiency, are underlined.

Cell culture

Experiments were performed in two *Mlc1*-expressing cell types, U373 MG glioblastoma-astrocytoma cells (ECACC 89081403), and a murine astrocyte cell line generated by transfecting primary astrocytes with an expression plasmid encoding the SV40 large T-antigen. Both cell types were grown in D-MEM High Glucose, supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine and 1 % penicillin/streptomycin (Pen/Strep) at 37 °C in a humidified atmosphere of 5 % CO_2. Cells were passaged at around 80 % confluency.

Transfection and stimulation

Cells were seeded at a density of 8 x 10^4 cells/ml in 24-well plates 24 hours prior to transfection. Transfection was done with Superfect (Qiagen) in a v/w ration of 10:1 (µl Superfect: µg DNA). 1 µg DNA contained 0.96 µg pGL3-luciferase constructs and 0.04 µg pGL4-74 (HRLUC/TK, *Renilla* luciferase; Promega). After 24 hours of incubation, cells were treated with one of the following: 50 µM dexamethasone, 50 µM Forskolin (For), 2 µM phorbol 12-myristate acetate for 24 hours, or with lipopolysaccharide (LPS) for 30 minutes.

Luciferase Assay

Cells were harvested in 50 µl Passive Lysis Buffer (PLB; Promega) 48 hours after transfection. Firefly and *Renilla* luciferase activities were measured sequentially with 10 µl of total cell lysates and 50 µl of luciferase assay reagent (LAR), followed by the addition of 50 µl of Stop & Glo reagent. The activity was determined by luminescence measurement for 10 sec in a liquid scintillation spectrophotometer (Berthold). The luminescence was given in Relative Light Units (RLU). Experiments were done in triplicates or quadruplicates from three independent trials. Promoter activities are given as ratio of firefly luciferase activity (RLU) divided by the activity of *Renilla* luciferase (RLU). While the firely luciferase activity indicates promoter activity, activity of *Renilla* luciferase is used as a control for transfection efficiency.

In silico analysis

PROSCAN Version 1.7 (http://www-bimas.cit.nih.gov/molbio/proscan/) was used for *in silico* analysis of the 5' region of *Mlc1*. Further *in silico* analysis was conducted for the region between 350 bp upstream of the first exon of *Mlc1* released in the ensembl database and the beginning of the TLSS in exon 2. Search for common promoter elements like TATA-, CCAAT- and GC-boxes was done according to the consensus sequences described by Suzuki and colleagues [90]. Sequences were considered as binding sites if they

showed ≥ 80 % identity to the given consensus sequences and fit 100 % to the core sequence (table 3). Consensus sequences for CCAAT- and GC-boxes were checked in both directions (forward and reverse complementary). The ratio of observed CpG to received CpG was calculated to identify CpG-islands. The used formula: CpG(O/R) = (CpG/(Cs*Gs))*N was taken from Gardiner and Garden [91]. CpG(O/R) means ratio of observed and received CpGs, N is the number of nucleotides investigated. Moving averages of % G+C content and ratio of observed CpG to received CpG were calculated with CpGPlot from EMBL-EBI (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) using a 200 bp window (N = 200) moving across the sequence at 1 bp intervals. In addition, a search by TRANSFAC Version 2009.1 was accomplished to search for potential transcription factor binding sites. The UCSC database (UCSC version, February 2009) was used to check for regions conserved between mouse and human of the *Mlc1*/*MLC1* 5' region up to the next 5' gene *Mov10l1*/*MOV10L1*.

Table 1: Primer pairs for expression analysis of putative first *Mlc1* exons

	primer sequence	**expected size for cDNA**	**gDNA**	**detected size**
***Mlc1* exon 1A**	for: 5'-CAGCAGTTCAAGGGCCAGC-3'	603 bp	1103 bp	not detected
	rev: 5'-TGTAGCTGCCTGGGTCCTGC-3'			
***Mlc1* exon 1B**	for: 5'-GAGGCCAGCTTTCCCAAC-3'	161 bp	661 bp	143 bp
	rev: 5'-TGTAGCTGCCTGGGTCCTGC-3'			
***Mlc1* exon 1C**	for: 5'-AGAAGCTCACCTCTGTTTGG-3'	177 bp	550 bp	not detected
	rev: 5'-TGTAGCTGCCTGGGTCCTGC-3'			
***Mlc1* exon 1E**	for: 5'-AGATGAAGGCTGAGTGTGCT-3'	172 bp	480 bp	480 bp
	rev: 5'-TGTAGCTGCCTGGGTCCTGC-3'			

Table 2: Primers for creating pGL3-luciferase constructs

Mus musculus	**primer sequence**	**primer position**
pGL3-K1	for: 5'-ATAACGCGTAAGCTTCCAAAGGCCTG-3'	-4183 bp to -4167 bp
	rev: 5'-ATTCTCGAGATTACTTGACGAAAATCTCC-3'	+77 bp to +96 bp
pGL3-K2	for: 5'-ATTACGCGTGTCAGTTGAGAGCCTAGAGG-3'	-871 bp to -852 bp
	rev: 5'-ATTCTCGAGGTTCTTGATTTTGGCAAAGC-3'	+32 bp to +51 bp
pGL3-K2rev	for: 5'-ATTCTCGAGGTCAGTTGAGAGCCTAGAGG-3'	-871 bp to -852 bp
	rev: 5'-ATTACGCGTGTTCTTGATTTTGGCAAAGC-3'	+32 bp to +51 bp
pGL3-mB	for: 5'-ATAACGCGTTTCAAGGGCCAGCTTTGC-3'	+21 bp to +38 bp
	rev: 5'-ATTCTCGAGCTGGCCTCTGGGTGATG-3'	+448 bp to +464 bp
pGL3-mE	for: 5'-ATAACGCGTCTCACCTCTGTTTGGGAC-3'	+573 bp to +590 bp
	rev: 5'-ATTCTCGAGCACACTCAGCCTTCATCT-3'	+638 bp to +655 bp
Homo sapiens	**primer sequence**	**primer position**
pGL3-hB	for: 5'-ATAACGCGTCCACACAGCTAAGCCGA-3'	+101 bp to +117 bp
	rev: 5'-ATTCTCGAGAAGAAGTATTCACAAATG-3'	+495 bp to +512 bp
pGL3-hE	for: 5'-ATAACGCGTCAGCGGGGGAGGTAAGT-3'	+557 bp to +573 bp
	rev: 5'-ATTCTCGAGCCTCCAGGTGCAACAC-3'	+706 bp to +721 bp

Table 3: Consensus sequence of TATA-, GC- and CCAAT-Box

binding site	**preferental binding position**	**consensus sequence**	**core sequence**	***in M. musculus***	***in H. sapiens***
TATA-Box	-40 bp to -23 bp	STATAAAWRN*	TATAA	-	-
GC-Box	-55 bp to +56 bp	NRGGGGCGGGGCNK*	GGCGG	-	-
CCAAT-Box	-105 bp to -70 bp	NNRRCCAATSA*	CCAAT	position -27 bp; position -114 bp	-

* N=A,G,C or T; R=A or G; S=C or G; W=A or T; K=G or T (consensus sequences are taken from Suzuki *et al.* [90])

Results

Structural characterization of the 5' region of Mlc1

As a first step to find any regulatory regions for *Mlc1*, an analysis with the promoter search PROSCAN Version 1.7 software was conducted. The closest putative promoter predicted by PROSCAN was located 4.3 kb upstream of *Mlc1*. Interestingly, this predicted promoter region of 345 bp seemed to work in a bi-directional modus. The sequence is flanked by *Mov10l1*, the adjacent annotated gene on the opposite strand with respect to *Mlc1,* and BB614475, located on the same strand as *Mlc1*. A database search for ESTs between *Mov10l1* and *Mlc1* provided two sequences, GenBank accession numbers BB614475 and AV253704, both being located on the same strand as *Mlc1* and part of the same cDNA clone (clone ID: 921504G13) (figure 1A). The expressed sequence of clone ID: 921504G13 AV253704 has already been reported by Steinke and colleagues [89]. They described a high sequence similarity to the *Homo sapiens* voltage-gated K channel beta subunit 4.1 mRNA (*Hspc014*), which is officially named proteasome maturation protein gene (*Pomp*). Additionally, they described an Alu-J-like element being located between AV253704 and *Mlc1* (figure 1). As the expression of BB614475 and AV253704 were known for testis (according to the RIKEN gene bank entry), we wanted to test whether 1) the whole sequence between BB614475 and AV253704 is transcribed; and 2) BB614475 and AV253704 were also expressed in the murine brain. Therefore, PCRs with murine testis and murine brain cDNA were conducted. The forward primer was located in BB614475 and the reverse primer in AV253704. The result showed that there is no expression in murine brain. For murine testis, the result showed one small PCR product of ~700 bp and one larger of 2015 bp. Direct sequencing of the smaller amplicon revealed two different splice variants of the *Pomp* pseudogene, showing the same size of 675 bp. Splicing occurred at repetitive sequences; therefore, it is not possible to provide the exact splicing positions. Sequencing of the 2015 bp amplicon confirmed the expected size and sequence of the murine genomic DNA. The last 707 bp of the larger amplicon show a 87 % identity to *Pomp*, but there was no similarity for the first 1308 bp.

Due to the partial similarity to *Pomp*, we have consequently termed this the *Pomp* pseudogene. As far as a contamination of the PCR with gDNA could be excluded, this experiment showed that there are at least three different *Pomp* mRNAs transcribed. Expression levels and sequences of the *Pomp* pseudogene splice variants are shown in figures 1B + 1C. Even if the *Pomp* pseudogene may have no influence on *Mlc1* expression, as it is not expressed in brain, this transcript seems to be an interesting finding because of its unconventional splicing.

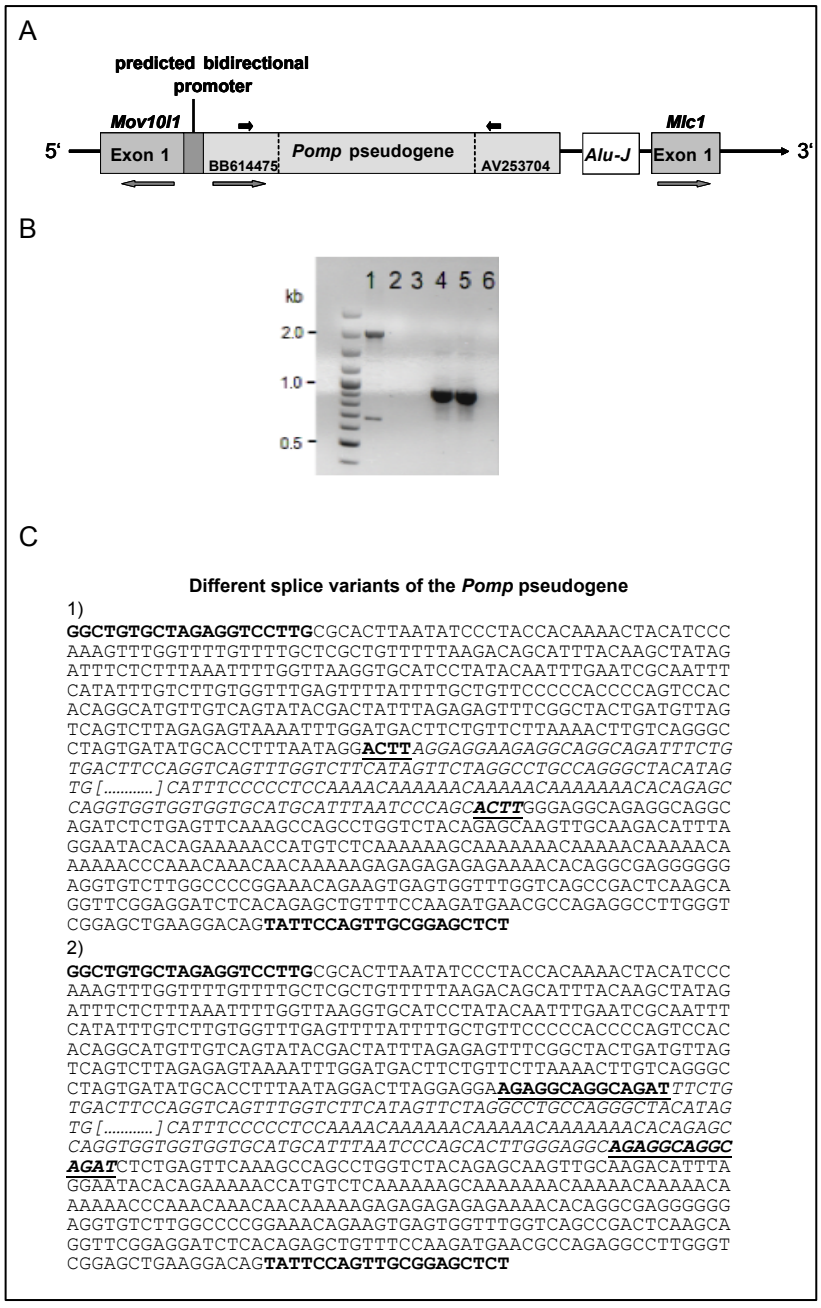


Figure 1:

Figure 1A shows a schemic overview of the 5' region of the murine *Mlc1* gene. The 5' region of *Mlc1* consists of an Alu-J-like element and a *Pomp* pseudogene. The sole promoter predicted by PROSCAN software is a bidirectional promoter 4.3 kb upstream of *Mlc1*, evidently acting for *Mov10l1* and the *Pomp* pseudogene. The grey arrow bars display the orientation of the genes and the black arrow bars display the position of the primers which were used to check for the *Pomp* pseudogene expression. Figure 1B shows the expression of the *Pomp* pseudogene in murine testis and brain. Expression of *Pomp* pseudogene was shown in testis (lane 1). Beside the expected fragment of 2015 bp, a smaller fragment of 675 bp was detected. No expression for the *Pomp* pseudogene was detected in brain tissue (lane 2). As positive control, *ActB* was used (lane 4: testis, lane 5: brain tissue). As negative controls, water was used instead of cDNA (lane 3 and 6). Figure 1C shows the different splice variants of the *Pomp* pseudogene in testis. Splicing occurred at certain repeats (bold and underlined). Bold letters represent the position of the primer pair for expression analysis. Italicized letters represent the sequence which was spliced out. Portion of unshown sequence is replaced by dots.

Expression studies

For the human *MLC1*, several alternative first exons are known, which are described in the database of transcriptional start sites (DBTSS). To investigate if alternative first exons of *Mlc1* in mice exist, we compared the sequence of the human *MLC1* first exons with the orthologous murine *Mlc1* sequence. Several *MLC1* first exons were released in the DBTSS, but only first exons with common acceptor- and donor-sites (GT/AG) were examined for sequence similarity to the murine *Mlc1* in the present study. Sequences of the first exons fulfilling this condition (exon 1A, exon 1B, exon 1C and exon 1E) were used for alignments with the orthologous murine sequence. Sequence identity varied from 47.8 % (exon 1E) to 76.7 % (exon 1B). To test the expression of the alternative first exons in mice, PCRs with cDNA from different murine brain regions and a murine astrocyte cell line were conducted. For this, the forward primer was located in the orthologous first exon sequence and the reverse primer was located in exon 2, downstream of the translation start site (TLSS). Used primers and expected amplicon sizes are shown in table 1. Expression was detected for *Mlc1* exon 1B and exon 1E only (figures 2A + 2B). Real time PCR experiments were conducted to compare the expression rate of both exons. The experiment failed to quantify the expression rate of exon1E, so no conclusion of the relationship between expression of exon1B and exon1E can be drawn. Expression for both first exons could be detected in amygdala, hippocampus, hypothalamus, cerebellum, cortex and the murine astrocyte cell line. Sequencing of exon 1B resulted in a 143 bp sequence with a splice position at +492 bp/+1010 bp. This diverged from the data provided by Ensembl, where the exon 2 of *Mlc1* starts at position +993 bp. All positions are according to the *Mlc1* exon 1 released in the Ensembl database version 56 (ENSMUSG 00000035805), where +1 is representing the transcriptional start site of exon 1. The murine exon 1E showed a size of 480 bp, which was assumed to be the identical size of the related genomic DNA sequence. The sequencing of exon 1E confirmed this assumption as the same sequence as the genomic DNA is shown. Sequencing data are available on request.

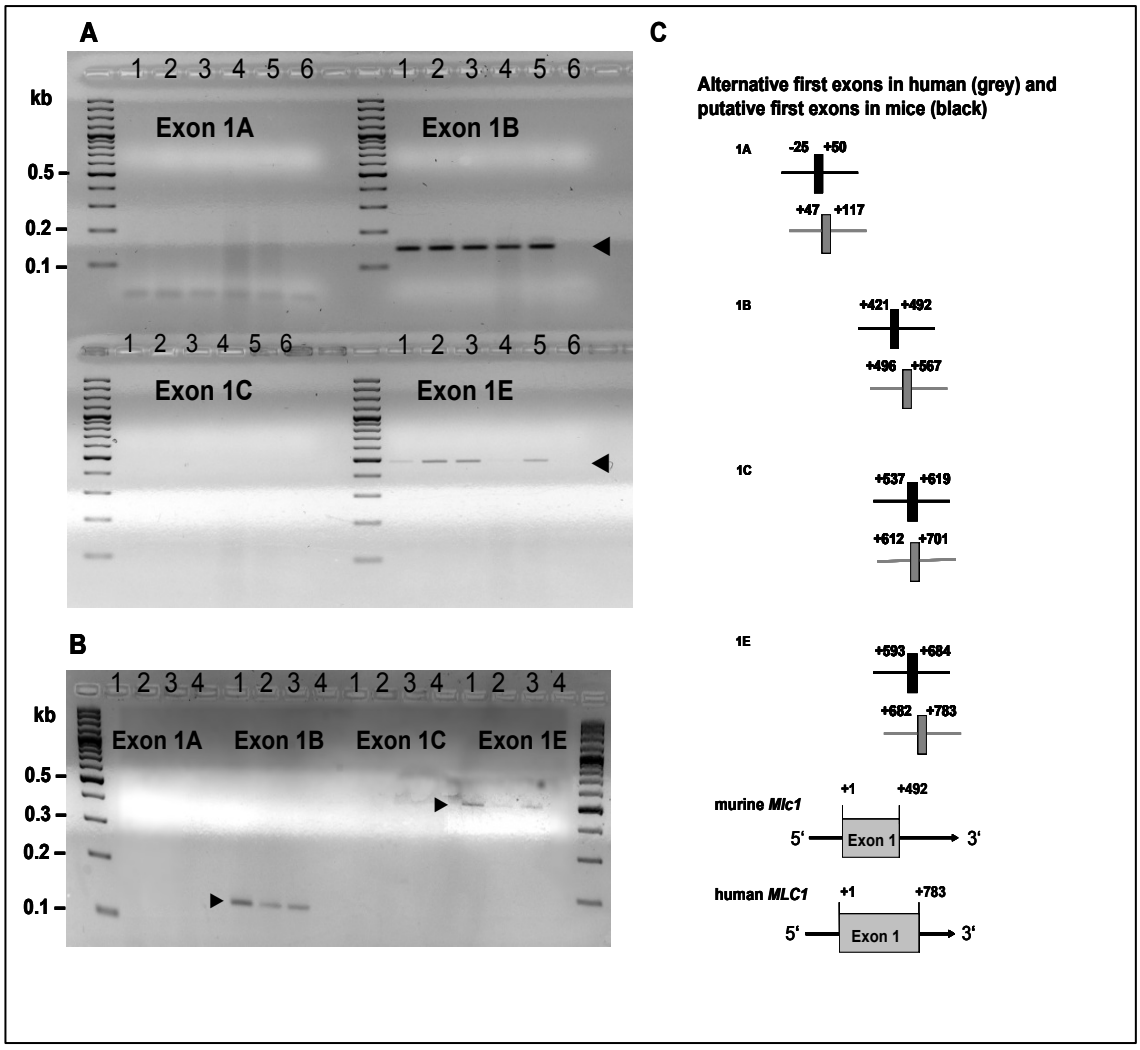


Figure 2:

A) Expression analysis of the alternative first exons for murine *Mlc1* in amygdalae (lane 1), hypothalamus (lane 2), hippocampus (lane 3), cerebellum (lane 4) and cortex (lane 5). Water instead of DNA was used as negative control (lane 6). Expression was detected in all tissues of exon 1B (143 bp band) and 1E (480 bp band). Expression of exon 1A and 1C could not be detected. B) Expression analysis of the alternative first exons for murine *Mlc1* in the astrocyte cell line shows expression of exon 1B and 1E (see arrows). Lanes 1 to 3 contain three independent preparations of cDNA from the same astrocyte cell line. Water instead of DNA was used as negative control (lane 4).

C) Schematic overview of the alternative first exons of the *MLC1* gene in human (in grey) and the putative first *Mlc1* exons in mice (in black). Positions of the alternative first exons of human *MLC1* were taken from the database for transcriptional start sites (DBTSS version 4.0) and all positions are referring to the transcriptional start site (+1) of *MLC1* exon 1 released in the Ensembl database version 56. Putative first exons of the murine *Mlc1* were chosen by sequence similarity to the human sequences. Figures are not to scale.

Luciferase reporter gene assay

In order to investigate the regulatory activity of the region upstream of the TSS of *Mlc1*, reporter gene plasmids based on pGL3-Basic (Promega) were constructed. Initially, two fragments, from position -4183 bp to +96 bp (pGL3-K1) and from position -871 bp to +51 bp (pGL3-K2) were inserted into pGL3-Basic. The positions are corresponding to the *Mlc1* exon 1 released in the Ensembl database (version 56). The aim was to find out whether the sole predicted promoter region 4.3 kb upstream of *Mlc1* shows any effect on gene expression, or if there is any regulatory element present downstream of the *Pomp* pseudogene, which was not predicted by the applied software. Measurement of luciferase activity of pGL3-K1 and pGL3-K2 did not show any promoter activity compared to pGL3-Basic (data not shown). The expression analysis of putative alternative first exons revealed only expression for exon 1B and exon 1E, hence further pGL3-luciferase constructs were assembled with the regions immediately upstream of the first exon 1B and 1E. Additionally, pGL3-luciferase constructs with the putative promoter regions of the human exons 1B and 1E were created (figure 3). All pGL3-luciferase constructs revealed lower activity levels compared to pGL3-Basic, which served as negative control (figures 3A + 3B). PGL3-K2, where no expression could be detected for the corresponding exon 1A, has been used as a further negative control. For these experiments, pGL3-Control was used as positive control. All cells were co-transfected with pGL4 to check for transfection efficiency. To investigate whether the promoter reacts under certain stimulations, activity was measured under treatment with dexamethasone, forskolin, PMA, or LPS. The results showed no sensitivity of the putative promoter regions to any of the tested stimulants under the applied conditions (data not shown).

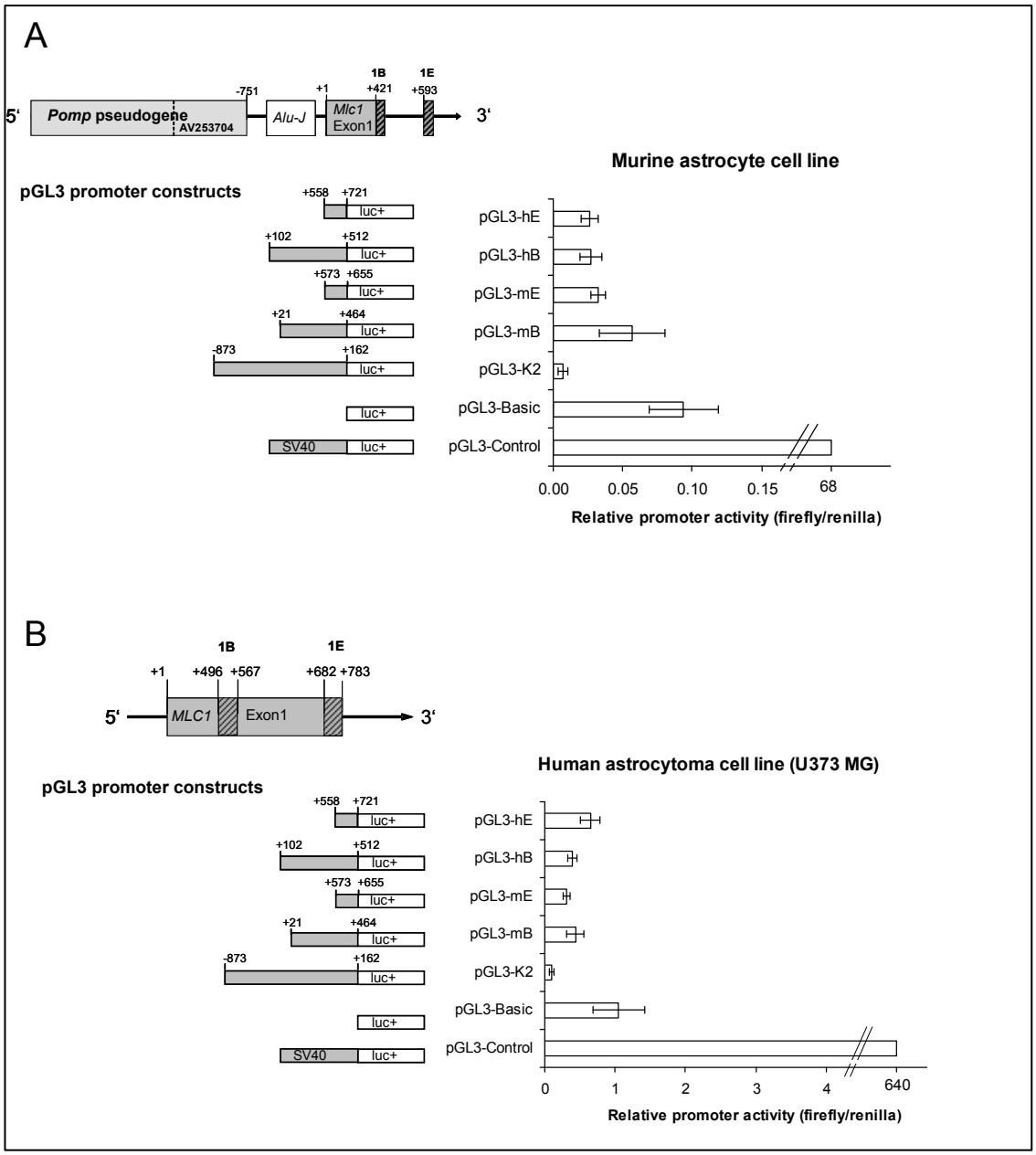


Figure 3:

Figure 3A shows the reporter gene constructs used and the relative promoter activity in the murine astrocyte cell line. All positions are referring to the transcriptional start site (+1) of *Mlc1* exon 1 released in the Ensembl database version 56. Ratio of Luciferase activity (RLU)/*Renilla* activity (RLU) was used to calculate for promoter activity. Data represent the mean ± SD of three independent triplicates under basal conditions. Murine pGL3-promoter constructs (pGL3-mB, pGL3-mE and pGL3-K2) and human pGL3-promoter constructs (pGL3-hB and pGL3-hE) showed lower activity than the negative control (pGL3-Basic), which contains no promoter. pGL3-Control contains a strong SV40 promoter and was used as positive control. Figure 3B shows the used reporter gene constructs and the relative promoter activity in the human astrocytoma cell line U373 MG. All positions are referring to the transcriptional start site (+1) of *MLC1* exon 1 released in the Ensembl database version 56. Ratio of Luciferase activity (RLU)/*Renilla* activity (RLU) was used to calculate for promoter activity. Data represent the mean ± SD of three independent triplicates under basal conditions. Murine pGL3-promoter constructs (pGL3-mB, pGL3-mE and pGL3-K2) and human pGL3-promoter constructs (pGL3-hB and pGL3-hE) showed lower activity than the negative control (pGL3-Basic), which contains no promoter. pGL3-Control contains a strong SV40 promoter and was used as positive control.

In silico study

For *in silico* analysis of the murine and the human putative *Mlc1* promoters, the whole sequence from position -350 bp to the TLSS was investigated. The human *MLC1* TLSS is located at position +1020 bp, whereas the murine TLSS is located at +1015 bp. Positions are according to *Mlc1* exon 1 from Ensembl database version 57. Consensus sequences were taken from Suzuki and colleagues, who studied over 1000 promoter regions [90]. They described GC-boxes for 97 %, CCAAT-boxes for 64 % and TATA-boxes for 32 % of the investigated promoter regions. In our study, we did not detect any GC-boxes or TATA-boxes consistent with the consensus sequences provided by Suzuki and colleagues (see table 3). The analysis revealed no CCAAT-boxes for human and only two putative CCAAT-boxes for mice, which had previously been described by Steinke and colleagues [89]. The CCAAT-boxes are located at positions -119 to -109 and -32 to -22. Additionally, a sequence analysis with TRANSFAC Version 2009.1 was performed for human and mouse to search for potential nuclear factor 1 (NF-1), specificity protein 1 (SP-1) and activating proteins (AP-1, AP-2) binding sites. Two NF-1 binding sites were found for the mouse. As NF-1 is known for binding at CCAAT-boxes, the first putative NF-1 binding site was identical to the CCAAT-box at position -119 to -109. The second putative NF-1 binding site was at position +279 to +289. The match to the consensus core sequence was 1.000, and the overall matrix sequence was 0.773 for the first NF-1 binding site and 0.911 and 0.754 for the second one. There were no predicted binding sites for AP-1, AP-2 and SP-1. Since the preferred regions of CCAAT-boxes involved in transcription are generally located -105 to -70 from the TSS, and the fact that *Mlc1* expression could not be detected for exon 1A but only for exon 1B and exon 1E in this study, the CCAAT-boxes are seemingly located somewhat further away from the identified TSS.

Next, the regions -350 to +1015 (for mouse) and -350 to +1020 (for human) were screened for CpG-islands. CpG-islands are known to occur often in promoter regions, especially of brain- specific genes, and may influence gene transcription [92]. Our results showed a GC-content of 46.5 % in human and

mouse. Ratios of observed CpGs to received CpGs (CpG(O/R)) were 0.2 for mouse and 0.5 for human *Mlc1*, respectively. As CpG-islands are conventionally defined as sequences (> 200 bp) with a high GC-content (> 50 %) and a CpG (O/R) ratio > 0.6 [91], the regions which have been investigated contained no CpG-island.

The comparison between the human and the murine *Mlc1* 5' region up to the next annotated gene *Mov10l1* revealed four blocks of conserved regions. The first block overlaps with the first exon of *Mlc1* and contains around 130 bp of the upstream adjacent Alu-J-like element, which has been described by Steinke *et al.* [89]. The second block (~160 bp) covers the space between the *Pomp* pseudogene and the Alu-J-like element, overlapping 15 bp of the Alu-J-like element. The third block contains around 780 bp and is located in the middle of the *Pomp* pseudogene as part of the intron. The fourth block showing conservation covers the promoter region of *Mov10l1*, including the flanking sequences of *Mov10l1* and around 200 bp of the flanking *Pomp* pseudogene. A schematic overview is given in figure 4.

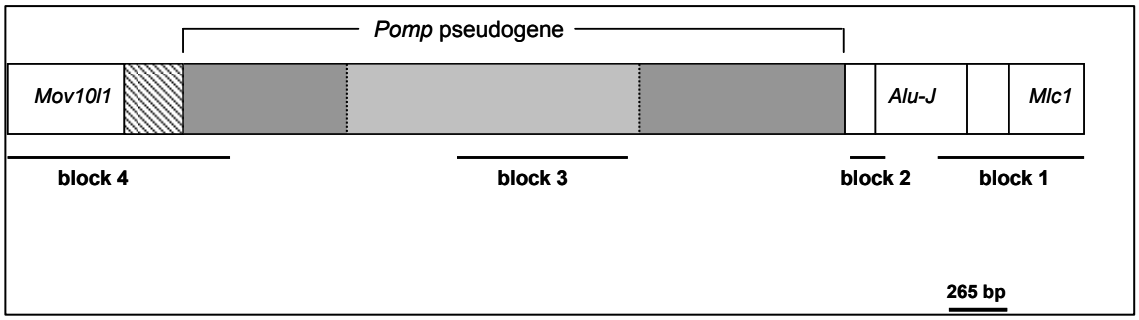


Figure 4:
Schematic overview of the murine *Mlc1* 5' region up to the adjacent gene *Mov10l1* and the regions showing conservation compared to the human sequence. The hatched box represents the promoter region of *Mov10l1* and the *Pomp* pseudogene. The *Pomp* pseudogene is marked in grey. The light grey coloured box represents the intron of the *Pomp* pseudogene. Black bars mark the different conserved blocks (block 1 to block 4).

Discussion

The brain-specific expression of the megalencephalic leucoencephalopathy with subcortical cysts gene *MLC1*, and its involvement in neurological disorders like MLC as well as mental disorders like schizophrenia and bipolar disorder, supports the importance of the role of *MLC1* for brain development and physiological brain function. A certain homology to ABC2-transporters, which are known to play a role in drug resistance [93], has been reported by Leegwater and colleagues [88]. As the membrane spanning MLC1 is predominantly located at astrocytic end-feet at the blood brain barrier and glial-limiting membrane, a functional role as a selective transport protein, most probably as an exporter, could be presumed. Furthermore, Ambrosini and colleagues described an association between the dystrophin-glycoprotein complex (DGC) and MLC1 [94]. DGC is essential to anchor the cell membrane to the extra cellular matrix and its need for the stability of the blood brain barrier [95]. Defects in MLC1 may affect stability of the DGC, causing instability and dysfunction of the blood brain barrier; the latter has been found in schizophrenic and depressive patients [96-98]. So far, neither the real function of the protein, nor the gene regulation of *MLC1* is known. Here, we studied the upstream region of the murine *Mlc1* to find regulatory elements and define a promoter region. As no promoter could be identified by promoter prediction software, *Mlc1* was investigated for promoter elements known to be essential for initiation of transcription. Suzuki and colleagues described binding sites for transcription factors appearing in most of the promoter regions they investigated [90]. They mentioned an appearance of GC-boxes for 97 %, CCAAT-boxes for 64 % and TATA-boxes for 32 % of the investigated promoter regions. Here, we detected three CCAAT-boxes in the mouse, which may act as NF-1 binding site. Beside NF-1, CCAAT-boxes are potential binding sites for the nuclear factor NF-Y, which requires a high conservation of the five core nucleotides [99]. NF-1 and NF-Y are known to be ubiquitous transcription factors acting on a variety of genes. While NF-1- mediated gene expression might be influenced by the organization of DNA into chromatin leading to a ~100 fold change in gene activity [100], NF-Y seems to be acting as

a switch between gene activation and repression by influencing deposition of positive and negative histone methyl marks [101,102]. Nevertheless, the CCAAT-boxes we found were not located within the usual region of about -105 to -70 bp from the TSS. TATA-boxes and GC-boxes were absent in the human and murine *Mlc1* promoter region. Carninci and colleagues mentioned that roughly 90 % of TATA-independent transcription initiation occurs within a CpG island [103]. Roider and colleagues also showed that for genes which are predominantly expressed in brain it is common to have CpG islands in their promoter region [92]. Beside the lack of TATA-boxes and GC-boxes, our study did not show any CpG-island within the promoter region, therefore, the promoter region of *Mlc1* is quite uncommon. Additionally, reporter gene assays with pGL3-luciferase-constructs showed no promoter activity, further supporting the lack of promoter-specific functional elements. Measurements of pGL3-mB, pGL3-mE, pGL3-hB and pGL3-hE revealed no significant differences in activity, independent of the presence or absence of CCAAT-boxes. Although no transcription for first exons of *MLC1*/*Mlc1* genes could be demonstrated by this method, neither under basal nor under stimulated conditions, *Mlc1* expression was detectable in the cells by polymerase chain reaction. It is known that there are post-transcriptional regulating processes, like miRNAs, which are able to bind at AUGs of the 5'UTR and inhibits translation of the mRNA [104]. This may be one explanation for finding no activity of our reporter gene. However, it has been published that MLC1 protein is expressed in astrocyte membranes and we had verified the gene expression [87]. Thus, it can be assumed that there is a translation process. Furthermore, we tested several promoter constructs, containing different parts of the 5'UTR and hence, sequences with different ATGs. It would be highly improbable if every construct would contain such a repressive ATGs. Therefore, a more probable assumption is that either a distal enhancer element or an activating co-factor is crucial for *Mlc1* expression, which is not included in the investigated promoter region. It was shown that other genes, like the Keratin 19 gene (K19), have an upstream promoter, which needs an enhancer element to be active. As an example, Hu and colleagues conducted a CAT-reporter gene assay showing the same low CAT-activity for the

plasmid with and without the K19 promoter, with the plasmid containing the K19 promoter and the 3' flanking sequence of the K19 revealing a 10-fold increase in expression level [105]. Other reports also found crucial enhancer elements for promoter activity, as shown for the importance of the 5'UTR intron of the Ubiquitin C gene for its expression [106]. Interestingly, there are also some reports on *MLC1* supporting the assumption that there might be a crucial enhancer element or an activating co-factor. Despite MLC being considered as an autosomal recessive disorder, Boor and colleagues described patients suffering from MLC but containing only one or no mutated *MLC1* allele. In these patients, linkage analysis revealed linkage to the MLC1-locus. 5'-UTR and 3'-UTR had been screened for mutations with a negative result. Boor and colleagues investigated expression of *MLC1* in lymphoblastoid cell lines of affected versus non-affected patients. The result showed that *MLC1* expression of patients with only one or no mutated *MLC1* allele compared to patients with homozygous MLC1 mutations revealed a reduced expression level of *MLC1*, leading to the assumption that there must be a second mutation somewhere near the *MLC1* gene which affects expression of MLC [107]. Leegwater and colleagues found a few MLC-affected patients with no mutated *MLC1* allele showing no linkage to the *MLC1* locus on chromosome $22q_{tel}$ [88,108], which indicates a second gene locus for the disease. Lopez-Hernandez and colleagues recently found the second gene locus causing MLC [109]. They describe mutations in *HEPCAM*, encoding for an IgG-like cell adhesion molecule that interacts with MLC1. If there is still another important gene locus affecting *MLC1* expression remains still unclear. However, we found no regulatory element upstream of *Mlc1*, which was capable of activating the gene's transcription. Furthermore, the predicted promoter 4.3 kb upstream of *Mlc1* does not influence activity of the investigated pGL3-K1. Since this predicted promoter fills the whole intergenic spacer between *Mov1l* and the *Pomp* pseudogene, and both genes are demonstrably expressed in testis, it is rather likely that this bi-directional promoter is specific for both genes but not for *Mlc1*. It is quite striking that a pseudogene, which obviously originated from retrotransposition because it lacks intronic regions, and an Alu-J-like element, that belongs to a family of

transposable elements, are located in the 5' prime region of the murine *Mlc1*. It can be assumed that the 5' region of *Mlc1* was a subject of repeated transposon integration events in former times. Therefore, it would make sense that this region is somehow repressed as a kind of protection. Even if there is no *Pomp* pseudogene or Alu-J-like element in the 5' prime region of human *MLC1*, there are two blocks showing conservation, which contains flanking parts of the Alu-J-like element described in mice. Interestingly, the 5' region of exon1A and 1B shows a low CpG content and contains no CpG-islands. A high CpG content correlates with repressed gene activity via methylation [110]. It was further observed that 70 % of all genes expressed in the brain contain CpG-islands [92]. Although *Mlc1* is expressed in brain, it does not harbour CpG-islands. In summary, the promoters of both human and murine *MLC1/Mlc1* genes display unusual structural and functional features, with yet undetected *cis*-acting elements and *trans*-acting co-factors likely playing a crucial role for *Mlc1* gene expression.

Chapter 4

Extrapolating genetic results from large families to the general population: Association study of SNPs in unrelated bipolar and schizophrenic individuals from results of prior large family studies

This chapter has been adapted from the manuscript (in preparation as of 5th September 2011) entitled:

The chromosome 15q14 locus for bipolar disorder and schizophrenia: Is *C15orf53* a major candidate gene?

Thorsten Kranz[1*], Savira Ekawardhani[1*], Michelle K. Lin[1], Simone R. Alt[2], Fabian Streit[3], Ulrike Schuelter[1], Hans Bauer[1], Darja Henseler[1], Jonathan D. Turner[2], Claude P. Muller[2], Andreas Reif[4], Andrea B. Schote[1] and Jobst Meyer[1]

* these authors contributed equally to this work

[1] Department of Neurobehavioral Genetics, Institute of Psychobiology, University of Trier, Germany;

[2] Institute of Immunology, Centre de Recherche Public-Santé, Laboratoire National de Santé, Luxembourg;

[3] Department of Genetic Epidemiology in Psychiatry, Central Institute for Mental Health, Mannheim, Germany;

[4] Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Germany.

Abstract

Family and twin studies revealed a high heritability for psychiatric conditions like bipolar disorder and schizophrenia. Both are complexly inherited disorders with currently unknown etiologies. Recently, two independent genome-wide association studies for bipolar disorder identified a small region on chromosome 15q14, containing the *C15orf53* gene [111,112]. Previously, this genomic region was also found to co-segregate with periodic catatonia (SCZD10, OMIM %605419), an unsystematic schizophrenia according to Leonhard's classification, in several of our multiplex SCZD10 families, thus pointing to overlapping etiologies of both conditions. Here, we narrowed down the susceptibility locus to a 4.5 Mb region on chromosome 15q14-15.1 between the genomic markers D15S1042 (centromeric) and D15S968 (telomeric) in seven unrelated German multiplex SCZD10 families. Mutation and segregation analyses of *C15orf53* in these families were accomplished. We further conducted an association analysis of *C15orf53* in 274 unrelated individuals affected by BD and SCZD10 and 230 controls (case-control sample were recruited in the same geographical region in Germany as all the multiplex SCZD10 pedigrees) as well as expression analysis in *post mortem* human brain tissues.

C15orf53 revealed no mutations in our SCZD10 family members but a segregation of two common haplotypes of *C15orf53* in our multiplex SCZD10 families were found. Further association analysis of these *C15orf53* haplotypes with BD and/or SCZD10 was carried out in our case-control samples but no significant association signal was detected. Expression of *C15orf53* could be demonstrated for immune system-derived cells but not for the *post mortem* human limbic brain tissues. We concluded that *C15orf53* is probably not causative for the etiologies of BD and SCZD10 in our samples.

Introduction

Psychiatric disorders such as schizophrenia and bipolar disorder (BD) are typically heterogeneous with respect to their etiologies and pathophysiologies, and show a significant genetic component [113,114]. The lifetime prevalence to develop schizophrenia or BD is about ~1 % across the world's populations [115,116]. The high heritability (71 % - 90 %) has been demonstrated in numerous twin and family studies, but only a small portion of that heritability could be linked to known genetic variations and explicit causative genetic variants remain unknown [117,118]. Similarly to the majority of physiological disorders, psychiatric and behavioural traits are usually of polygenic nature, where the effect of many genes comes about together to cause a particular phenotype to manifest. Dissecting the numerous layers of genetic pathways of complex disorders is never easy as compared to monogenic disorders, whereby only one gene of major effect is able to cause a particular phenotype. Interestingly, there exist many exceptions for such a straightforward polygenic *versus* monogenic categorization of genetic traits, where a seemingly polygenic trait may be a monogenic trait in multi-generational pedigrees comprising of multiple members affected with the particular trait being studied. Therefore, despite psychiatric diseases being commonly considered to be complexly inherited, rare Mendelian instances have also been described as in our schizophrenic (periodic catatonia, SCZD10, OMIM %605419) multi-generational pedigrees [119].

SCZD10, a condition phenotypically similar to BD, is a clinical subtype of unsystematic schizophrenias according to Leonhard's classification system [120]. Patients diagnosed with SCZD10 are most likely to be diagnosed with bipolar I disorder by the application of DSM-IV criteria [121]. SCZD10 patients exhibit subtle derangements of facial expression and gestures, so-called psychomotor disturbances. They express a highly variable phenotype comprising of hyperkinesia with stereotypies and parakinetic movements, increased anxiety, impulsivity and aggressiveness as well as akinetic negativism, immobility and mutism. In most cases, hallucinations and

delusions occur during the acute psychotic episodes. In the remission phase, there remains a catatonic residual state with psychomotorical weakness of mimics. SCZD10 is highly heritable with a morbidity risk of ~27 % among first-degree relatives [122].

The results of genetics and heritability studies in human disorders rest heavily on the experimental sample, with issues like ethnicities and environmental influences having the power to shift the experimental results in misleading directions (i.e. false-negative and/or false-positive results). The point of a homogenous population in the basic genetic approach is to try to ensure that in the event of a significant finding, it is due to linkage or association of particular genetic loci to the disorder being studied but not because of inter-ethnic genomic differences (i.e. variations in parts of the human genome due to difference in ethnicities). In our attempt to ensure a homogeneous ethnic study sample throughout the study, the case-control population of our unrelated individuals stems from the same geographical region (around Wuerzburg) in Germany, hence ethnically homogeneous, as with our multiplex SCZD10 families [123]. We therefore reasoned that despite being unrelated to the multiplex SCZD10 families, all involved study subjects (both from the case-control population and the multiplex families) share closer recent common ancestors than across other European populations. Hence, rare genetic variations or mutations that exert a major effect in the causation of the disorder specific to the families may have a higher probability in showing up in the general population of the case-control sample group. Here, we executed the classical two-stage genetic approach, from linkage analysis in the large SCZD10 families to association analysis of the candidate gene, *C15orf53*, in the general population of unrelated case-control subjects.

Previously, through a genome-wide linkage analysis in twelve of our unrelated SCZD10 families of German descent, we found significant linkage on chromosome 15q15 to SCZD10 [124]. In the present study, we carried out fine-mapping of the 15q15 region in our twelve SCZD10 families and a 4.5 Mb region on chromosome 15q14 – 15q15.1 was found to be co-segregating with

the SCZD10 status in seven of the SZCD10 multi-generational families. Within this region lie several candidate genes and one of these genes, *C15orf53*, was chosen for further mutation analysis. No mutation of the *C15orf53* gene was found in the SCZD10-affected members who were analysed. We continued with the genotyping of three SNPs, rs7171233, rs7165988 and rs2172835, which appeared in our mutation analysis effort of our candidate gene *C15orf53*, in the SCZD10 pedigrees. Two haplotypes of *C15orf53*, A-G-T and T-C-C, were revealed to be co-segregating among all SCZD10 subjects in our SCZD10 multiplex families. We proceeded on with association analyses of these SNPs in 203 BD and 71 SCZD10 subjects, who were matched with 230 healthy subjects in a case-control study design. However, no significant association was reached for these two *C15orf53* haplotypes in the BD and SCZD10 case-control study of unrelated individuals. We also carried out a gene expression analysis of *C15orf53* in various *post mortem* human brain tissues and several immune cell lines but were only able to detect *C15orf53* expression in the immune cell lines. Taking these findings into account, we concluded that *C15orf53* is highly unlikely to play a major role in the genetic pathway of BD and SCZD10 in our samples.

Material & Methods

Clinical samples

Members of large SCZD10 families were recruited around Wuerzburg, Germany, as previously described in detail [125]. From all patients, genomic DNA was isolated and used for further analysis. From twelve multiplex pedigrees, 57 SCZD10 patients from the first genome-wide scan study [119] were included in the fine-mapping. Extensive clinical evaluation confirmed the diagnosis according to Leonhard's classification system and the ICD-10. Additional information was collected from different sources, including case history, medical records and/or family informants.

In addition, 203 BD affected patients, previously described by us [123] and 71 patients diagnosed with SCZD10 were included in the present study. None of the subjects showed neurological comorbidities, epilepsy, mental retardation, or a history of substance abuse. The control sample consisted of 230 healthy subjects without any history of psychiatric disorder and from the same geographic region (Wuerzburg, Germany) as the patient group. Mean age of patients was 46±15 years, of controls 32±10.5 years. Only patients and healthy volunteers who have given written informed consent after oral and written explanation about the aim and scope of the investigation were enrolled in this study. The ethics committee of the University of Wuerzburg, Germany, approved the study.

Selection of SNPs, microsatellite markers and primer design

Microsatellite markers, single nucleotide polymorphisms (SNPs) markers and primer selections for *C15orf53* were based on annotations derived from Ensembl (May 2009) and UCSC (May 2004). Primer pairs were designed using the software Primer3 [126].

Fine-mapping of chromosome 15 in multiplex SCZD10 families

Twenty-five microsatellite markers were chosen for the fine-mapping of chromosome 15q11.2-15q15. A 17 Mb genomic area was covered, encompassed by the microsatellite markers D15S1035 (chr15: 22 908578-22 908827) and D15S1044 (chr15: 39 668975-39 669161). Marker amplifications were accomplished on GeneAmp 9700 thermal cyclers (PerkinElmer, USA) and fragment analysis was done on ABI Prism® 310 Genetic Analyzer (supplementary information (SI) 1A).

Linkage analysis in multiplex SCZD10 families

Nonparametric, model-free multipoint linkage analyses were performed for chromosome 15-linked pedigrees (seven pedigrees) using the SIMWALK

program [127] with the easyLinkage program as the interface [128]. Statistical significance was estimated based on shared allele's identical-by-descent (IBD) of all affected persons.

Mutation analysis of C15orf53

The segregating region contains four genes (*SPRED1, FAM98B, RASGRP1* and *C15orf53*), of which *C15orf53* was further investigated. Primers encompassing the proximal promoter region, 5´-UTR, coding regions and intron-exon boundaries of *C15orf53* were used for genomic DNA amplification from seven SCZD10-affected patients. DNA was amplified by polymerase chain reaction (PCR) and subsequently sequenced (SI 1B and 1C).

Genotyping of C15orf53 in SCZD10 multiplex families, and bipolar disorder and SCZD10 case-control samples

Genomic regions encompassing the SNPs rs7171233, rs7165988 and rs2172835 were amplified by PCR (SI 1D). The subsequent genotypes of the SNPs rs7171233 and rs7165988 were determined by restriction digestion with *Rsa*I and those of SNP rs2172835 with *Hin*1II, all incubated at 37 °C for 4 hours. Restriction products were separated and visualized on 2 % agarose gels.

Association analysis in bipolar disorder and SCZD10 case-control samples

Linkage disequilibrium among the three SNPs was determined and haplotypes were estimated using Haploview 4.2 [129]. Only haplotypes with a frequency of over two percent were included in the analyses. We tested the haplotypes for association in a case-control design for the following groups: a) BD-affected (203 subjects) versus controls (230 subjects), b) SCZD10-affected (71 subjects) versus controls, and c) pooled sample of BD-affected and SCZD10-affected versus controls.

cDNA from human brain post mortem tissues, human cell lines and immune cells

Post mortem limbic brain tissues (amygdala, hippocampus, inferior gyrus, cingulate gyrus and nucleus accumbens) from six donors free of central nervous system (CNS) diseases and without a history of longterm psychotropic medication were obtained from the Dutch Brain Bank (Netherlands Institute of Neuroscience). All patients have provided *pre mortem* consent for brain autopsy and use of brain material for research purposes as previously reported [130].

Isolation of $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ cells and peripheral blood mononuclear cells (PBMCs) from fresh blood of a healthy donor was performed using the appropriate MACS® MicroBeads Kits (Miltenyi Biotech, Germany) according to the manufacturer's protocol. Total RNA was purified with the High Pure RNA Isolation Kit (Roche, Germany) by following the manufacturer's instructions. cDNA synthesis was realised using standard protocols.

C15orf53 expression analysis

For expression analysis of *C15orf53*, quantitative PCR (qPCR) was performed in duplicates (SI 1E). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was included as an internal control. The qPCR products were separated and visualized on 2 % agarose gel. The amplicons were directly sequenced and confirmed.

Results

Fine-mapping of chromosome 15 in multiplex SCZD10 families

Twelve German large families with SCZD10-affected individuals were investigated. For further comparison, the data of family F30 was taken from literature [131]. Figure 1a shows the genomic region on chromosome 15 of the seven large SCZD10 families (F5, F9, F11, F13, F17, F19, and F24), which was segregating in SCZD10-affected individuals. After refinement, the segregating region (15q14-15q15.1) was found to span 4.5 Mb and is defined by the polymorphic markers D15S1042 (centromeric border) and D15S968 (telomeric border). Within this region, the candidate gene *C15orf53* is located. Non-parametric linkage (NPL) scores for the regions surrounding these two bordering markers were 2.45 and 1.99 respectively (figure 1b).

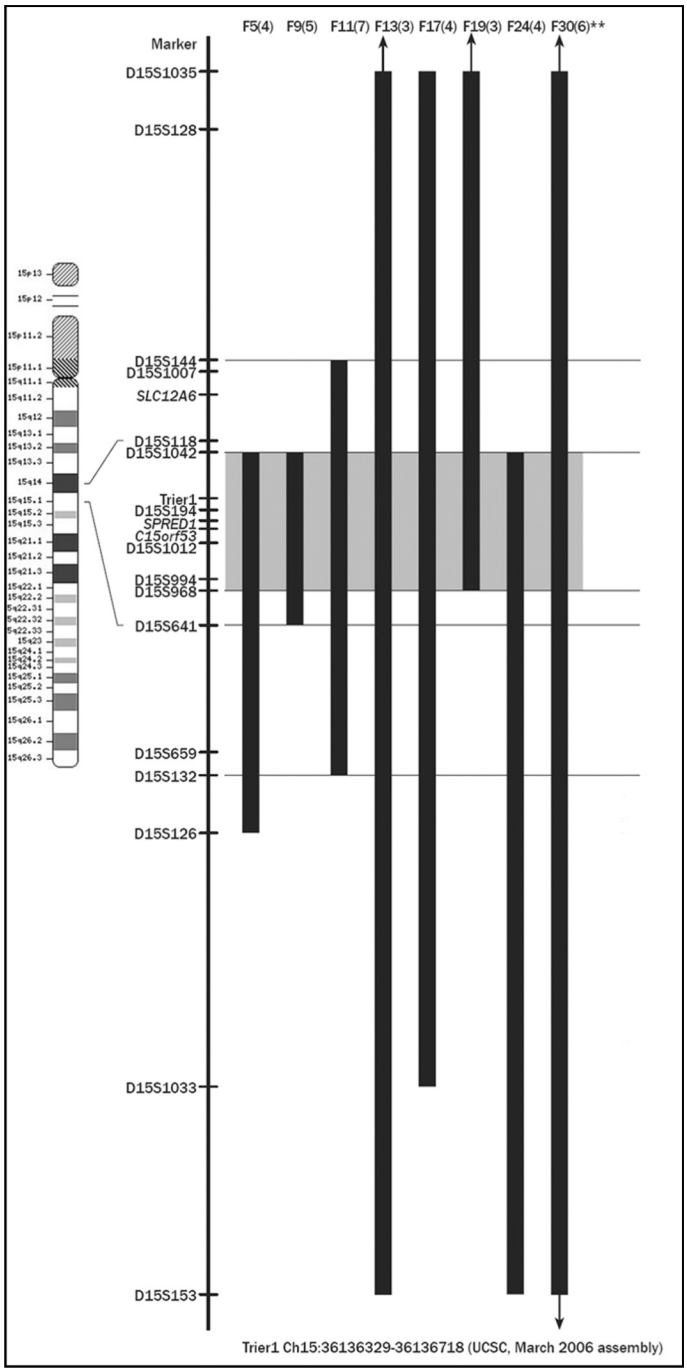


Figure 1a: Overlapping intervals on chromosome 15 that segregate with psychosis in German families with SCZD10. Black bars represent chromosomal regions segregating with SCZD10 in each family, and the ends of the bars represent markers defining the boundaries of the linked haplotypes. Arrows indicate that the susceptibility region is extending further to proximal or distal. Numbers of affected family members are depicted in brackets.

**Data for F30 was taken from the literature [131]

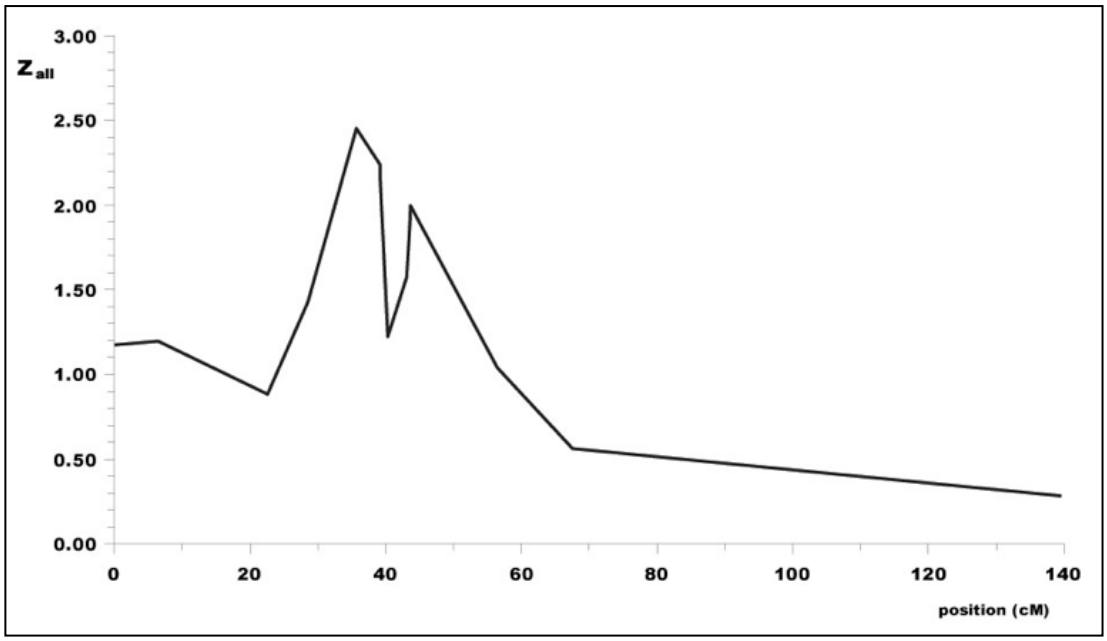


Figure 1b: Non-parametric linkage analysis results for seven SCZD10 pedigrees with confirmed linkage to chromosome 15. The X-axis shows the position on chromosome 15 in centiMorgan and the Y-axis shows the NPL linkage score.

The gene, *C15orf53*, which lies in the segregating region, was selected for further mutation analysis.

Mutation analysis of C15orf53

None of the patients affected by SCZD10 showed any mutations in exons or splice donor and acceptor sites in *C15orf53*. Apart from this, only already described SNPs were detected in all sequenced patients (data not shown). We were next interested if there was any segregation of the three SNPs (rs7171233 (T/A), rs7165988 (C/G) and rs2172835 (C/T)), which were identified in our mutation analysis, with the disease status among the affected members in our multiplex SCZD10 families, thus proceeded on with segregation analysis of SNPs in the candidate gene, *C15orf53*.

Genotyping of candidate gene C15orf53 in multiplex SCZD10 families

Genotyping of the three SNPs, rs7171233 (T/A), rs7165988 (C/G) and rs2172835 (C/T), in our candidate gene *C15orf53* in multiplex family members

with SCZD10 revealed two haplotypes, T-C-C and A-G-T, while no other haplotypes were observed (table 1). The common haplotype T-C-C segregated with the SCZD10 status in three families (F13, F19, F24). The rare haplotype A-G-T segregated with the SCZD10 status in the other four (F5, F9, F11, and F17).

Family	* non-affected / affected	segregating haplotype in SCZD10 affected individuals
F5	3 / 4	A - G - T
F9	8 / 5	A - G - T
F11	14 / 7	A - G - T
F17	10 / 4	A - G - T
F13	6 / 3	T - C - C
F19	4 / 3	T - C - C
F24	11 / 4	T - C - C

* All mentioned individuals (non-affected / affected) were available at the date of DNA sampling

Table 1: Genotyping of candidate gene *C15orf53* (consisting of SNPs rs7171233 (T/A), rs7165988 (C/G) and rs2172835 (C/T)) in SCZD10-affected large families. The common haplotype (T-C-C) segregated with the SCZD10 status in three (F13, F19, F24), and the rare haplotype (A-G-T) in four (F5, F9, F11, F17) of the families.

Genotyping of candidate gene C15orf53 and association analysis in BD and SCZD10 case-control samples

The three investigated SNPs, rs7171233 (T/A), rs7165988 (C/G) and rs2172835 (C/T) of *C15orf53* were in high linkage disequilibrium in each investigated cohort (BD & SCZD10 patients) and the combined samples (data not shown). Within all three cohorts, the common haplotype T-C-C was prevalent with more than 61 % occurrence and the rare haplotype A-G-T showed a prevalence of about 32 % (table 2). The T variant of rs7171233 and the C variants of rs7165988 and rs2172835 represent the major alleles according to the most recent HapMap-CEU data (HapMap Public Release #28, August 2010). We tested *C15orf53* haplotypes for association in 203 patients diagnosed with BD and 71 patients diagnosed with SCZD10 and the

pooled SCZD10 and BD samples (n = 274) versus 230 controls without any psychiatric history. None of the haplotypes found fulfilled the criteria for statistical significance ($p < 0.05$) with respect to association. The Hardy-Weinberg equilibrium was confirmed for all three variants in the patient groups and the control group. None of the single markers showed significant association with BD, SCZD10 or the combination of both phenotypes (data not shown).

(a) BD versus controls

Haplotypes	Haplotype frequencies	Frequencies Cases, Controls	Chi Square	p-value
Block 1				
T - C - C	0.640	268.0 : 134.0, 288.0 : 172.0	1,546	0.2137
A - G - T	0.329	124.9 : 277.1, 158.9 : 301.1	1,164	0.2806

(b) SCZD10 versus controls

Haplotypes	Haplotype frequencies	Frequencies Cases, Controls	Chi Square	p-value
Block 1				
T - C - C	0.614	82.0 : 60.0, 287.9 : 172.1	1,077	0.2993
A - G - T	0.350	52.0 : 90.0, 158.9 : 301.1	0.206	0.6502

(c) Combined samples versus controls

Haplotypes	Haplotype frequencies	Frequencies Cases, Controls	Chi Square	p-value
Block 1				
T - C - C	0.633	349.0 : 195.0, 286.9 : 173.1	0.337	0.5616
A - G - T	0.334	176.9 : 367.1, 158.9 : 301.1	0.456	0.4997

Table 2: Association analysis of *C15orf53* SNPs, rs7171233 (T/A), rs7165988 (C/G) and rs2172835 (C/T) in a) BD, b) SCZD10 and c) combined BD and SCZD10 sample versus control sample.

Among all investigated samples, the haplotype frequency was very similar. Haplotypes with an abundance of less than 2 % were not depicted. All three haplotypes showed a similar distribution within the BD and SCZD10 samples and the combination of both. None of them was associated with BD or with SCZD10.

C15orf53 is expressed in immune cells but not in human brain tissues

Since nothing was known about the physiological role of *C15orf53*, we carried out an expression analysis in a variety of *post mortem* human brain tissues, and human brain and immune cell lines. *C15orf53* was exclusively expressed in the human immune cell lines tested but not in the human brain cell lines (data not shown). *C15orf53* was expressed in the following immune cells: PBMCs, $CD4^+$ and $CD14^+$ cells. The *post mortem* human brain regions amygdala, hippocampus, inferior prefrontal gyrus, cingulate gyrus and nucleus accumbens tested did not show any detectable expression of *C15orf53* (figure 2).

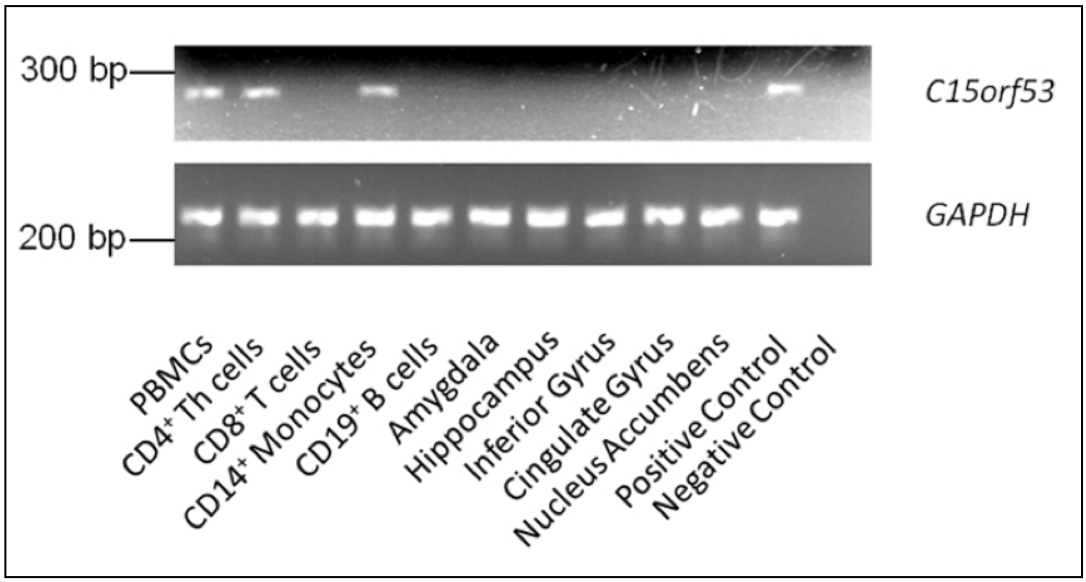


Figure 2: Expression of *C15orf53* in different immune cells and brain areas. *C15orf53* is expressed in peripheral blood mononuclear cells (PBMCs), $CD4^+$ and $CD14^+$ cells. In none of the examined *post mortem* human brain areas could we detect expression. *GAPDH* was used as a housekeeping gene to control DNA quality, quantity and the validity of the method.

Discussion

A genome-wide linkage analysis followed by fine-mapping with microsatellite markers revealed a 4.5 Mb region on chromosome 15q14 that is co-segregating with the SCZD10-affected members in seven of our multiplex SCZD10 families. Coincidentally, the chromosomal region 15q11-14 has been the focus of numerous studies investigating a variety of neuropsychiatric disorders like schizophrenia, bipolar disorder, autism and mental retardation [131-133]. Chromosome 15q11-13 also happens to be a major susceptibility locus for the Prader-Willi/Angelman imprinting syndrome, which was found to overlap with schizophrenia and other psychoses in a recent large-scale study [134]. On our part, we have previously demonstrated that rare variants of the potassium chloride co-transporter 3 gene (*KCC3*), located on chromosome 15q14, which co-segregate with SCZD10 in our multiplex SCZD10 families, are significantly associated with BD [123]. Chromosome 15q14 as a susceptibility region for BD is further supported by a recent BD genome-wide association study (GWAS) on European American and African American samples [111]. Another recently published GWAS on 4387 BD and 6209 control subjects by Ferreira *et al.* showed a strong association with BD on chromosome 15q14, with the highest association signal ($p = 7.5 \times 10^{-7}$) by a SNP (rs 2172835) on C15orf53 [112]. Taking into account unpublished results on functional studies carried out by Ekici and colleagues on the other SCZD10 candidate genes located in the chromosome 15q14 region and Ferreira's work, we have decided to select *C15orf53* as a BD/SCZD10 candidate gene for further study.

With the ever-enlarging sample sizes in the majority of psychiatric genetic studies (amounting to even tens of thousands of test subjects), which usually involve heterogeneous population samples consisting of mixed ethnicities, we tried to use a suitably yet moderately sized study sample to replicate a significant signal in our BD/SCZD10 study. In order to do so, instead of using a larger and more genetically heterogeneous European population in our case-control sample, we used a much smaller but more genetically homogeneous study sample from the same geographical region (around

Wuerzburg) in Germany as with our multiplex SCZD10 families [123]. In doing so, rare genetic variants or mutations that exert a major effect in the causation of the BD/SCZD10, specific to the families, may have a higher probability of showing up in the general population of the case-control sample group.

Here, we report that mutation and segregation analyses for *C15orf53* revealed neither a mutation nor a rare haplotype of our candidate gene *C15orf53* in our SCZD10 families. In the subsequent case-control study, possible association of *C15orf53* with either SCZD10 or BD in separate and combined patient samples were investigated. We showed that all three SNPs, rs7171233, rs7165988 and rs2172835 of *C15orf53* identified by mutation analysis, were in high linkage disequilibrium. We tested the respective haplotypes for association with SCZD10 or BD but found no association of *C15orf53,* neither in the separate BD/SCZD10 samples nor in the combined samples in comparison to our control group. Since there is currently no information about *C15orf53*, except that the databases revealed its expression in human spleen tissue, we decided to carry out an expression analysis of *C15orf53* in various human immune and brain cell lines and human *post mortem* brain tissues. In our study, we showed for the first time that *C15orf53* is expressed in the human immune system but not in the brain.

The reported results indicate a high probability of excluding *C15orf53* in playing a major role causing BD/SCZD10 in our sample. Despite our well selected (in terms of ethic-group homogeneity) pedigreed and case-control samples, we were unable to detect any association signal of our proposed candidate gene. Several other explanations may be plausible aside from the exclusion of *C15orf53* as BD/SCZD10 causative gene: 1) Even though we have taken care to include genetically well matched samples, we did not consider the effect of environment influences on genes in the current study. Factors such as epigenetics and incomplete penetrance may also influence the probability of the manifestation of the disorder; 2) the sample size in our case-control may indeed be insufficient as the minor allele frequency (MAF) of the alleles being studied is exceptionally low, hence the next probable step

would be to execute a power calculation in predicting a statistically more suitable case-control sample size; and 3) we have so far focused on genes as plausible candidates for the causation of BD/SCZD10 but recent psychiatric genetic studies showed that structural variations in the genome, affecting more than 50 bp of sequence, not necessarily involving genes (i.e. may occur over gene deserts in the genome) [135,136], and gene expression trait loci (eQTL), which influence the expression of genes located a distance away on the genome [137,138], may also affect the regulation and expression of causative genes.

Supplementary information (SI)

SI 1A: *Genetic marker analysis*

Marker amplifications were performed using either FAMTM or HEXTM labeled primers (MWG Biotech, Germany). Depending on the amplification products' intensity, 3 µl were mixed with 0.5 µl of 500 ROXTM standard (Applied Biosystems, USA) and 12 µl Hi-Di formamide (Applied Biosystems, USA). The mixes were heated to 94 ^{o}C for 2 min and subsequently separated on an ABI PrismTM 310 Genetic Analyzer according to the amplification products' sizes (Applied Biosystems, USA). Allele size calling was performed using the GeneScan® analysis software (Applied Biosystems, USA) and checked by operational procedures by an operator blinded to phenotype. Alleles of all genetic markers were appropriately assembled to haplotypes, which were used for segregation analysis of the SCZD10-affected multiplex families.

SI 1B: *Sequencing primer pairs for C15orf53*

	fragment	forward (5' - 3')	reverse (5' - 3')
C15orf53	1	TGTGGTTGAGAACCCATCA	CAGGCTGGAGGTGTCTGAAT
	2	GCAGGGTTCTCAGTTTCTGC	CCACACTTGCAAAATCCTGTT
	3	GACAGAAAGATGCCCACTCC	CCAAAACTTTAGCAGCTTAAAACA
	4	AGAGCTGCCACCAAGGAAC	CCTCAGCTTTCCTCCCTGTA
	5	GTCAGTCAGTGGCTCCATCC	CCTAGTTTGGGTGGGGAAAG
	6	GCATGCCAATCTCTCCTCTC	CAAGCCAGGGATGAAACAAT
	7	GTTCCTTCTGGGGTCACTCA	ATGTTTCCGGTAACCAGCAG
	8	CCCAGACTTCTTGGATGGAC	CCCACTGCATTTCTTCACCT
	9	TTTGCCAAACTGGAACACAA	TCTCCCTCCTGCCTGACTAA

SI 1C: *Mutation analysis*

For mutation analysis of *C15orf53*, a 50 µl PCR reaction containing 100 ng of SCZD10 affected patient's genomic DNA, 10 pmol gene-specific primers (MWG Biotech, Germany), 200 µM dNTPs (MBI Fermentas, Germany), 0.75 to 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25 °C), 0.0025 mg/ml BSA, 0.025 % Tween-20 and 1 U of Taq Polymerase (MBI Fermentas, Germany) was conducted. Amplicons were separated on 2% agarose gel, purified using a gel purification kit (PeqLab, Germany) and directly

sequenced. Sequencing reactions were performed for both DNA strands with the sequencing primers depicted in supplementary information 1B using the CEQ DTCS Quick Start Kit (Beckman Coulter, Germany) according to the manufacturer's protocol and analyzed using the ChromasLite program (Technelysium Ltd) and the reference sequence from the Ensembl genome browser (May 2009).

SI 1D: *PCR conditions for genotyping of C15orf53*

PCR reactions were carried out in a total volume of 50 µl with 100 ng of genomic DNA, 10 pmol of each primer, 200 µM dNTPs, 2.0 or 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25 °C), 0.0025 mg/ml BSA, 0.025 % Tween-20 and 2.5 U High-Fidelity Taq Polymerase (MBI Fermentas, Germany) and 5 % (v/v) dimethylsulfoxide (DMSO). PCR reaction 1 (rs7171233 and rs7165988) was performed with forward primer 5'-TGTGGTTGAGAACCCATCA-'3 and reverse primer 5'-CAGGCTGGAGGTGTCTGAAT-3'. The SNP rs2172835 was amplified using forward primer 5'-AGAGCTGCCACCAAGGAAC-3' and reverse primer 5'-CCTCAGCTTTCCTCCCTGTA-3'. All PCR reactions were performed in a MultigeneThermocyler™ TC9600 (Labnet, USA). Conditions for PCR reactions were: 5 min at 94 °C followed by 40 cycles of 45 s at 94 °C, 45 s at 56 °C or 58 °C, 45 s at 72 °C and a final extension for 7 min at 72 °C. PCR products were separated and visualized on 1.5 % agarose gel.

SI 1E: *Expression analysis of C15orf53*

Quantitative PCR reactions were conducted in a total volume of 25 µl containing 50 mM KCl, 200 mM dNTPs, 20 mM Tris-HCl (pH 8.3 at 25 °C), 2.5 U Platinum Taq Polymerase (Invitrogen, USA) and 1x concentrated SYBR® green I (Cambrex, Belgium). The exon- spanning primers used for the amplification of *C15orf53* were forward primer 5'-GCGTCTGTTTGGATGGCTGGAGG-3' and reverse primer 5'-GGAAGTGGTGAACGGCGGGG-3'. For *GAPDH* amplification we used

forward primer 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse primer 5'-GAAGATGGTGATGGGATTTC-3'. The thermal cycling was carried out in DNA Engine Opticon® 2 (Biorad, USA).

Chapter 5

Feasibility of replicating genetic results from large family studies in general population of unrelated individuals for complex psychiatric disorders and behavioral traits: From eQTL linkage to association analyses in vervets

This chapter is presented as an adaptation of the manuscript (in preparation as of 5th September 2011) entitled:

A non-human primate system for large-scale genetic studies of complex traits

Anna J. Jasinska [1], Michelle K. Lin [1,2], Susan Service [1], Oi-Wa Choi [1], Joseph DeYoung [1], Olivera Grujic [1], Sit-yee Kong [1], Matthew J. Jorgensen [2], Lynn A. Fairbanks [1], Trudy Turner [3], J. David Jentsch [4] and Nelson B. Freimer [1].

[1] Center for Neurobehavioral Genetics, University of California Los Angeles, United States of America;

[2] Department of Neurobehavioral Genetics, Institute of Psychobiology, University of Trier, Germany;

[3] Department of Pathology, Section on Comparative Medicine, Wake Forest University Health Sciences, Winston-Salem, NC, United States of America;

[4] Department of Psychology, University of California Los Angeles, United States of America.

Abstract

A classical two-stage genetic approach utilizing both pedigree and population samples of non-human primate species, on which strategies that are infeasible in human studies can be applied, can provide powerful means for genetically dissecting complex traits. We describe here a proof of concept of an approach for genetically dissecting transcriptomic variation in tissues that are largely inaccessible in humans (i.e. brain tissues), using their more readily available counterparts (i.e. blood tissues) as substitutes. We have access to 347 related vervet monkeys (*Chlorocebus aethiops sabaeus*) from the UCLA/Wake Forest University Vervet Research Colony (VRC) and 299 unrelated vervets from the feral vervet population from St. Kitts, Carribean. Through linkage and association analyses of gene expression patterns in peripheral blood from vervet monkeys, we have genetically mapped a candidate regulatory locus for *B3GALTL* expression in the brain. We have hereby proven our concept that 1) the classical multistage genetic approach is applicable in mapping expression quantitative trait loci (eQTL), which regulate gene expression; and 2) gene expression patterns in inaccessible tissues can possibly be studied using those of accessible tissues as substitutes.

Introduction

Non-human primates (NHP) fill a critical need for biomedical models that 1) are more directly relevant to human biology and disease than rodents or other commonly employed model systems and 2) permit longitudinal or invasive investigations that are infeasible in humans [139]. The advent of next generation sequencing has raised interest in genetic and genomic investigations of NHP as a means of illuminating diverse human traits including HIV/AIDS, cardiometabolic diseases, and disorders of brain and behavior. Yet, the perceived lack of suitably sized NHP samples has thus far inhibited the implementation of such investigations. Here, we describe a strategy for large-scale genetic mapping studies utilizing genetically homogeneous populations of feral vervet monkeys (also termed African green monkeys, *Chlorocebus aethiops sabaeus*) inhabiting the Caribbean islands of St. Kitts and Nevis, together with pedigreed monkeys descended from these populations, in the UCLA/Wake Forest University Vervet Research Colony (VRC).

During the early colonial era (16^{th}-17^{th} centuries), very small numbers of West African vervets were introduced to the Caribbean islands of St. Kitts, Nevis, and Barbados, and they rapidly established feral populations (figure 1). In the absence of predators, these populations expanded dramatically, reaching estimates as high as 100,000 for the total vervet population on the three islands. The multi-generational VRC pedigree, founded in the 1970s with 57 monkeys from St. Kitts and Nevis, is presumed to be broadly representative of the populations of St. Kitts and Nevis from which it derives. We therefore reasoned that variants implicated in quantitative traits within the VRC would likely be associated with the same traits in the feral population of these islands, but would be contained in much shorter genome segments of shared identity by descent (IBD) among feral monkeys compared to pedigree monkeys.

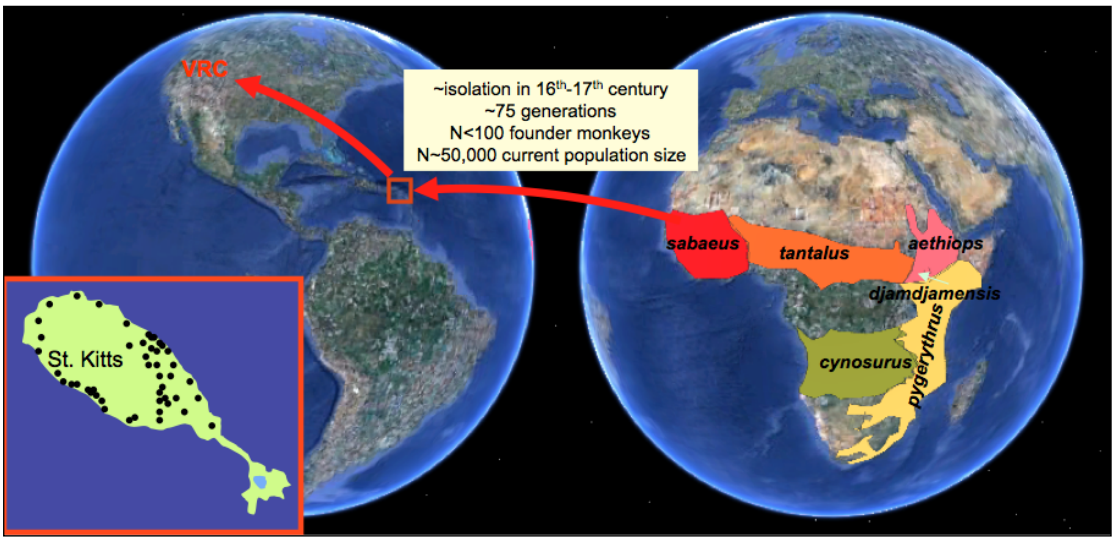


Figure 1: Geographical location of the different sub-species of vervets in Africa. This study utilized the *Chlorocebus aethiops sabaeus* (geographical location in red), which were brought to the Caribbean islands (e.g. St. Kitts). The VRC's *Chlorocebus aethiops sabaeus* is derived from the Caribbean populations.

We have conducted a proof of concept study, demonstrating the feasibility of 1) a multistage approach to genetically map loci regulating the level of expression in blood of transcripts identified by genome-wide microarray analysis, i.e. expression quantitative trait loci (eQTL), which are segments of the genome that regulate the expression of genes; and 2) the use of readily accessible tissues as substitutes for less accessible tissues for gene expression studies. We have previously described a series of analyses in the VRC animals which enabled us to identify transcripts for which gene expression patterns in blood are stable, heritable and correlate particularly strongly with gene expression patterns in the brain [140]. In the current study, we present the genetic mapping by linkage analysis in the pedigreed animals of the VRC of a *cis* eQTL for one of these transcripts – *B3GALTL*, a glycosyltransferase which, when recessively mutated results in Peters plus syndrome (OMIM #261540), a developmental delay disorder [141] – and our subsequent use of association analysis in independently ascertained feral animals from St. Kitts to localize the *B3GALTL* expression quantitative trait variant (QTV) to a region of about 170 kb on the vervet chromosome 3.

Materials and Methods

Study samples

Two groups of vervet monkeys were investigated: related members of the large inbred pedigree that constitutes the VRC; and apparently unrelated individuals from the vervet population of St. Kitts. Pedigree samples consisted of 347 individuals two years and older at the time of sample collection (December 2007 - January 2008) and were described in Jasinska *et al*. [140]. The St. Kitts population sample consisted of 299 Caribbean vervets sampled at the Saint Kitts Biomedical Research Foundation, *SKBRF,* (273 females and 26 males with mean (sd) ages of 11.95 (4.5) and 11.62 (4.8) years, respectively), 279 of whom passed SNP genotyping quality control metrics and provided high quality gene expression measurements using real time-quantitative polymerase chain reaction (RT-qPCR).

Biological material

Genomic DNA samples were obtained from peripheral blood preserved in ACD (acid-citrate-dextrose) anticoagulant or PaxGene DNA tubes using an automated Gentra Autopure LS system (Qiagen, USA) and Gentra Puregene chemistry (Qiagen, USA) or a standard phenol/chloroform extraction procedure.

The PaxGene RNA system (PreAnalyticX) was used to preserve blood for RNA extraction, which was subsequently performed using a PAXgene Blood RNA Kit (Qiagen, USA) or PAXgene 96 Blood RNA Kit (Qiagen, USA). RNA quality was determined with a Bioanalyzer 2100 (Agilent, USA) and RNA quantity was determined using Nanodrop 8000 (Thermo Scientific, USA) or PicoGreen (Invitrogen, USA).

Expression microarray analysis

Microarray gene expression measurements for eQTL mapping were performed using an Illumina HumanRef-8 v2 chip in 347 individuals from the VRC pedigree, as described previously [140].

Filtering criteria for selecting the best eQTL candidates

A detailed description of the strategy to identify the most promising candidate transcripts for eQTL mapping was presented in Jasinska *et al.* [140]. Briefly, to identify eQTL for which expression patterns in blood are highly correlated with expression patterns in the brain, we created two datasets: 1) a set of matched tissues from eight brain regions and peripheral blood from 12 individuals (figure 2a); and 2) a set of duplicate blood samples from 18 individuals, all of whom were sampled at two different time points (figure 2b). We developed three criteria based on Spearman rank correlation (SRC) and variance components analysis: 1) correlation between at least one brain region and blood expression profiles SRC > 0.55; 2) the proportion of variability (PV) of expression attributable to inter-individual differences, as compared to between tissues within individual, for at least one brain-blood comparison > 0.55; and 3) the PV of expression attributable to inter-individual differences, as compared to between replicate samples within individual > 0.55. Finally, we focused only on probes, which were detected in all samples from both data sets.

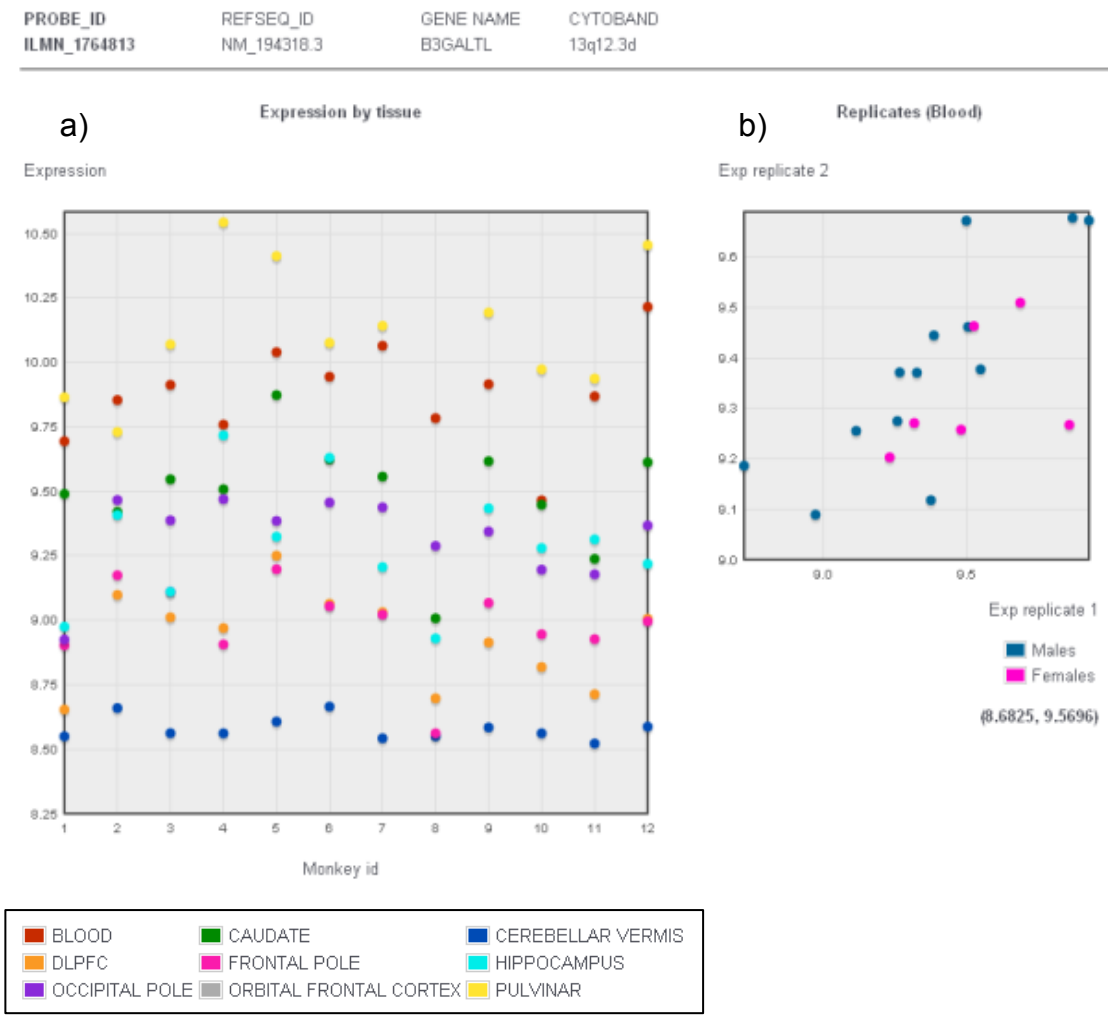


Figure 2: a) Expression of probe ILMN1764813 signal, representing *B3GALTL* gene expression, in different brain tissues and blood of 12 VRC animals; and b) a set of duplicate blood samples from 18 VRC animals, whom were sampled at two different time points.

eQTL linkage mapping in pedigreed VRC samples

Microarray probe intensities were analyzed for linkage to 261 genome-wide STR (microsatellite) markers [142] using SOLAR [143]. As in Jasinska *et al.* [140], covariates of age, sex, and batch were considered in each analysis and those significant at p=0.10 were retained.

Correction for multiple testing of expression traits was calculated in the same manner as a correction for multiple models based on the formula $3+\log_{10}(N)$, where N is the number of tested gene expression traits. 29 tested transcripts with genome-wide significance threshold were determined at the level of 4.46.

A total of seven *cis* and one *trans* eQTL passed this threshold (supplementary table 1). In total, twelve eQTL had significant linkage of LOD score > 3 (data not shown).

Assay design

Vervet-specific RT-qPCR assays and single nucleotide polymorphism (SNP) genotyping were designed using vervet sequences available from whole-genome sequencing of a member of the VRC pedigree (ID: 1994-021). For this purpose, we used sequence from the 454 short reads (~4x coverage) of the individual 1994-021, sequenced by 454 Life Sciences (USA), mapped to the human reference genomic assembly, as the vervet assembly is not yet completed. Given the Caribbean origin of the VRC founders, we expected that the sequence variation discovered from this single individual would be present in both the VRC and population samples from Caribbean. Candidate SNPs were selected based on a minimum of two reads indicating each of two alleles in heterozygous loci.

Validation of the microarray experiment with RT-qPCR

The most robust expression traits for eQTL analyses were selected by performing RT-qPCR validation of microarray results for a group of nine transcripts (*B3GALTL*, *STOM*, *TMEM14C*, *SMOX*, *TMED3*, *CDNKA1A*, *TUBA1B, CDKN1A* and *TSPAN14*) out of twelve showing significant eQTL linkage scores (LOD > 3) and which more complete vervet genome sequence was available around the transcript region. The validation sample set for each transcript included individuals with extreme expression values from the microarray experiment. RT-qPCR expression levels were measured for twenty individuals for each transcript; ten with the highest and ten with the lowest transcript expression levels from the microarray data were selected for each transcript being tested.

For RT-qPCR experiments, 500 ng of blood-isolated RNA was converted into cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's protocol. RT-qPCR reactions were prepared using the SYBR® PCR Master Mix from Applied Biosystems (USA) and oligos from Invitrogen (USA), and analyzed with the 7900HT Fast Real Time PCR System (Applied Biosystems, USA), according to the manufacturers' protocols. Amplicons used ranged from 80 to 120 bp in size. For cDNA amplification, 25-50 ng of cDNA and 300 nM primers were used per sample reaction and each sample was analyzed in triplicates. The amount of cDNA used was according to optimized conditions per transcript; all average Ct values were less than 30 cycles. Expression changes for each transcript were quantified relative to *GAPDH*, selected as an endogenous control, using the Pfaffl method [144]. The medians of expression from the bottom tail and top tail as defined by microarray results were compared with RT-qPCR quantification results using a non-parametric Wilcoxon test.

SNP genotyping in the St. Kitts population samples

For fine-mapping the selected eQTL, we focused on the regions centered on a target gene and approximately 100 kb flanks. A total of 92 candidate SNPs localized in target genes (*B3GALTL, STOM and SMOX*) and their flanks were genotyped in these population samples (table 2). SNP genotyping was performed with the Sequenom iPLEX system technology (Sequenom, USA). Three multiplex PCR assays (36-, 33-, 23-plex) consisting a total of 92 SNPs were designed using the MassARRAY software (v3.1), with default settings of the software and no-masking of repeats. Oligos for the PCR reactions and mass-extend reactions were ordered from Integrated DNA Technologies, USA. Reactions for the SNP genotyping were prepared according to the MassARRAY® iPLEX® Gold SNP Genotyping protocol from Sequenom, USA. Multiple positive, negative and non-amplification controls were included. The success rate of SNP discovery was 45.6% (42 out of 92 genotyped SNPs showed common polymorphism in Hardy-Weinberg equilibrium and were used for association analysis).

Association fine-mapping

Correlation between blood expression levels of three transcripts *B3GALTL, STOM* and *SMOX* measured by RT-qPCR and local SNPs were measured in a population of St. Kitts vervets. We tested for association between SNP genotypes and overall gene expression (from both alleles) at a given locus using regression analysis. Each genotype was coded as 0, 1 or 2 copies of the minor allele, and expression levels regressed on this variable, assuming an additive model of action of the minor allele.

Bonferroni correction was used to adjust for multiple testing in association analysis. Taking into consideration the correction for 42 informative SNPs, which were used for association studies, the genome-wide significance threshold was determined as 0.05/42=0.0012.

Results

Using 347 animals from the VRC, we conducted genome-wide linkage mapping of 29 gene expression traits. The animals had been genotyped for the 261 microsatellite markers that constitute the first generation vervet genetic map [142] and gene expression in peripheral blood was assayed in these monkeys using the Illumina HumanRef-8 v2 chip. As previously described in Jasinska *et al*. [140], these 29 expression measures showed significant heritability in the VRC, and represented transcripts whose expression pattern in blood was stable across at least two time points and was highly correlated with their expression pattern in brain tissues in twelve VRC animals. Twelve significant eQTL (LOD > 3) were identified from the 29 transcripts; ten *cis* and two *trans* in the eQTL linkage mapping. Out of the twelve transcripts, we selected nine with the most robust expression for eQTL analyses for validation using RT-qPCR. Six transcripts – *B3GALTL*, *STOM*, *TMEM14C*, *SMOX*, *TMED3* and *CDNKA1A* – were validated successfully

(non-parametric Wilcoxon test p-value less than 0.05), providing experimental evidence indicating that the expression levels measured using the microarray accurately reflected inter-individual variation in expression of these genes within the VRC (table 1).

Table 1: Characteristics of RT-qPCR assay design and validation of microarray results with RT-qPCR

Gene	Primer sequence **(Forward 5' to 3'; Reverse 5' to 3')**	R^2	Slope	Efficiency	Validation p-value*
TMEM14C	AGCTGTCTCAGGATCCAAGG GCAGGCATGAATTTTCCAGA	0.99	-3.34	0.993	1.08E-05
B3GALTL	CTTTCAAGTGGGTGATGAGC AATGCGTCTGGGAGTCAATC	0.95	-3.39	0.971	1.08E-05
TMED3	CTTTCAAGTGGGTGATGAGC AATGCGTCTGGGAGTCAATC	0.95	-3.43	0.955	0.0015
TUBA1B	AGCTCATCACAGGCAAGGAA GCTGTGGAAAACCAAGAAG	1	-3.54	0.917	1
STOM	AGCGTGTGGAAATCAAGGAT ATGGAGGCTTCTTTCAGA	0.99	-3.51	0.927	4.33E-05
SMOX	CTCTTCCAAGTGCCCAGAAC CTGCTGGAAGAGGTCTCG	0.98	-3.34	0.991	1.08E-05
CDKN1A	TGGAGACTCTCAGGGTCGAA TAGGGCTTCCTCTTGGAGAA	1	-3.29	1.011	0.00013
TSPAN14	CTCCCGCGCAACATTTAC CCCTCAACTCTGCTCCTCAG	1	-3.39	0.971	0.063
TMEM111	AGCAGATGACGGGAGCAG CCATGAGCTCTTCTTCGAC	1	-3.41	0.966	0.0789
*GAPDH***	CAGGTGGTCTCCTCTGACTTCAA GTTGCTGTAGCCAAATTCGTTGT	1	-3.47	0.945	

* p-value for non-parametric Wilcoxon test

** reference gene

A particularly strong linkage (peak LOD score of 8.65) was detected for a transcript within *B3GALTL* to the marker D13S1233 (supplementary table 1, supplementary figure 1 and 5a) in the eQTL linkage analytical stage, which expression was also validated. Hence, using this transcript from *B3GALTL* as an example, we continued on to the second stage of the classical two-stage genetic approach where we further tested the utility of the feral vervet population samples to refine the QTL findings from the pedigreed VRC. SNPs in the region of *B3GALTL* were obtained from the Vervet Genome Sequencing Project (VGSP). The VGSP generates a reference assembly by combining short-read whole-genome sequencing of a VRC animal (ID: 1994-021) previously used to create a vervet BAC library (CHORI-252). Given the Caribbean origin of the VRC, we expected that sequence variation discovered from this single individual would be present both in the VRC pedigree and in the natural Caribbean population. We identified 26 putative SNPs over an interval that incorporated the entire extent of *B3GALTL*, including approximately 100 kb at both the 5' and 3' flanks in a SNP discovery effort specific to the vervet genome (supplementary figure 2). We used 15 SNPs that were polymorphic in this sample to genotype 279 feral-born and presumably unrelated animals from St. Kitts, all of whom had been assayed for *B3GALTL* gene expression using the same vervet-specific RT-qPCR assay previously employed in the VRC (table 2, supplementary figure 3). Four of *B3GALTL* SNPs displayed significant association at $p < 0.0001$. Twenty vervet SNPs of *STOM* and seven SNPs of *SMOX* were also tested for association in the Caribbean population but no significant association of these two eQTL was found (table 2).

Table 2: Association fine-mapping of three eQTL in the Caribbean population*

Vervet Chr.	Human Chr.	Position in hg19 [bp]	SNP ID	BETA	SE	R^2	T	p-value
12	9	124,001,931	STOM_0	-0.02514	0.1436	0.000111	-0.1751	0.8611
12	9	124,002,158	STOM_0.1	-0.1316	0.1637	0.00233	-0.8043	0.4219
12	9	124,007,325	STOM_1	-0.1151	0.1633	0.001812	-0.7052	0.4813
12	9	124,010,003	STOM_2	-0.1316	0.1637	0.00233	-0.8043	0.4219
12	9	124,041,231	STOM_4	-0.1279	0.1733	0.00197	-0.7381	0.4611
12	9	124,043,835	STOM_5	-0.05244	0.1406	0.000511	-0.373	0.7095
12	9	124,046,606	STOM_7	0.1291	0.1178	0.004377	1.096	0.2743
12	9	124,051,514	STOM_8	0.0849	0.1145	0.001989	0.7416	0.4589
12	9	124,071,275	STOM_10	0.08046	0.1183	0.001668	0.6802	0.4969
12	9	124,084,428	STOM_11	-0.00062	0.1385	7.31E-08	-0.0045	0.9964
12	9	124,089,662	STOM_12	-0.155	0.1099	0.007131	-1.411	0.1595
12	9	124,118,313	STOM_15	-0.1064	0.1535	0.001731	-0.693	0.4889
12	9	124,121,411	STOM_16	-0.1064	0.1535	0.001731	-0.693	0.4889
12	9	124,121,753	STOM_17	-0.1064	0.1535	0.001731	-0.693	0.4889
12	9	124,123,227	STOM_18	-0.1415	0.1784	0.002275	-0.7933	0.4283
12	9	124,171,428	STOM_22	-0.02877	0.1167	0.00022	-0.2465	0.8055
12	9	124,204,897	STOM_23	-0.02382	0.1183	0.000147	-0.2014	0.8405
12	9	124,206,767	STOM_24	-0.02217	0.1192	0.000128	-0.186	0.8526
12	9	124,219,342	STOM_25	-0.01244	0.1175	4.05E-05	-0.1059	0.9158
12	9	124,224,081	STOM_26	-0.00987	0.1165	2.64E-05	-0.08474	0.9325
3	13	31,676,914	B3GALTL_0	-0.1438	0.0592	0.02086	-2.429	0.01576
3	13	31,689,187	B3GALTL_2	0.2865	0.06264	0.07092	4.573	**7.28E-06**
3	13	31,758,316	B3GALTL_3	-0.1159	0.05773	0.01445	-2.008	0.04565
3	13	31,758,727	B3GALTL_4	0.2773	0.0629	0.06558	4.409	**1.49E-05**
3	13	31,761,667	B3GALTL_5	-0.09483	0.1279	0.001988	-0.7415	0.459
3	13	31,834,058	B3GALTL_11	0.1201	0.07922	0.008235	1.517	0.1305
3	13	31,836,303	B3GALTL_12	0.2623	0.0602	0.06416	4.358	**1.85E-05**
3	13	31,837,552	B3GALTL_13	-0.03341	0.08569	0.000551	-0.3899	0.6969
3	13	31,855,094	B3GALTL_15	0.1059	0.06451	0.009813	1.642	0.1018
3	13	31,860,398	B3GALTL_16	0.2342	0.05671	0.05918	4.129	**4.86E-05**
3	13	31,906,160	B3GALTL_19	-0.0279	0.1278	0.000172	-0.2184	0.8273

3	13	31,932,565	B3GALTL_20.1	-0.0279	0.1278	0.000172	-0.2184	0.8273
3	13	31,948,748	B3GALTL_21	-0.02533	0.13	0.000138	-0.1949	0.8456
3	13	31,953,645	B3GALTL_22	-0.04565	0.06007	0.00208	-0.7599	0.448
3	13	32,005,195	B3GALTL_24	-0.1161	0.06103	0.01293	-1.902	0.05827
2	20	4,116,864	SMOX_2	-0.1264	0.1107	0.004681	-1.141	0.2547
2	20	4,180,580	SMOX_26	-0.09028	0.102	0.00284	-0.8851	0.3769
2	20	4,192,067	SMOX_32.1	-0.00697	0.07669	2.99E-05	-0.09094	0.9276
2	20	4,203,943	SMOX_35	-0.1223	0.1105	0.004417	-1.107	0.2695
2	20	4,214,861	SMOX_36	-0.06741	0.0668	0.00369	-1.009	0.3138
2	20	4,226,923	SMOX_37	0.00616	0.05588	4.39E-05	0.1102	0.9123
2	20	4,230,806	SMOX_38	-0.07791	0.07023	0.004423	-1.109	0.2682

* shown association p-values were calculated for regression of expression on SNP genotype, assuming an additive model. Beta is the regression coefficient, SE is the standard error of beta, R^2 is the coefficient of determination from the regression model, and T is the t-statistic testing the null hypothesis that Beta = 0. Genomic regions investigated are chromosome 12: *STOM*, chromosome 3: *B3GALTL* and chromosome 2: *SMOX*. Significant association p-values ($p<0.0012$) are shown in boldface.

The peak association ($p = 7.28x10^{-6}$) observed at B3GALTL_SNP_2 (table 2), corresponds to position 31,689,187 bp on the human chromosome 13, located ~85 kb upstream of *B3GALTL* [hg19] (supplementary figure 4).

This gene is located ~15 kb downstream of an additional significantly-associated SNP and also contains two significantly-associated intronic SNPs ($p<0.00005$) (supplementary figure 5b). Information on the linkage and association mapping of *B3GALTL* can be found in the supplementary materials section (supplementary figure 5).

Discussion

The overall workflow of this study's concept is summarized in figure 3 (on the left) – from selection of candidate eQTL with good brain-blood gene expression correlation, to linkage analysis of these eQTL in 347 related animals, to association analysis in 279 unrelated animals from the feral vervet population.

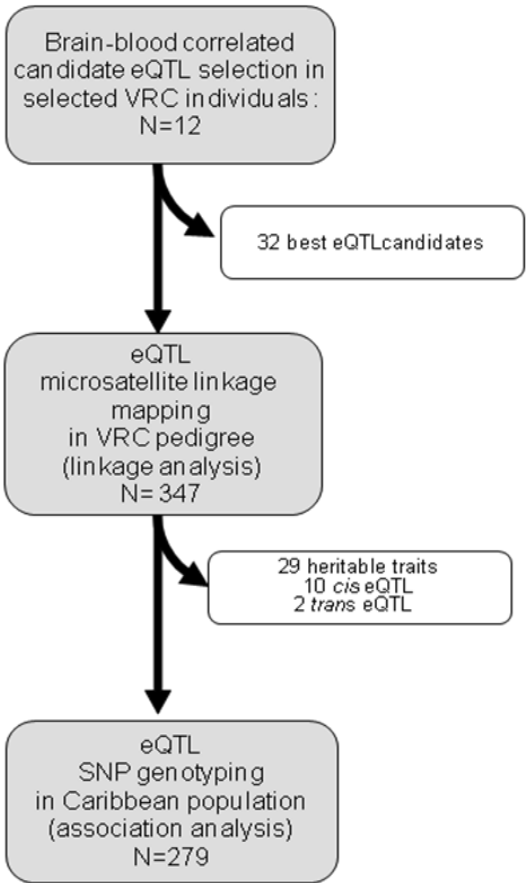


Figure 3: Proposed concept of a classical two-stage genetic approach (from linkage to association) using tissues substitution to dissect complex genetics.

The results described represent a proof of concept for large-scale genomic investigations in the vervet system, illustrating several potential advantages of this system, and similar non-human primate (NHP) systems, for genetic dissection of complex traits, particularly those relevant to brain and behavior. Although it has long been established that QTL analysis in pedigrees is a powerful means for initial localization of variants contributing to complex phenotypes, pinpointing the signal identified through such analyses has proven to be a major challenge. The availability of large vervet pedigree and population samples descended from the same genetically-isolated Caribbean population offers the possibility of conducting both linkage and association analyses for a given set of phenotypes in a setting of minimal genetic heterogeneity. This opportunity for combining closely related pedigree and population samples also exists for other NHP systems, including the

cynomolgous macaques from the island of Mauritius [145] and vervets of the *pygerytherus* subspecies in the Western Cape Province of South Africa [146].

Traditionally, genetic investigations of NHP have been limited to relatively small samples that could be maintained in primate colonies. The development of technologies for tracking and repeat identification of wild or feral NHP populations now enables a wide range of longitudinal investigations of various biomedically important traits. Given the size of such populations, the vervet population in sub-Saharan Africa likely includes several million monkeys, and even large-scale genetic investigations in such systems may be relatively inexpensive compared to human research.

Such investigations are now possible given the rapid development of tools for NHP genomic research. The current study was conducted with relatively rudimentary genomic information; it required the utilization of human genomic information and reagents, including the genome assembly, sparse microsatellite sets, and gene expression arrays, which diminished the opportunities for identifying both linkage and association signals. The imminent availability of the vervet reference genome assembly, genome-wide genetic variants, and tissue-specific transcriptome databases will greatly expand the scope of genetic investigation in this system. In particular, the vervet will permit large-scale implementation of a strategy for systematic functional genomic characterization of inaccessible tissues, which is largely infeasible in humans.

The combination of genome-wide gene expression and genetic variation data has proven invaluable in the genetic dissection of a wide range of complex traits [147]. eQTL mapping provides a link to specific genetic markers that are associated with the trait being studied and the expression of specific gene/s. For example, SNPs that represent eQTL are more likely than other SNPs to identify significant association in GWAS [148]. The study of eQTL can complement GWAS data in picking out unobvious candidate genes. A successful example was shown in an asthma study, which initial strong

association signal spanning over 200 kb on chromosome 17q23 containing 19 genes but no obvious candidate for asthma pathology [149]. Subsequent eQTL study was carried out and the eQTL data was used with the association data, which indicated that the genetic variants regulating *ORMDL3* gene expression determine the susceptibility to childhood asthma. Another successful example is a GWAS carried out for Crohn's disease, in which results showed genetic markers with strong association signals located in a gene desert on chromosome 5p13.1 [150]. However, a comparison with eQTL database revealed that this associated region modulates the gene expression of *PTGER4*, which is located 270 kb away.

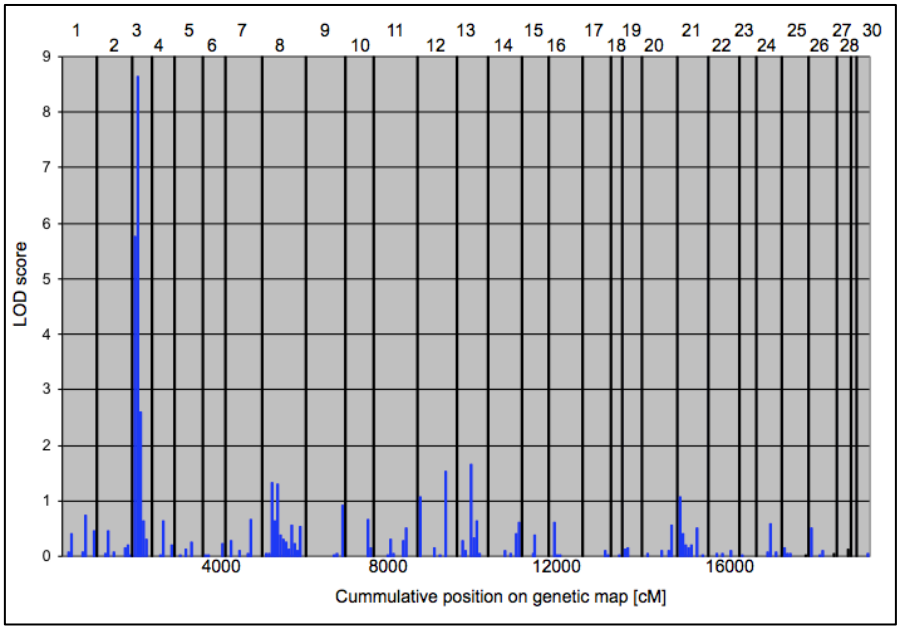
In human, gene expression studies is generally limited to readily accessible tissues, particularly blood and lymphoblastoid cell lines (LCLs), and the use of such tissues has enabled insights into the functional impact of genetic variation for several genes implicated in complex brain-specific phenotypes. Yet, many phenotypes depend on functional variation that is distinctive to – or in some cases limited to – particular tissues, for example, the effects of a substantial proportion of eQTL may be cell-type dependent [151]. The possibility of assaying virtually all tissue types with the vervet samples that are also suitable for large-scale genetic investigations offers a unique opportunity to initiate primate systems biology.

Supplementary Materials

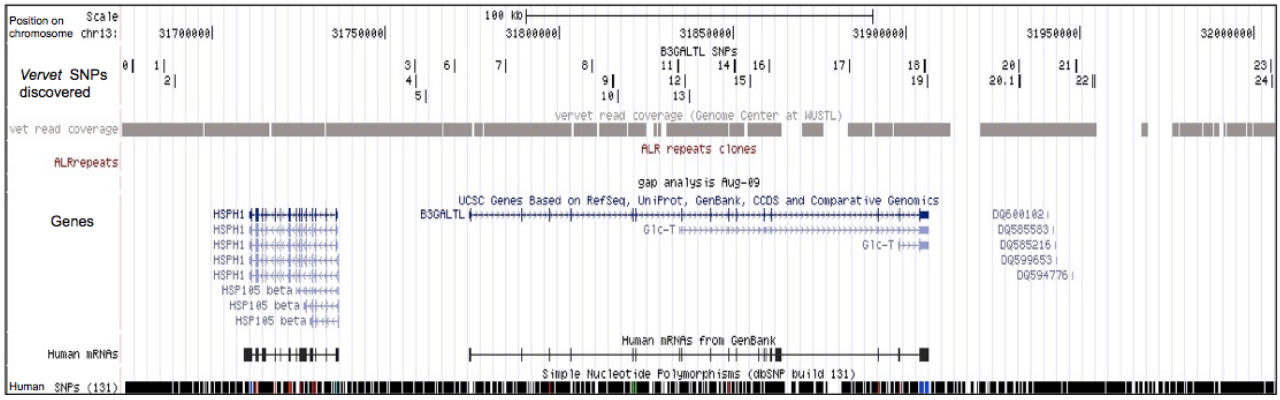
Supplementary table 1: Microsatellite eQTL mapping results with significant linkage evidence (LOD > 4.46) in the VRC.

Gene Symbol	H2r pval	H2r	H2q1	Max LOD	Marker
TMEM14C	6.80E-22	0.7	0.43	6.18	D6S1653
B3GALTL	7.30E-13	0.73	0.51	8.65	D13S1233
TMED3	2.20E-13	0.64	0.43	7.62	D15S206
BAT1	2.50E-12	0.6	0.61	21.82	D6S273
TMEM111	2.60E-13	0.76	0.58	20.55	D3S3589
TUBA1B	2.80E-10	0.62	0.64	20.24	D12S1701
CDKN1A	2.10E-08	0.46	0.38	5	D6S273
TSPAN14	1.40E-04	0.39	0.38	9.05	D10S1677

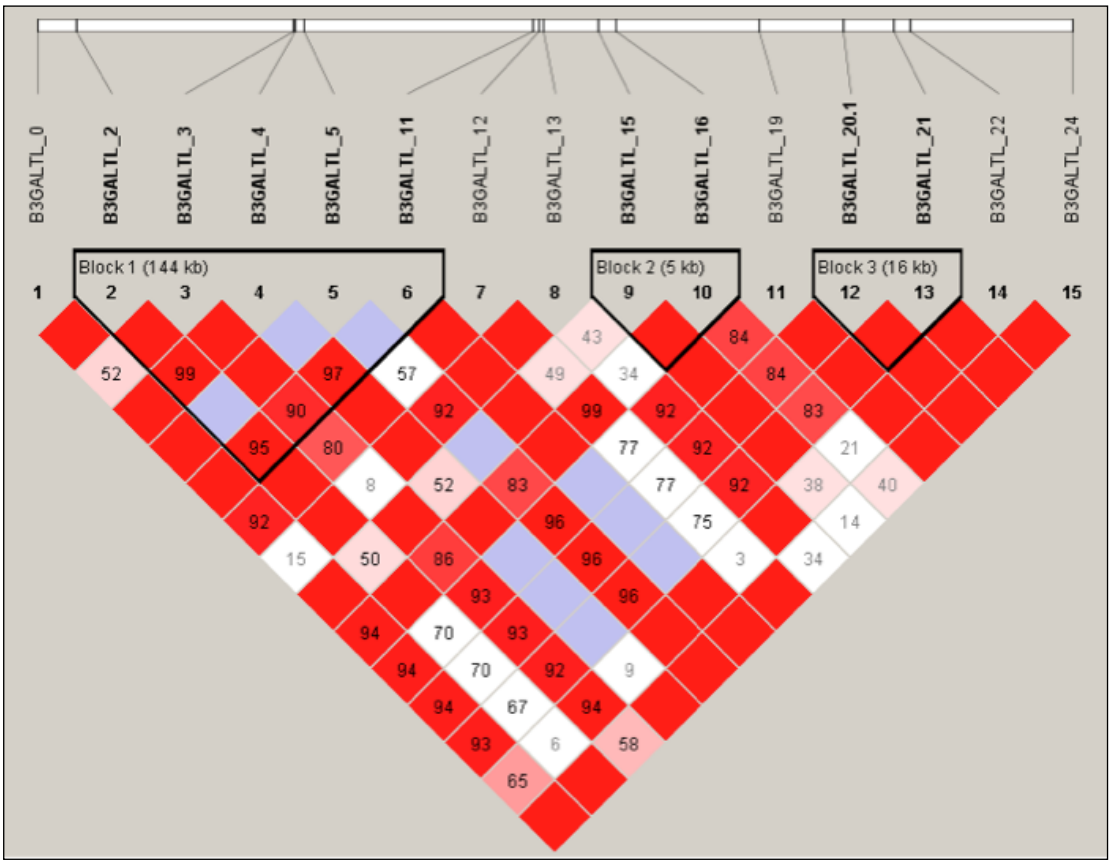
H2r pval: heritability p-value; H2r: total heritability; H2q1: locus specific heritability; Max LOD: maximum LOD score for eQTL linkage mapping in the pedigree using microsatellite genome scan data; Marker: microsatellite marker with maximum LOD score.



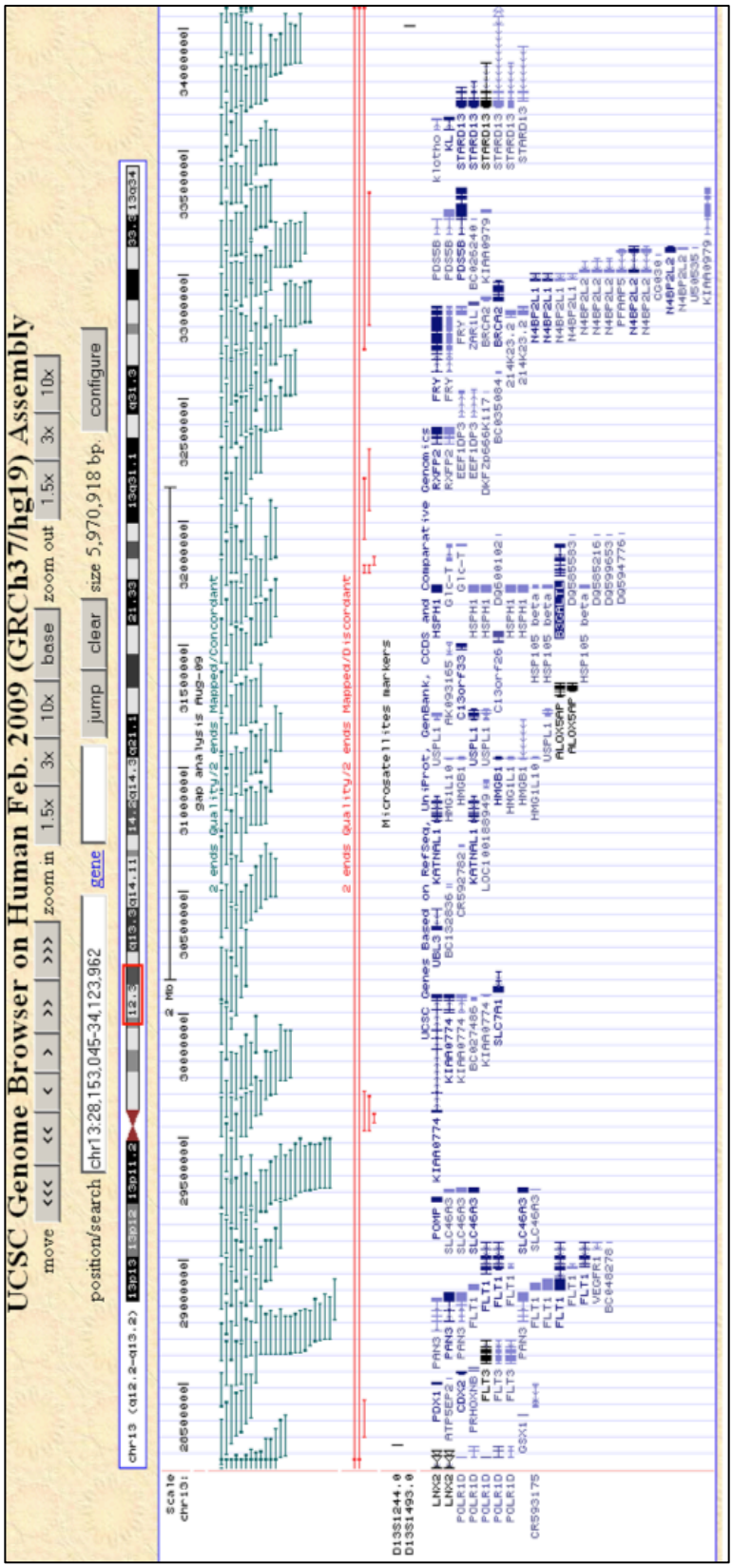
Supplementary figure 1: a) Microsatellite whole-genome linkage scan for a *B3GALTL* regulatory locus. Each blue bar on the plot represents the linkage signal between genome-wide microstallite markers and the microarray signal from probe ILMN1764813 representing *B3GALTL* gene. The linkage signal in LOD score units is presented on the Y-axis. The position of each marker is plotted on the X-axis according to genetic distances (from the vervet genomic map) for all vervet autosomes. Numbers above the plot indicate each chromosome; black bars separate distinct chromosomes.



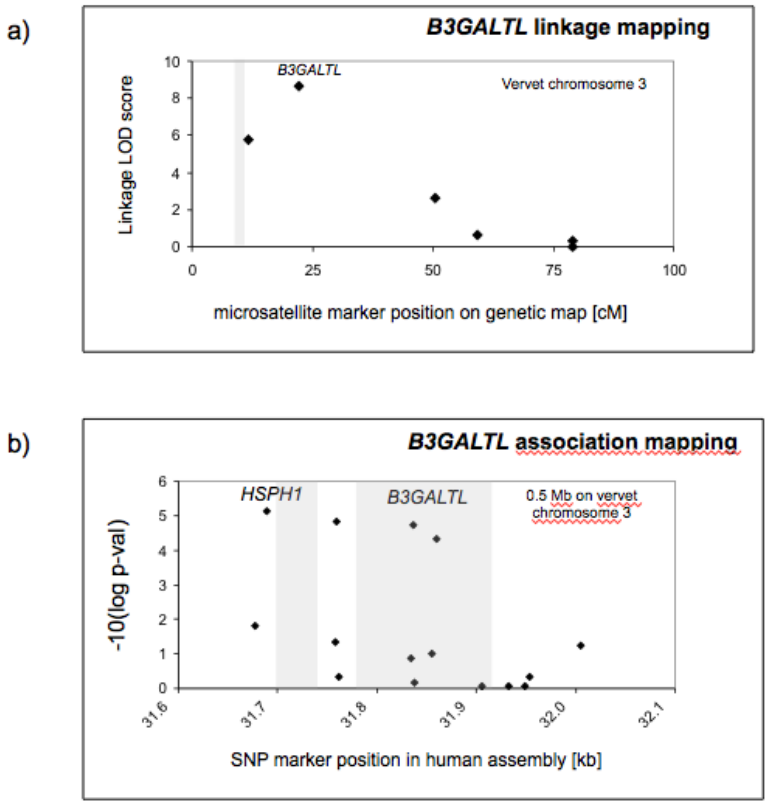
Supplementary figure 2: SNP discovery around *B3GALTL* gene in the vervet genome. 26 putative SNPs were discovered (numbered).



Supplementary figure 3: The linkage disequilibrium (LD) pattern in the *B3GALTL* gene region. The Haploview LD plot shows LD estimates between the 15 *B3GALTL* SNPs in the St. Kitts vervet population. The color scheme for the figure is: LOD<2 & D'<1 (white), LOD<2 & D'=1 (blue), LOD>2 & D'<1 (various shades of pink) and LOD>2 & D'=1. The LOD here evaluates the significance of the estimate of D'. The numeric value within a cell is the estimate of D', estimates of D'=1 are left blank for clarity.



Supplementary figure 4: Synteny between vervet and human in the *B3GALTL* region. The 500 kb of human chromosome 13 is embedded in larger 6 Mb region flanked by markers D13S1244 and D13S1493, where we have complete BAC level synteny. Snapshot of the UCSC human genome browser [hg19] shows alignment of the vervet genomic sequence in custom tracks: gray – 4x coverage of the 454 reads from the reference vervet animal from the VRC (animal ID: 1994-021), green – concordantly mapped BAC ends, red – discordantly mapped BAC ends. The view of the vervet genome data is available from the website http://www.genomequebec.mcgill.ca/compgen/submit_db/vervet_project/vervet_vs_human_hg19.



Supplementary figure 5: a) Linkage mapping on vervet chromosome 3. Linkage LOD score is shown on the Y-axis and microsatellite marker position in cM is shown on the X-axis. Black squares depict the linkage signal of each probe tested; b) Association mapping on vervet chromosome 3. Association p-value is shown on the Y-axis and microsatellite marker position in kb on the human genome assembly is shown on the X-axis. Black squares depict the association signal of each SNP tested.

Chapter 6
Tip of the iceberg --- What's next?

From linkage mapping in large families with Mendelian pattern of inheritance to association studies in the general population, the earlier chapters, 2 to 4, attempt to cover the classical genetic approach in search for genes or genetic variations, which are qualitative factors, influencing psychiatric diseases. Rounding up the bulk of the previous chapters, chapter 5 not only investigates the feasibility of applying the classical two-stage genetic approach to psychiatric and behavioral disorders/traits, it also introduces another genetic perspective, which is expression quantitative trait loci (eQTL), a quantitative factor, to consider aside from the traditional candidate gene studies. eQTL are segments of the genome which regulate gene expression, found to be usually located a distance away from the gene that they are regulating [152].

Aside from the typical gene mutations, variants and expression profiling presented in the previous chapters, there are other genetic aspects one can consider in elucidating the genetic architecture of psychiatric and behavioral phenotypes, in which large families can endow the experimental power more than non-familial-based setups. Some popular genetic aspects in recent psychiatric and behavioral studies, in which large family-based investigation can be useful, are briefly introduced below:

1) Genome structural variations

Structural variants are genomic rearrangements that affect more than 50 bp of sequence, which include deletions, insertions, inversions, duplications, transpositions and translocations [153]. Copy number variations (CNVs) are defined as structural variants resulting in varying numbers of base pairs in the genome. A recent *Nature Genetics* review on genome structural variation has summarized from a number of studies that human genomes differ more as a

consequence of structural variations than single base-pair differences; and that rare CNVs are collectively quite common in the general population, with significant differences in neurocognitive and neuropsychiatric diseases [154]. As described in chapter 1, rare genetic occurrences (e.g. genetic mutations and variants), which can also include structural variations, can be more easily discovered from large family-based studies. An example of such has been reported in one of our attention-deficit/hyperactivity disorder (ADHD) families in chapter 2, where a seemingly monogenic CNV is revealed to be co-segregating with ADHD by our collaborators' efforts [155].

2) Endophenotypes

According to Gottesman, an endophenotype should be a heritable factor that co-segregates with a psychiatric disorder and can be found in non-affected family members at a higher frequency than in the general population [156]. It has been proposed that it is easier to analyze the genetic basis of endophenotypes than the very psychiatric disorders themselves because of the belief that endophenotypes provide more definite biological components compared to that of the diagnostic criteria used, hence the attractiveness of endophenotyping large families in psychiatric genetics research. However, a review compiled by Flint and Munafo about the endophenotype concept in psychiatric genetics showed that the genetic architecture of endophenotypes is not necessary easier to dissect than all other psychiatric disorders [157]. Nevertheless, endophenotyping large families can better aid in garnering more information about psychiatric disorders such as their disease susceptibility and discovering possible biomarkers correlating to the disorders.

3) Phenomics

An emerging field in neuropsychiatric research is phenomics, which is the systematic study of phenotypes on a genome-wide scale [158]. Better

characterization of phenotypes will facilitate genetic studies of the phenotypes. It can allow better insights to how genomic variants can affect phenotypes, how one gene may affect more than one phenotype (pleiotropy), and help decipher complex disorders and traits [159]. Large families can assist phenomics by enabling the Mendelian randomization approach, which allows studies of both the genomes and phenomes of individuals in separate familial populations [160]. Mendelian randomization refers to the natural genetic setting whereby the genetic segregation within families and the variation in environmental exposures among the individuals during their lives can be studied to understand how the genes segregating in certain populations can contribute to understanding the environment factors that cause complex disorders/traits [161]. A more detailed categorization of phenotypes from phenomics can provide sharper diagnostic criteria, which will aid in many psychiatric and behavioral studies as discussed in the sections on cross-disorders and subtyping of disorders in chapter 1.

The previous chapters all explore the theme on large family studies and how they can benefit psychiatric and behavioral research. However, even if large families are recruited, there is still no guarantee that one will be fortunate enough to sample the families for which a major genetic determinant is co-segregating with the disorder or trait being investigated. Despite this, they still offer a higher probability in detecting rare genetic factors of disorders/traits. Past successes in many physiological disorders and traits have proven that studying multiplex families allow geneticists to delve into the complexities of multifactorial disorders/traits and unfold the genetic architecture, through piecing together the missing puzzles of the genetic mechanism, rare variant by rare variant [15].

References

1. Comings DE: Why different rules are required for polygenic inheritance: lessons from studies of the DRD2 gene. *Alcohol* 1998; **16:** 61-70.

2. Burmeister M, McInnis MG, Zollner S: Psychiatric genetics: progress amid controversy. *Nat Rev Genet* 2008; **9:** 527-540.

3. Sullivan PF: The psychiatric GWAS consortium: big science comes to psychiatry. *Neuron* 2010; **68:** 182-186.

4. Burbridge D: Francis Galton on twins, heredity and social class. *Br J Hist Sci* 2001; **34:** 323-340.

5. Gottesman II, Shields J, Meehl PE: *Schizophrenia and genetics; a twin study vantage point*. New York,: Academic Press, 1972.

6. Rosenthal D: Searches for the mode of genetic transmission in schizophrenia: reflections and loose ends. *Schizophr Bull* 1977; **3:** 268-276.

7. Lander ES: The new genomics: global views of biology. *Science* 1996; **274:** 536-539.

8. Cirulli ET, Goldstein DB: Uncovering the roles of rare variants in common disease through whole-genome sequencing. *Nat Rev Genet* 2010; **11:** 415-425.

9. Cichon S, Craddock N, Daly M *et al*: Genomewide association studies: history, rationale, and prospects for psychiatric disorders. *Am J Psychiatry* 2009; **166:** 540-556.

10. Goldstein DB: Common genetic variation and human traits. *N Engl J Med* 2009; **360:** 1696-1698.

11. Ginns EI, Ott J, Egeland JA *et al*: A genome-wide search for chromosomal loci linked to bipolar affective disorder in the Old Order Amish. *Nat Genet* 1996; **12:** 431-435.

12. Allen-Brady K, Robison R, Cannon D *et al*: Genome-wide linkage in Utah autism pedigrees. *Mol Psychiatry* 2010; **15:** 1006-1015.

13. Devlin B, Bacanu SA, Roeder K *et al*: Genome-wide multipoint linkage analyses of multiplex schizophrenia pedigrees from the oceanic nation of Palau. *Mol Psychiatry* 2002; **7:** 689-694.

14. Mitchell KJ, Porteous DJ: Rethinking the genetic architecture of schizophrenia. *Psychol Med* 2011; **41:** 19-32.

15. Peltonen L, Perola M, Naukkarinen J, Palotie A: Lessons from studying monogenic disease for common disease. *Hum Mol Genet* 2006; **15 Spec No 1:** R67-74.

16. Raj A, Rifkin SA, Andersen E, van Oudenaarden A: Variability in gene expression underlies incomplete penetrance. *Nature* 2010; **463:** 913-918.

17. Bassett AS, Bury A, Hodgkinson KA, Honer WG: Reproductive fitness in familial schizophrenia. *Schizophr Res* 1996; **21:** 151-160.

18. Uher R: The role of genetic variation in the causation of mental illness: an evolution-informed framework. *Mol Psychiatry* 2009; **14:** 1072-1082.

19. Merikangas KR: Assortative mating for psychiatric disorders and psychological traits. *Arch Gen Psychiatry* 1982; **39:** 1173-1180.

20. Teltsh O, Kanyas K, Karni O *et al*: Genome-wide linkage scan, fine mapping, and haplotype analysis in a large, inbred, Arab Israeli pedigree suggest a schizophrenia susceptibility locus on chromosome 20p13. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B:** 209-215.

21. Mansour H, Klei L, Wood J *et al*: Consanguinity associated with increased risk for bipolar I disorder in Egypt. *Am J Med Genet B Neuropsychiatr Genet* 2009; **150B:** 879-885.

22. Rommelse NN, Franke B, Geurts HM, Hartman CA, Buitelaar JK: Shared heritability of attention-deficit/hyperactivity disorder and autism spectrum disorder. *Eur Child Adolesc Psychiatry* 2010; **19:** 281-295.

23. Lichtenstein P, Yip BH, Bjork C *et al*: Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *Lancet* 2009; **373:** 234-239.

24. Huang J, Perlis RH, Lee PH *et al*: Cross-disorder genomewide analysis of schizophrenia, bipolar disorder, and depression. *Am J Psychiatry* 2010; **167:** 1254-1263.

25. Angst J: Psychiatric diagnoses: the weak component of modern research. *World Psychiatry* 2007; **6:** 94-95.

26. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (http://www.psych.org/MainMenu/Research/DSMIV/DSMIVTR/DSMIVvsDSMIVTR.aspx).

27. World Health Organization. International Classification of Diseases (http://www.who.int/classifications/icd/en/).

28. Peltonen L, Jalanko A, Varilo T: Molecular genetics of the Finnish disease heritage. *Hum Mol Genet* 1999; **8:** 1913-1923.

29. Peltonen L, Palotie A, Lange K: Use of population isolates for mapping complex traits. *Nat Rev Genet* 2000; **1:** 182-190.

30. Selch S, Strobel A, Haderlein J *et al*: MLC1 polymorphisms are specifically associated with periodic catatonia, a subgroup of chronic schizophrenia. *Biol Psychiatry* 2007; **61:** 1211-1214.

31. Lilenfeld LR, Kaye WH, Greeno CG *et al*: A controlled family study of anorexia nervosa and bulimia nervosa: psychiatric disorders in first-degree relatives and effects of proband comorbidity. *Arch Gen Psychiatry* 1998; **55:** 603-610.

32. Strober M, Freeman R, Lampert C, Diamond J, Kaye W: Controlled family study of anorexia nervosa and bulimia nervosa: evidence of shared liability and transmission of partial syndromes. *Am J Psychiatry* 2000; **157:** 393-401.

33. Bulik CM, Sullivan PF, Wade TD, Kendler KS: Twin studies of eating disorders: a review. *Int J Eat Disord* 2000; **27:** 1-20.

34. Wang K, Zhang H, Bloss CS *et al*: A genome-wide association study on common SNPs and rare CNVs in anorexia nervosa. *Mol Psychiatry* 2011; **16:** 949-959.

35. Kral TV, Rauh EM: Eating behaviors of children in the context of their family environment. *Physiol Behav* 2010; **100:** 567-573.

36. Moises HW, Yang L, Kristbjarnarson H *et al*: An international two-stage genome-wide search for schizophrenia susceptibility genes. *Nat Genet* 1995; **11:** 321-324.

37. A framework for interpreting genome-wide association studies of psychiatric disorders. *Mol Psychiatry* 2009; **14:** 10-17.

38. Geller DS, Zhang J, Wisgerhof MV, Shackleton C, Kashgarian M, Lifton RP: A novel form of human mendelian hypertension featuring nonglucocorticoid-remediable aldosteronism. *J Clin Endocrinol Metab* 2008; **93:** 3117-3123.

39. Froguel P, Velho G, Passa P, Cohen D: Genetic determinants of type 2 diabetes mellitus: lessons learned from family studies. *Diabete Metab* 1993; **19(1):** 1-10.

40. Panhuysen CI, Karban A, Knodle Manning A *et al*: Identification of genetic loci for basal cell nevus syndrome and inflammatory bowel disease in a single large pedigree. *Hum Genet* 2006; **120(1):** 31-41.

41. Lee SL, Murdock DG, Mccauley JL *et al*: A Genome-wide Scan in an Amish Pedigree with Parkinsonism. *Ann Hum Genet* 2008; **72(Pt 5):** 621-629.

42. Yang S, Wang K, Gregory B *et al*: Genomic landscape of a three-generation pedigree segregating affective disorder. *PLos One* 2009; **4(2):** e4474.

43. Teltsh O, Kanyas K, Karni O *et al*: Genome-wide linkage scan, fine mapping, and haplotype analysis in a large, inbred, Arab Israeli pedigree suggest a schizophrenia susceptibility locus on chromosome 20p13. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B(2):** 209-215.

44. Polanczyk G, de Lima MS, Horta BL, Biederman J, Rohde L: The worldwide prevalence of ADHD: A systemic review and metaregression analysis. *Am J Psychiatry* 2007; **164:** 942-948.

45. Kessler RC, Adler LA, Barkley R *et al*: Patterns and predictors of attention-deficit/hyperactivity disorder persistence into adulthood: results from the national comorbidity survey replication. *Biol Psychiatry* 2005; **57:** 1442-1451.

46. Faraone SV, Perlis RH, Doyle AE *et al*: Molecular genetics of attention-deficit/hyperactivity disorder. *Biol Psychiatry* 2005; **57(11):** 1313-1323.

47. Sprich S, Biederman J, Crawford MH, Mundy E, Faraone S: Adoptive and biological families of children and adolescents with ADHD. *J Am Acad Child Adolesc Psychiatry* 2000; **39(11):** 1432-1437.

48. Jacob CP, Romanos J, Dempfle A *et al*: Co-morbidity of adult attention-deficit/hyperactivity disorder with focus on personality traits and related disorders in a tertiary referral center. *Eur Arch Psychiatry Clin Neurosci* 2007; **257(6):** 309-317.

49. Boomsma DI, Saviouk V, Hottenga JJ *et al*: Genetic epidemiology of attention deficit hyperactivity disorder (ADHD index) in adults. *PLoS One* 2010; **5:** e10621.

50. Romanos M, Freitag C, Jacob C *et al*: Genome-wide linkage analysis of ADHD using high density SNP arrays: novel loci at 5q13.1 and 14q12. *Mol Psychiatry* 2008; **13:** 522-530.

51. Kaufman J, Birmaher B, Brent D *et al*: Schedule for affective disorders and schizophrenia fro school-age children - present and lifetime version (K-SADS-PL): initial reliability and validity data. *J Am Acad Child Adolesc Psychiatry* 1997; **36:** 980-988.

52. Schneider S, Margraf J, Unnewehr S: Kinder-DIPS Diagnostisches Interview bei Psychischen Stoerungen von Kindern. *Berlin: Springer* 1995.

53. Achenbach T, Rescorla L: Manual for the Child Behavior Checklist/6-18. *University of Vermont, Department of Psychiatry, Burlington, VT* 2001.

54. Rösler M, Retz W, Retz-Junginger P *et al*: Tools for the diagnosis of attention-deficit/hyperactivity disorder in adults. Self-rating behavior questionnaire and diagnostic checklist. *Nervenarzt* 2004; **75:** 888-895.

55. Doepfner M, Lehmkukl G: Diagnostik-System fuer psychische Stoerungen im Kindes- und Jungenalter nach ICD-10/DSM-IV (DISYPS-KJ). 2nd corrected and extended edition. *Bern: Hans Huber* 2003.

56. Wittchen H-U, Wunderlich U, Gruschwitz S, M Z: *SKID I*. Göttingen: Hogrefe, 1997.

57. Fydrich T, Renneberg B, Schmitz B, H-U W: *SKID II*. Göttingen: Hogrefe, 1997.

58. Retz-Junginger P, Retz W, Blocher D *et al*: Wender Utah rating scale. The short version for the assessment of the attention-deficit hyperactivity disorder in adults. *Nervenarzt* 2002; **73:** 830-838.

59. Cattell RB, Weiss R, Osterland J: Grundintelligenztest Skala 1-CFT1. *Goettingen: Hogrefe* 1997.

60. Weiss R: Grundintelligenztest Skala 2-CFT20. *Goettingen: Hogrefe* 1998.

61. Lehrl S, Triebig G, B F: Multiple choice vocabulary test MWT as a valid and short test to estimate premorbid intelligence. *Acta Neurol Scand* 1995; **91:** 335-345.

62. Sobel E, Lange K: Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker sharing statistics. *Am J Hum Genet* 1996; **58:** 1323-1337.

63. Lindner TH, Hoffmann K: easyLINKAGE: a PERL script for easy and automated two-/multi-point linkage analyses. *Bioinformatics* 2005; **21(3):** 405-407.

64. Thiele H, Nuernberg P: HaploPainter: a tool for drawing pedigrees with complex haplotypes. *Bioinformatics* 2005; **21(8):** 1730-1732.

65. Broman KW, Murray JC, Sheffield VC, White RL, Weber J: Comprehensive human genetic maps: Individual and sex-specific variation in recombination. *Am J Hum Genet* 1998; **63:** 861-869.

66. Abecasis GR, Cherny SS, Cookson WO, Cardon L: Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002; **30(1):** 97-101.

67. Amin N, Aulchenko YS, Dekker MC *et al*: Suggestive linkage of ADHD to chromosome 18q22 in a young genetically isolated Dutch population. *Eur J Hum Genet* 2009; **7:** 958-966.

68. Saviouk V, Hottenga JJ, Slagboom EP *et al*: ADHD in Dutch adults: heritability and linkage study. *Am J Med Genet B Neuropsychiatr Genet* 2011; **156B:** 352-362.

69. Sobreira NLM, Cirulli ET, Avramopoulos D *et al*: Whole-genome sequencing of a single proband together with linkage anlaysis identifies a Mendelian disease gene. *PLos Genet* 2010; **6(6):** e1000991.

70. Risch N, Merikangas K: The future of genetic studies of complex human diseases. *Science* 1996; **273(5281):** 1516-1517.

71. Neale BM, Medland S, Ripke S *et al*: Case-Control Genome-Wide Association Study of Attention-Deficit/Hyperactivity Disorder. *J Am Acad Child Adolesc Psychiatry* 2010; **49(9):** 906-920.

72. Zhou K, Dempfle A, Arcos-Burgos M *et al*: Meta-analysis of genome-wide linkage scans of attention deficit hyperactivity disorder. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B(8):** 1392-1398.

73. Lesch KP, Selch S, Renner TJ *et al*: Genome-wide copy number variation analysis in attention-deficit/hyperactivity disorder: association with neuropeptide Y gene dosage in an extended pedigree. *Mol Psychiatry* 2010; **[Epub ahead of print]**.

74. Vegt R, Bertoli-Avella AM, Tulen JH *et al*: Genome-wide linkage analysis in a Dutch multigenerational family with attention deficit hyperactivity disorder. *Eur J Hum Genet* 2010; **18(2):** 206-211.

75. Arcos-Burgos M, Castellanos FX, Pineda D *et al*: Attention-Deficit/Hyperactivity Disorder in a Population Isolate: Linkage to Loci at 4q13.2, 5q33.3, 11q22, and 17p11. *Am J Hum Genet* 2004; **75(6):** 998-1014.

76. Arcos-Burgos M, Jain M, Acosta MT *et al*: A common variant of the latrophilin 3 gene, LPHN3, confers susceptibility to ADHD and predicts effectiveness of stimulant medication. *Mol Psychiatry* 2010; **15 (11):** 1053-1066.

77. Varilo T, Peltonen L: Isolates and their potential use in complex gene mapping efforts. *Curr Opin Genet Dev* 2004; **14(3):** 316-323.

78. Banerjee TD, Middleton F, Faraone S: Environmental risk factors for attention-deficit hyperactivity disorder. *Acta Paediatr* 2007; **96(9):** 1269-1274.

79. van der Knaap MS, Barth PG, Stroink H *et al*: Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. *Ann Neurol* 1995; **37:** 324-334.

80. Meyer J, Huberth A, Ortega G *et al*: A missense mutation in a novel gene encoding a putative cation channel is associated with catatonic schizophrenia in a large pedigree. *Mol Psychiatry* 2001; **6:** 302-306.

81. Verma R, Mukerji M, Grover D *et al*: MLC1 gene is associated with schizophrenia and bipolar disorder in Southern India. *Biol Psychiatry* 2005; **58:** 16-22.

82. Rubie C, Lichtner P, Gartner J *et al*: Sequence diversity of KIAA0027/MLC1: are megalencephalic leukoencephalopathy and schizophrenia allelic disorders? *Hum Mutat* 2003; **21:** 45-52.

83. Spijker S, Van Zanten JS, De Jong S *et al*: Stimulated gene expression profiles as a blood marker of major depressive disorder. *Biol Psychiatry* 2010; **68:** 179-186.

84. Boor PK, de Groot K, Waisfisz Q *et al*: MLC1: a novel protein in distal astroglial processes. *J Neuropathol Exp Neurol* 2005; **64:** 412-419.

85. Schmitt A, Gofferje V, Weber M, Meyer J, Mossner R, Lesch KP: The brain-specific protein MLC1 implicated in megalencephalic leukoencephalopathy with subcortical cysts is expressed in glial cells in the murine brain. *Glia* 2003; **44:** 283-295.

86. Teijido O, Martinez A, Pusch M *et al*: Localization and functional analyses of the MLC1 protein involved in megalencephalic leukoencephalopathy with subcortical cysts. *Hum Mol Genet* 2004; **13:** 2581-2594.

87. Teijido O, Casaroli-Marano R, Kharkovets T *et al*: Expression patterns of MLC1 protein in the central and peripheral nervous systems. *Neurobiol Dis* 2007; **26:** 532-545.

88. Leegwater PA, Yuan BQ, van der Steen J *et al*: Mutations of MLC1 (KIAA0027), encoding a putative membrane protein, cause megalencephalic leukoencephalopathy with subcortical cysts. *Am J Hum Genet* 2001; **68:** 831-838.

89. Steinke V, Meyer J, Syagailo YV *et al*: The genomic organization of the murine Mlc1 (Wkl1, KIAA0027) gene. *J Neural Transm* 2003; **110:** 333-343.

90. Suzuki Y, Tsunoda T, Sese J *et al*: Identification and characterization of the potential promoter regions of 1031 kinds of human genes. *Genome Res* 2001; **11:** 677-684.

91. Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. *J Mol Biol* 1987; **196:** 261-282.

92. Roider HG, Lenhard B, Kanhere A, Haas SA, Vingron M: CpG-depleted promoters harbor tissue-specific transcription factor binding signals--implications for motif overrepresentation analyses. *Nucleic Acids Res* 2009; **37:** 6305-6315.

93. Dean M: ABC transporters, drug resistance, and cancer stem cells. *J Mammary Gland Biol Neoplasia* 2009; **14:** 3-9.

94. Ambrosini E, Serafini B, Lanciotti A *et al*: Biochemical characterization of MLC1 protein in astrocytes and its association with the dystrophin-glycoprotein complex. *Mol Cell Neurosci* 2008; **37:** 480-493.

95. Cohn RD: Dystroglycan: important player in skeletal muscle and beyond. *Neuromuscul Disord* 2005; **15:** 207-217.

96. Gudmundsson P, Skoog I, Waern M *et al*: The relationship between cerebrospinal fluid biomarkers and depression in elderly women. *Am J Geriatr Psychiatry* 2007; **15:** 832-838.

97. Muller N, Ackenheil M: Immunoglobulin and albumin content of cerebrospinal fluid in schizophrenic patients: relationship to negative symptomatology. *Schizophr Res* 1995; **14:** 223-228.

98. Schwarz MJ, Ackenheil M, Riedel M, Muller N: Blood-cerebrospinal fluid barrier impairment as indicator for an immune process in schizophrenia. *Neurosci Lett* 1998; **253:** 201-203.

99. Mantovani R: A survey of 178 NF-Y binding CCAAT boxes. *Nucleic Acids Res* 1998; **26:** 1135-1143.

100. Blomquist P, Belikov S, Wrange O: Increased nuclear factor 1 binding to its nucleosomal site mediated by sequence-dependent DNA structure. *Nucleic Acids Res* 1999; **27:** 517-525.

101. Donati G, Gatta R, Dolfini D, Fossati A, Ceribelli M, Mantovani R: An NF-Y-dependent switch of positive and negative histone methyl marks on CCAAT promoters. *PLoS One* 2008; **3:** e2066.

102. Mantovani R: The molecular biology of the CCAAT-binding factor NF-Y. *Gene* 1999; **239:** 15-27.

103. Carninci P, Sandelin A, Lenhard B *et al*: Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet* 2006; **38:** 626-635.

104. Ajay SS, Athey BD, Lee I: Unified translation repression mechanism for microRNAs and upstream AUGs. *BMC Genomics* 2010; **11:** 155.

105. Hu L, Gudas LJ: Activation of keratin 19 gene expression by a 3' enhancer containing an AP1 site. *J Biol Chem* 1994; **269:** 183-191.

106. Bianchi M, Crinelli R, Giacomini E, Carloni E, Magnani M: A potent enhancer element in the 5'-UTR intron is crucial for transcriptional regulation of the human ubiquitin C gene. *Gene* 2009; **448:** 88-101.

107. Ilja Boor PK, de Groot K, Mejaski-Bosnjak V *et al*: Megalencephalic leukoencephalopathy with subcortical cysts: an update and extended mutation analysis of MLC1. *Hum Mutat* 2006; **27:** 505-512.

108. Leegwater PA, Boor PK, Yuan BQ *et al*: Identification of novel mutations in MLC1 responsible for megalencephalic leukoencephalopathy with subcortical cysts. *Hum Genet* 2002; **110:** 279-283.

109. Lopez-Hernandez T, Ridder MC, Montolio M *et al*: Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. *Am J Hum Genet* 2011; **88:** 422-432.

110. Chevalier-Mariette C, Henry I, Montfort L *et al*: CpG content affects gene silencing in mice: evidence from novel transgenes. *Genome Biol* 2003; **4:** R53.

111. Smith EN, Bloss CS, Badner JA *et al*: Genome-wide association study of bipolar disorder in European American and African American individuals. *Mol Psychiatry* 2009; **14:** 755-763.

112. Ferreira MA, O'Donovan MC, Meng YA *et al*: Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. *Nat Genet* 2008; **40:** 1056-1058.

113. McGuffin P, Owen MJ, Farmer AE: Genetic basis of schizophrenia. *Lancet* 1995; **346:** 678-682.

114. Potash JB, DePaulo JR, Jr.: Searching high and low: a review of the genetics of bipolar disorder. *Bipolar Disord* 2000; **2:** 8-26.

115. Saha S, Chant D, Welham J, McGrath J: A systematic review of the prevalence of schizophrenia. *PLoS Med* 2005; **2:** e141.

116. Perala J, Suvisaari J, Saarni SI *et al*: Lifetime prevalence of psychotic and bipolar I disorders in a general population. *Arch Gen Psychiatry* 2007; **64:** 19-28.

117. Sullivan PF, Kendler KS, Neale MC: Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch Gen Psychiatry* 2003; **60:** 1187-1192.

118. Smoller JW, Finn CT: Family, twin, and adoption studies of bipolar disorder. *Am J Med Genet C Semin Med Genet* 2003; **123C:** 48-58.

119. Stober G, Pfuhlmann B, Nurnberg G *et al*: Towards the genetic basis of periodic catatonia: pedigree sample for genome scan I and II. *Eur Arch Psychiatry Clin Neurosci* 2001; **251 Suppl 1:** I25-30.

120. Leonhard K, H B: *Classification of endogeneous psychoses and their differential etiology*, 2nd edn. Vienna and New York: Springer, 1999.

121. Taylor MA, Fink M: Catatonia in psychiatric classification: a home of its own. *Am J Psychiatry* 2003; **160:** 1233-1241.

122. Stober G, Franzek E, Lesch KP, Beckmann H: Periodic catatonia: a schizophrenic subtype with major gene effect and anticipation. *Eur Arch Psychiatry Clin Neurosci* 1995; **245:** 135-141.

123. Meyer J, Johannssen K, Freitag CM *et al*: Rare variants of the gene encoding the potassium chloride co-transporter 3 are associated with bipolar disorder. *Int J Neuropsychopharmacol* 2005; **8:** 495-504.

124. Stober G, Saar K, Ruschendorf F *et al*: Splitting schizophrenia: periodic catatonia-susceptibility locus on chromosome 15q15. *Am J Hum Genet* 2000; **67:** 1201-1207.

125. Stober G, Meyer J, Nanda I *et al*: Linkage and family-based association study of schizophrenia and the synapsin III locus that maps to chromosome 22q13. *Am J Med Genet* 2000; **96:** 392-397.

126. Rozen S, Skaletsky H: Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; **132:** 365-386.

127. Sobel E, Lange K: Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics. *Am J Hum Genet* 1996; **58:** 1323-1337.

128. Hoffmann K, Lindner TH: easyLINKAGE-Plus--automated linkage analyses using large-scale SNP data. *Bioinformatics* 2005; **21:** 3565-3567.

129. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21:** 263-265.

130. Alt SR, Turner JD, Klok MD *et al*: Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. *Psychoneuroendocrinology* 2010; **35:** 544-556.

131. Stober G, Seelow D, Ruschendorf F, Ekici A, Beckmann H, Reis A: Periodic catatonia: confirmation of linkage to chromosome 15 and further evidence for genetic heterogeneity. *Hum Genet* 2002; **111:** 323-330.

132. Ben-Shachar S, Lanpher B, German JR *et al*: Microdeletion 15q13.3: a locus with incomplete penetrance for autism, mental retardation, and psychiatric disorders. *J Med Genet* 2009; **46:** 382-388.

133. Dykens EM, Sutcliffe JS, Levitt P: Autism and 15q11-q13 disorders: behavioral, genetic, and pathophysiological issues. *Ment Retard Dev Disabil Res Rev* 2004; **10:** 284-291.

134. Ingason A, Kirov G, Giegling I *et al*: Maternally derived microduplications at 15q11-q13: implication of imprinted genes in psychotic illness. *Am J Psychiatry* 2011; **168:** 408-417.

135. Buizer-Voskamp JE, Muntjewerff JW, Strengman E *et al*: Genome-Wide Analysis Shows Increased Frequency of Copy Number Variation Deletions in Dutch Schizophrenia Patients. *Biol Psychiatry* 2011.

136. Williams NM, Zaharieva I, Martin A *et al*: Rare chromosomal deletions and duplications in attention-deficit hyperactivity disorder: a genome-wide analysis. *Lancet* 2010; **376:** 1401-1408.

137. Richards AL, Jones L, Moskvina V *et al*: Schizophrenia susceptibility alleles are enriched for alleles that affect gene expression in adult human brain. *Mol Psychiatry* 2011.

138. Liu C: Brain expression quantitative trait locus mapping informs genetic studies of psychiatric diseases. *Neurosci Bull* 2011; **27:** 123-133.

139. Bearden CE, Jasinska AJ, Freimer NB: Methodological issues in molecular genetic studies of mental disorders. *Annu Rev Clin Psychol* 2009; **5:** 49-69.

140. Jasinska AJ, Service S, Choi OW *et al*: Identification of brain transcriptional variation reproduced in peripheral blood: an approach for mapping brain expression traits. *Hum Mol Genet* 2009; **18:** 4415-4427.

141. Lesnik Oberstein SA, Kriek M, White SJ *et al*: Peters Plus syndrome is caused by mutations in B3GALTL, a putative glycosyltransferase. *Am J Hum Genet* 2006; **79:** 562-566.

142. Jasinska AJ, Service S, Levinson M *et al*: A genetic linkage map of the vervet monkey (Chlorocebus aethiops sabaeus). *Mamm Genome* 2007; **18:** 347-360.

143. Almasy L, Blangero J: Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* 1998; **62:** 1198-1211.

144. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29:** e45.

145. Mee ET, Badhan A, Karl JA *et al*: MHC haplotype frequencies in a UK breeding colony of Mauritian cynomolgus macaques mirror those found in a distinct population from the same geographic origin. *J Med Primatol* 2009; **38:** 1-14.

146. de Vries A, Holmes MC, Heijnis A *et al*: Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal axis function. *J Clin Invest* 2007; **117:** 1058-1067.

147. Goring HH, Curran JE, Johnson MP *et al*: Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes. *Nat Genet* 2007; **39:** 1208-1216.

148. Nicolae DL, Gamazon E, Zhang W, Duan S, Dolan ME, Cox NJ: Trait-associated SNPs are more likely to be eQTLs: annotation to enhance discovery from GWAS. *PLoS Genet* 2010; **6:** e1000888.

149. Moffatt MF, Kabesch M, Liang L *et al*: Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 2007; **448:** 470-473.

150. Libioulle C, Louis E, Hansoul S *et al*: Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genet* 2007; **3:** e58.

151. Dimas AS, Deutsch S, Stranger BE *et al*: Common regulatory variation impacts gene expression in a cell type-dependent manner. *Science* 2009; **325:** 1246-1250.

152. Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M: Mapping complex disease traits with global gene expression. *Nat Rev Genet* 2009; **10:** 184-194.

153. Durbin RM, Abecasis GR, Altshuler DL *et al*: A map of human genome variation from population-scale sequencing. *Nature* 2010; **467:** 1061-1073.

154. Alkan C, Coe BP, Eichler EE: Genome structural variation discovery and genotyping. *Nat Rev Genet* 2011; **12:** 363-376.

155. Lesch KP, Selch S, Renner TJ *et al*: Genome-wide copy number variation analysis in attention-deficit/hyperactivity disorder: association with neuropeptide Y gene dosage in an extended pedigree. *Mol Psychiatry* 2011; **16:** 491-503.

156. Gottesman, II, Gould TD: The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* 2003; **160:** 636-645.

157. Flint J, Munafo MR: The endophenotype concept in psychiatric genetics. *Psychol Med* 2007; **37:** 163-180.

158. Bilder RM: Phenomics: building scaffolds for biological hypotheses in the post-genomic era. *Biol Psychiatry* 2008; **63:** 439-440.

159. Houle D, Govindaraju DR, Omholt S: Phenomics: the next challenge. *Nat Rev Genet* 2010; **11:** 855-866.

160. Rockman MV: Reverse engineering the genotype-phenotype map with natural genetic variation. *Nature* 2008; **456:** 738-744.

161. Davey Smith G, Ebrahim S: 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol* 2003; **32:** 1-22.

Bibliography of presented work during doctoral studies (October 2007 – December 2010)

Invited Talks

1) ADHD Symposium (Wuerzburg, Germany, June 2008)
   'Linkage analysis in multiplex families with ADHD'

2) IMpACT Symposium on adult ADHD and complex psychiatric genetics (Wuerzburg, Germany, June 2010)
   'Linkage analysis and fine-mapping in multiplex ADHD pedigrees'

Oral Presentations

1) German Society of Neurogenetics (Luebeck, Germany, September 2008)
   'Fine-mapping of multiplex families with ADHD'

2) World Congress on Psychiatric Genetics (San Diego, USA, October 2009)
   'Haplotype segregation in multiplex ADHD families at different chromosomal loci'

3) IRTG Autumn School (Luxembourg, November 2009)
   'ADHD, Stress and Genes'

Poster Presentations

1) World Congress on Psychiatric Genetics (Osaka, Japan, October 2008)
   'Linkage analysis in multiplex families with ADHD'

2) European Human Genetics Conference (Vienna, Austria, May 2009)
   'Monogeneous inheritance in ADHD? Report from a fine-mapping study'

3) International Research Day (Trier, Germany, December 2010)
   'Genetics of blunted stress response: Perspectives from ADHD study'

Acknowledgements

Heartfelt gratitude to my mentor, Professor Jobst Meyer, with whose guidance helped and encouraged each little step in my academic pursuit these past years.

Many thanks to:

Professor Claude Muller, Ulrike Schuelter and all colleagues from the Department of Neurobehavioral Genetics (Institute of Psychobiology, University of Trier), International Research Training Group (Trier-Leiden) and collaborating institutes for their professional support and moral encouragement;

Professor Hartmut Schaechinger for his generosity in making a fruitful academic exchange with the Semel Institute at UCLA possible;

Professor Nelson Freimer and Dr. Anna Jasinska for their hospitality and guidance during my academic exchange stint with them;

and the many impressive scientists, whom I have met along the way, for their invaluable advices on scientific matters and kind words when the going gets tough.

Michelle Lin
Trier, September 2011

ERKLÄRUNG

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

1. .....

2. .....

3. .....

Weitere Personen waren an der inhaltlich-materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich versichere die Richtigkeit der vorangegangenen Erklärung und bin mir der strafrechtlichen Folgen einer Falschaussage bewusst.

---------- ----------

Ort, Datum

Unterschrift
(Michelle Lin)