THE CONTRIBUTION OF COMMON AND RARE VARIANTS TO THE COMPLEX GENETICS OF PSYCHIATRIC DISORDERS

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I. INTRODUCTION

V. APPENDIX

B. LIST OF SCIENTIFIC PUBLICATIONS

1. Selch S, Strobel A, Haderlein J, Meyer J, Jacob CP, Schmitt A, Lesch KP, Reif A. (2007). "**MLC1 polymorphisms are specifically associated with periodic catatonia, a subgroup of chronic schizophrenia."** Biol Psychiatry 61 (10): 1211-4.

2. Veenema AH, Reber SO, Selch S, Obermeier F, Neumann ID. (2008). "**Early life stress enhances the vulnerability to chronic psychosocial stress and experimental colitis in adult mice."** Endocrinology 149 (6): 2727-36.

3. Lesch KP*, Selch S*, Renner TJ*, Jacob C, Nguyen TT, Romanos M, Shoichet S, Dempfle A, Heine M, Boreatti-Hümmer A, Walitza S, Romanos J, Zerlaut H, Allolio B, Fassnacht M, Wultsch T, Reif A, Schäfer H, Warnke A, Ropers HH, Ullmann R. (2010) **"Genome-wide copy number variation analysis in ADHD: association with neuropeptide Y gene dosage in an extended pedigree."** Mol Psychiatry (Epub ahead of print)
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C. LECTURES

1. Selch S. (Dec 2005) **"Behavioral Phenotyping."** 2nd Würzburg Brain and Behaviour Days: *A critical evaluation of available method,* meeting of the Graduate College (GRK) 1156 "*From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Science.

2. Selch S. (Apr 2007) **"A genomwide duplication and deletion analysis on patients with ADHD."** 4th Würzburg Brain and Behaviour Days: *Presentation of the latest results*, meeting of the Graduate College (GRK) 1156: "*From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Science.

3. Selch S. (May 2007) **"Untersuchungen zu ADHS mit Hilfe des *Microarray-based comparative genomic hybridization* (a-CGH).**" Scientific neurobiological meeting, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

4. Selch S. (Dec 2007) **"Molekularbiologische Untersuchungen zu MLC1 – ein Kandidatengen für Schizophrenie."** Scientific neurobiological meeting, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

D. PRESENTATIONS AT CONFERENCES

1. Selch S, Fritzen S, Schmitt A, Lesch KP, Reif A. (Poster) **"Neural stem cell proliferation is significantly reduced in schizophrenic, but not in affective psychoses."** (2005) FENS (Federation of European Neuroscience), Vienna, Austria

2. Selch S, Lesch KP, Romanos M, Walitza S, Hemminger U, Warnke A, Romanos J, Renner T, Jacob C, Ropers HH, Ullmann R. (Poster) **"A genomwide duplication- and deletion analysis on patients with ADHD."** (2007) ECNP (European College of Neuropharmacology) workshop in neuropsychopharmacology for young scientists, Nice, France

3. Selch S, Kreutzfeldt M, Hall FS, Perona M, Ortega G, Hofmann M, Nietzer S, Sora I, Uhl GR, Lesch KP, Gerlach M, Grünblatt E, Schmitt A. (Poster) **"ADHD and Latrophilin3: Are there reasons to pay attention?"** (2008) FENS (Federation of European Neuroscience), Geneva, Switzerland

E. CURRICULUM VITAE

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Professional career

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Education

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10/2000 – 04/2005	Study of **Biology**, University of Regensburg, Germany
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F. ABSTRACT

Attention deficit/hyperactivity disorder (ADHD), one of the most frequent childhood-onset, chronic and lifelong neurodevelopmental diseases, affects 5 - 10% of school – aged children and adolescents, and 4% of adults. The classified basic symptoms are - according to the diagnostic system DSM-VI - inattentiveness, impulsivity and hyperactivity. Also daily life of patients is impaired by learning problems, relationship crises, conflicts with authority and unemployment, but also comorbidities like sleep - and eating problems, mood - and anxiety disorders, depression and substance abuse disorders are frequently observed. Although several twin and family studies have suggested heritability of ADHD, the likely involvement of multiple genes and environmental factors has hampered the elucidation of its etiology and pathogenesis. Due to the successful medication of ADHD with dopaminergic drugs like methylphenidate, up to now, the search for candidate genes has mainly focused on the dopaminergic and - because of strong interactions - the serotonergic system, including the already analyzed candidate genes *DAT1*, *DRD4* and *5*, *DBH* or *5-HTTLPR*.

Recently, DNA copy number changes have been implicated in the development of a number of neurodevelopmental diseases and the analysis of chromosomal gains and losses by Array Comparative Genomic Hybridization (Array CGH) has turned out a successful strategy to identify disease associated genes. Here we present the first systematic screen for chromosomal imbalances in ADHD using sub-megabase resolution Array CGH.

To detect micro-deletions and -duplications which may play a role in the pathogenesis of ADHD, we carried out a genome-wide screen for copy number variations (CNVs) in a cohort of 99 children and adolescents with severe ADHD. Using high-resolution aCGH, a total of 17 potentially syndrome-associated CNVs were identified. The aberrations comprise four deletions and 13 duplications with approximate sizes ranging from 110 kb to 3 Mb. Two CNVs occurred *de novo* and nine were inherited from a parent with ADHD, whereas five are transmitted by an unaffected parent. Candidates include genes expressing acetylcholine-metabolising butyrylcholinesterase (*BCHE*), contained in a *de novo* chromosome 3q26.1 deletion, and a brain-specific pleckstrin homology domain-containing protein (*PLEKHB1*), with an established function in primary sensory neurons, in two siblings carrying a 11q13.4 duplication inherited from their affected mother. Other genes potentially influencing ADHD-related psychopathology and involved in aberrations inherited from affected parents are the

genes for the mitochondrial NADH dehydrogenase 1 alpha subcomplex assembly factor 2 (*NDUFAF2*), the brain-specific phosphodiesterase 4D isoform 6 (*PDE4D6*), and the neuronal glucose transporter 3 (*SLC2A3*). The gene encoding neuropeptide Y (*NPY*) was included in a ~3 Mb duplication on chromosome 7p15.2-15.3, and investigation of additional family members showed a nominally significant association of this 7p15 duplication with increased NPY plasma concentrations (empirical FBAT, p = 0.023). Lower activation of the left ventral striatum and left posterior insula during anticipation of large rewards or losses elicited by fMRI links gene dose-dependent increases in NPY to reward and emotion processing in duplication carriers. Additionally, further candidate genes were examined via Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). This method enables the analysis of SNPs directly from human genomic DNA without the need for initial target amplification by PCR.

All these findings implicate CNVs of behavior-related genes in the pathogenesis of ADHD and are consistent with the notion that both frequent and rare variants influence the development of this common multifactorial syndrome.

The second part of this work concentrates on MLC1, a gene associated with Megalencephalic leukoencephalopathy with subcortical cysts, located on chromosome 22q13.33. To get more insight in the disease itself, a targeting vector for a conditional knockout mouse was constructed using homologous recombination.

Furthermore, MLC1 has been suggested as a risk gene for schizophrenia, especially the periodic catatonia subtype. An initially identified missense mutation was found to be extremely rare in other patient cohorts; however, a recent report again argued for an association of two intronic MLC1 SNPs with schizophrenia and bipolar disorder. A case-control study of these polymorphisms as well as SNPs in the transcriptional control region of MLC1 was conducted in 212 chronic schizophrenic patients, 56 of which suffered from periodic catatonia, 106 bipolar patients, and 284 controls. Both intronic and promoter polymorphisms were specifically and significantly associated with periodic catatonia but not schizophrenia or bipolar disorder in general. A haplotype constructed from all polymorphisms was also associated with periodic catatonia. The MLC1 variation is associated with periodic catatonia; whether it constitutes a susceptibility or a modifier gene has to be determined.

G. ZUSAMMENFASSUNG

Aufmerksamkeitsdefizit/Hyperaktivitätssyndrom (ADHS) ist eine bereits im Kindesalter beginnende, chronische und lebenslängliche psychische Krankheit, die zu 5 - 10% Kinder und Jugendliche sowie zu 4% Erwachsene betrifft. Die klassifizierten Grundsyndrome sind laut dem diagnostischen System DSM-IV Unaufmerksamkeit, Impulsivität und Hyperaktivität. Auch der Alltag der Patienten ist aufgrund von Lernschwierigkeiten, Konflikten in der Beziehung, Autoritätsproblemen und Arbeitslosigkeit beeinträchtigt. Zudem werden häufig Komorbiditäten wie Schlaf- und Essprobleme, Stimmungs- und Angsterkrankungen, Depressionen sowie Alkohol- und Drogenmissbrauch beobachtet. Obwohl Zwillings- und Familienstudien auf die Vererbbarkeit von ADHS hinweisen, erschweren mehrere Gene und Umweltfaktoren die Aufklärung der Ätiologie und Pathogenese. Aufgrund der erfolgreichen Behandlung von ADHS mit dopaminergen Medikamenten wie Methylphenidat liegt der Fokus bei der Suche nach neuen Kandidatengenen hauptsächlich beim dopaminergen und, aufgrund der starken Interaktionen, beim serotonergen System, einschließlich der bereits analysierten Gene *DAT1*, *DRD4* und *5*, *DBH* oder *5-HTTLPR*.

Copy Number Changes sind in die Entstehung einer Vielzahl von Krankheiten mit einer Störung der Entwicklung des zentralen Nervensystems impliziert. Die Analyse von chromosomalen Deletionen oder Duplikationen durch *Array Comparative Genomic Hybridization* (Array CGH) hat sich als eine erfolgreiche Strategie herausgestellt, um krankheitsassoziierte Gene zu identifizieren. Diese Arbeit ist der erste systematische Screen für den Nachweis von chromosomalem Ungleichgewicht bei ADHS mit Hilfe von Array CGH.

Um Mikrodeletionen und -duplikationen zu entdecken, die in der Pathogenese von ADHS eine Rolle spielen könnten, haben wir einen genomweiten Screen für *Copy Number Variations* (CNVs) an einer Gruppe mit 99 an ADHS erkrankten Kindern und Jugendlichen durchgeführt. Durch Hochauflösungs-Array CGH wurden insgesamt 17 potentielle Syndrom assoziierte CNVs identifiziert. Diese Aberrationen beinhalten vier Deletionen und 13 Duplikationen mit einer Größe von etwa 100 kb bis zu 3 Mb. Zwei CNVs sind *de novo*, neun wurden von einem ebenfalls an ADHS erkrankten Elternteil vererbt und fünf von einem nicht betroffenen Elter übertragen. Kandidatengene sind u. a. die Acetylcholin metabolisierende Butyrylcholonesterase (*BCHE*), welche *de novo* in einer Deletion auf Chromosom 3q26.1 auftritt, und das Gehirn spezifische *Pleckstrin homology domain-containing* Protein (*PLEKHB1*) mit einer bekannten Funktion in den primären sensorische Neuronen, welches

von der an ADHS erkrankten Mutter an zwei Geschwister in einer 11q13.4 Duplikation vererbt wurde. Weitere Gene, die möglicherweise die Psychopathologie von ADHS beeinflussen und von einem betroffenen Elternteil in einer Aberration vererbt wurden, sind die Gene für die mitrochondriale NADH Dehydrogenase 1 *Alpha Subcomplex Assembly Factor* 2 (*NDUFAF2*), die Gehirn spezifische Phosphodiesterase 4D Isoform 6 (*PDE4D6*) und der neuronale Glukosetransporter 2 (*SLC2A3*). Das Gen, welches Neuropeptid Y (*NPY*) codiert, wurde in einer ~3 Mb großen Duplikation auf Chromosom 7p15.2-15.3 gefunden. Eine Untersuchung zusätzlicher Familienmitglieder zeigte eine nominell signifikante Assoziation dieser 7q15 Duplikation mit einer gesteigerten NPY Plasmakonzentration (empirischer FBAT, p = 0.023). Zusätzlich wurden weitere Kandidatengene durch Matrix-unterstützte Laser-Desorption/Ionisation-Massenspektrometrie (MALDI-TOF MS) untersucht. Diese Methode ermöglicht die Analyse von SNPs direkt von der humanen genomischen DNS ohne vorherige Target Amplifikation durch PCR.

All diese Ergebnisse schließen CNVs von verhaltensverbundenen Genen in die Pathogenese von ADHS mit ein und stimmen außerdem mit der These überein, dass sowohl häufige wie auch seltene Variationen die Entwicklung dieses häufig auftretenden, multifaktoriellen Syndroms beeinflussen.

Der zweite Teil dieser Arbeit beschäftigt sich mit dem Gen *MLC1*, das mit „Megalenzephaler Leukoenzephalopathie mit subkortikalen Cysten“ assoziiert und auf Chromosom 22q13.33 lokalisiert ist. Um mehr Einblick in diese Krankheit zu erlangen wurde ein spezieller Zielvektor für eine konditionale Knockout Maus durch homologe Rekombination erstellt. Zusätzlich wird angenommen, dass MLC1 ein Risikogen für Schizophrenie sein könnte, v. a. für den periodisch katatonischen Subtyp. Eine früher identifizierte *Missense* Mutation wurde extrem selten in anderen Patientenkohorten gefunden. Ein kürzlich veröffentlichter Bericht hingegen plädiert für eine Assoziation von zwei intronischen MLC1 SNPs mit Schizophrenie und manisch-depressiver Erkrankung. Eine Fall-Kontroll-Studie über diese Polymorphismen sowie über die SNPs der transkriptionalen Kontroll-Region von MLC1 wurde an 212 chronischen Schizophrenie-Patienten durchgeführt, von denen 56 an periodischer Katatonie leiden und 106 manisch-depressiv waren, sowie an 284 Kontrollen. Sowohl die intronischen Polymorphismen als auch die der Promotorregion waren spezifisch und signifikant mit periodischer Katatonie assoziiert, allerdings nicht mit Schizophrenie oder manisch-depressiver Erkrankung im Allgemeinen. Ein Haplotyp aus allen Polymorphismen konnte

ebenfalls mit periodischer Katatonie assoziiert werden. Diese MLC1 Variation scheint somit mit periodischer Katatonie verknüpft zu sein. Ob es ein Suszeptibilitäts- oder ein Modifikatorgen darstellt, muss allerdings noch genauer bestimmt werden.

I. INTRODUCTION

1. ATTENTION-DEFICIT/HYPERACTIVITY DISORDER (ADHD)

1.1. CLINICAL PHENOTYPE

Attention-deficit/hyperactivity disorder (ADHD) belongs to the most common neurobehavioral disorders with a childhood onset. It is characterized by the behavioral symptoms hyperactivity, inattention and impulsivity (DSM-IV). By the recent diagnostic system DSM-IV affected children are classified in three subtypes, the predominantly inattentive or hyperactive-impulsive type as well as the combined type.

Inattention is a broad concept and involves much more than simply not paying attention for a long period of time. The affected person also has persisting difficulties in the organization and planning of tasks and following instructions, as well as working memory problems. Not only one but the interaction of diverse, related cognitive functions falls in the category of “inattention”. Impulsivity is characterized by abrupt and imprudent actions. These are mostly precipitous and without assessment of possible risks. Consequently, the number of injuries is higher-than-average in children with ADHD (Diagnosis 2000). Motor activity often appears uncoordinated and handwriting is often not legible. Hyperactivity delineates an excess of uncoordinated motor activity. Affected children often fidget with hands or feet, squirm in their seat and/or have difficulty playing or engaging in leisure activities quietly. This motor activity is one of the most conspicuous abnormalities of ADHD. In adulthood these symptoms are often confined to a subjective feeling of agitation.

In children, as well as in adults, there is a high degree of co-morbidity. Children suffer frequently from aggressive or antisocial behavior. Up to 20% of children with ADHD have a conduct disorder, a pattern of repetitive behavior with symptoms of verbal and physical aggression, destructive behavior or vandalism. Another 30 - 45% of the patients also have oppositional defiant disorder (ODD) (Arcos-Burgos, Castellanos et al. 2004) which is described as an ongoing, hostile, and defiant behavior towards authorities. Adolescents and

adults exhibit mainly anxiety and depressive disorders; substance abuse and alcoholism come often along with antisocial personality disorder (Retz, Thome et al. 2002).

1.2. TREATMENT

In spite of the heterogeneous character of ADHD and still not clarified pathomechanisms, psychostimulants like amphetamine or amoxetine have been applied for many years. Amphetamines exert their behavioral effects by increasing the level of several key neurotransmitters including serotonin, norepinephrine (NE) and dopamine (DA) in the brain. Methylphenidate (MPH, known as ”Ritalin®”) i. e. increases the level of dopamine by partially blocking the dopamine receptor. This inhibition blocks the reuptake of dopamine into the presynaptic neuron, thereby increasing the amount of dopamine in the synaptic cleft. Amphetamines also bind to the NE transporter (NET) and to the serotonin transporter (SERT), but to a smaller amount than to the DA transporter (DAT).

Amoxetine is characterized by a different mode of action, as it is a selective NE reuptake inhibitor increasing the concentration of NE in the prefrontal cortex, but not in the striatum. Originally, amoxetine was used as an antidepressant but soon its effectiveness in the treatment of ADHD emerged in controlled trials.

In summary, pharmacological effects depend on the relative concentration of DAT and NET in the diverse brain regions. Indeed, the precise modes of action are still not clarified. So it is possible that other neurotransmitter systems are equally involved by the impact of these drugs.

1.3. NEUROBIOLOGICAL FUNDAMENTALS

A complex multigenetic etiology with a contribution of genes (see chap. 2) influencing different neuronal functions and intermediate phenotypes are thought to form the genetic basis of ADHD. Several brain areas, neurocircuits, and transmitter systems have been implicated. Pharmacological and functional neuroimaging studies in human and animal

models have consistently linked the prefrontal/anterior cingulated cortex and various connected association cortices to the modulation of attention, cognition, and motor response-related processes as well as to those influencing executive and motor circuits or inhibit behavior and decision making. A schematic image of the brain is shown in Fig. 1.

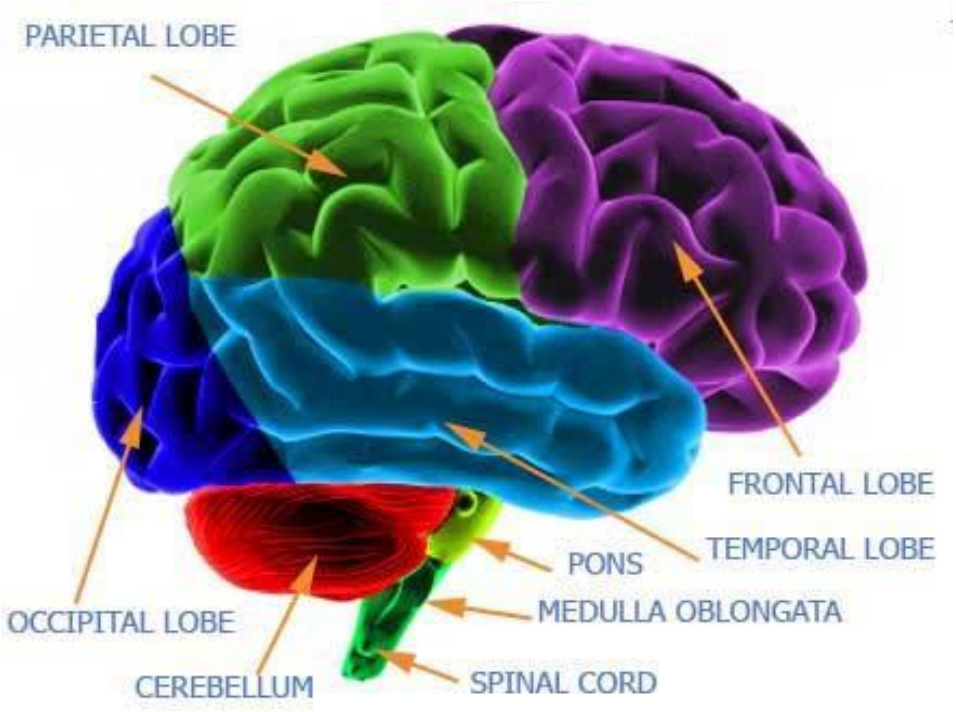


Fig. 1: Schematic picture of the human brain.

Brain structures that most frequently have been implicated in ADHD are, amongst others, the prefrontal cortex or the cerebellum. ADHD research showed that the brain regions with the most significant decrease in brain activity were the superior prefrontal cortex and the premotor cortex. (https://docyoung.com/adhd-science)

Prefrontal cortex

Of particular interest is the prefrontal cortex (PFC), especially the dorsolateral part. The PFC uses representational knowledge, i. e. working memory, and attention as well as movement. It is divided into three functional subgroups: the prefrontal (orbital, dorsolateral and mesial), the premotor and motor regions (Fuster 1989). Patients with lesions in the PFC are easily distracted, have poor concentration and organization and can be impulsive, because these lesions impair the ability to sustain attention and reduce the ability to regulate sensory input (Arnsten 2006). Therefore the PFC has particular relevance to ADHD; in support of this, imaging studies indicated that ADHD patients often have smaller PFC volumes, mainly on the right side (Casey, Castellanos et al. 1997; Sowell, Thompson et al. 2003). Furthermore, nine independent MRI studies in children with ADHD detected a reduced prefrontal volume either in the right or the left hemisphere (Seidman, Valera et al. 2005).

Dorsal anterior cingulated cortex

The dorsal anterior cingulated cortex (dACC) is located above the frontal lobe and exhibits strong associations to the dorsolateral PFC, basal forebrain and the limbic structures. It appears to play a role in complex rational cognitive processes, such as reward anticipation, decision-making, modulation of emotional response (empathy and emotion), motivation, problem solving and error detection (Bush, Vogt et al. 2002; Schneider, Retz et al. 2006). There are some structural studies of dACC in ADHD. One study suggested a reduced volume of the right posterior cingulate in children with ADHD (Overmeyer and Taylor 2000). Several functional studies consistently argue for a hypoactivity of the dACC, especially in adult patients (Schneider, Retz et al. 2006).

Striatum

The basal ganglia (putamen, pallidum, caudate nucleus) are essential for executive functions (Dubois, Defontaines et al. 1995; Casey, Castellanos et al. 1997). On the one hand, the striatum is an origin of dopaminergic synapses (Dougherty, Bonab et al. 1999) and dopamine itself plays an important role in the regulation of striatal function. It is known that excitatory drugs such as MPH increase extracellular dopamine in the striatum (Volkow, Fowler et al. 2002). On the other hand, an injury of the striatum seems to be associated with ADHD. Lou

has shown in 1996 for the first time that ADHD symptoms are associated with striatal damage (Lou 1996). Experimental lesions of the striatum of mice lead to hyperactivity and memory decline (Alexander, DeLong et al. 1986). If an uni - or bilateral volume reduction of the nucleus caudatus could be one of the determining factors for the development of ADHD is still under review (Seidman, Valera et al. 2005; Schneider, Retz et al. 2006). Until now no evidence for basal ganglia volume reduction in adult ADHD has been reported. A possible explanation is that differences between controls and ADHD disappear with increasing age during brain development (Castellanos, Lee et al. 2002).

Cerebellum

Although the cerebellum was originally thought to be primarily involved in motor control, both research and clinical findings show cerebellar involvement in many cognitive and affective processes, which leads to an increased interest in ADHD research. Middleton & Strick (Middleton and Strick 2001) have demonstrated cerebellar-cortical connections that provide an anatomical substrate for a cerebellar-prefrontal circuit in the pathophysiology of ADHD. Additionally, several groups studied the cerebellum in ADHD children. I. e. Castellanos (Castellanos, Lee et al. 2002) compared regional brain volumes in male and female ADHD patients and healthy controls. Mainly, the cerebellar volume was significantly smaller in children with ADHD. Furthermore, the volumes were significantly and negatively correlated with ratings of attentional problems. More recently, Durston (Durston, Hulshoff Pol et al. 2004) found smaller overall right cerebellar volumes in a group of 30 ADHD children.

Corpus callosum

The corpus callosum (CC), composed of mostly myelinated axons, connects homotypic regions of the two cerebral hemispheres. Injury of callosal structures can lead to problems in holding sustained attention with associated deficits in learning and memory (Schneider, Retz et al. 2006). Abnormalities of the CC have been reported in a number of morphometric studies of children with ADHD (Seidman, Valera et al. 2005). Because different measures were used, the results cannot be easily compared. Nevertheless, fairly consistent evidence indicates that abnormalities in ADHD children are found particularly in the posterior regions linked to temporal and parietal cortices in the splenium (Seidman, Valera et al. 2005).

2. CANDIDATE GENES

Family, adoption, and twin studies revealed that ADHD is a highly heritable disorder (h^2 = 70 - 80%) (Thapar, Holmes et al. 1999) with a multifactorial pattern of inheritance most likely due to multiple genes of small size effect. Twin studies support this hypothesis by demonstrating a high concordance rate of 70% in monozygotic and 30% in dizygotic twins. Furthermore, the worldwide prevalence is estimated to affect 5 - 10% of children and 4% of adults (Biederman 2005). Genome-wide linkage analyses identified several susceptibility loci on different chromosomes, like 4q13.2, 5q33.3, 11q22 or 17p11 (Arcos-Burgos, Castellanos et al. 2004).

Due to the multiple character of ADHD it is also assumed that gene-gene as well as gene-environment interactions have a role in this disorder. Environmental risk factors may include perinatal and postnatal complications, low birth weight, maltreatment during childhood, alcohol or cigarette consumption of the mother may exert influence on the development and etiopathology of the disease (Banerjee, Middleton et al. 2007; Thapar, Langley et al. 2007).

There are many genes, which were analyzed with regard to ADHD, but only those showing an association to the disorder are mentioned in the following chapters.

2.1. DOPAMINERGIC SYSTEM

Pharmacological and neuroimaging studies are consistently suggestive of the notion that dopamine (DA) is one of the most important neurotransmitters in the etiology of ADHD. DA has many functions in the central nervous system (CNS), including important roles in behavior and cognition, motor activity, motivation and reward, sleep, mood, attention and learning. Dopaminergic neurons are present in the ventral tegmental area (VTA) of the midbrain. The dopaminergic neurons exist mainly in the substantia nigra and the ventral tegmental area and project axons to large areas of the brain through the mesocortical, mesolimbic, nigrostratial and tuberinfundibular pathway. Also in the vegetative peripheral nervous system DA regulates the blood circulation of the viscera and influences the extrapyramidal motor function.

DA is biosynthesized mainly by nervous tissue and the medulla of the adrenal glands. Its biological precursor is the amino acid L-tyrosine, which is hydroxylated to L-dihydroxyphenylalanine (L-DOPA) via the enzyme tyrosine-3-monooxygenase, also known as tyrosine hydroylase. Afterwards L-DOPA is decarboxylated to DA by the aromatic L-amino acid decarboxylase, which is often referred to as dopa decarboxylase. The whole reaction is illustrated in Fig. 2.

Whereas DA fails to cross the blood brain barrier and hence is ineffective as therapy for patients who have DA deficiencies (i.e. Parkinson's disease), its amino acid precursor L-DOPA is transported across this barrier and provides a substrate for DA synthesis (Ahlskog 2001). In neurons, DA is packaged after synthesis into vesicles, which are then released by Ca^{2+}-induced exocytosis into the synaptic cleft in response to a presynaptic action potential. There it interacts with five different DA receptors *DRD1-5* (see chap. 2.2.) (Fig. 3).

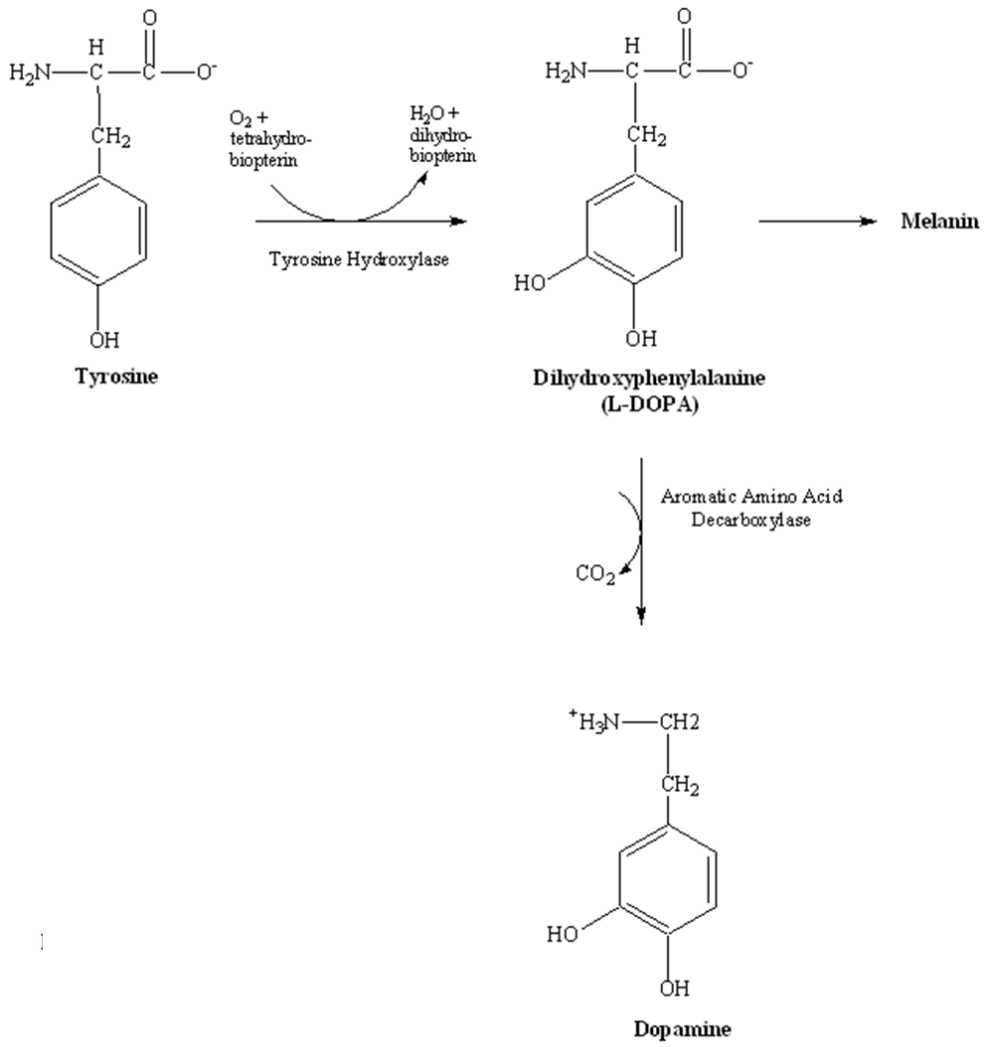


Fig. 2: The dopamine synthesis pathway.

Tyrosine is converted to L-dopa by the enzyme tyrosine hydroxylase (TH), a reaction that also requires the TH cofactor 6-tetrahydrobiopterin (BH_4). Guanosine triphosphate cyclohydrolase I (GTPCHI) is the rate-limiting enzyme involved in BH_4 synthesis. Conversion of L-dopa to dopamine requires the enzyme aromatic acid decarboxylase. (www.rpi.edu/~bellos/new_page_2.htm)

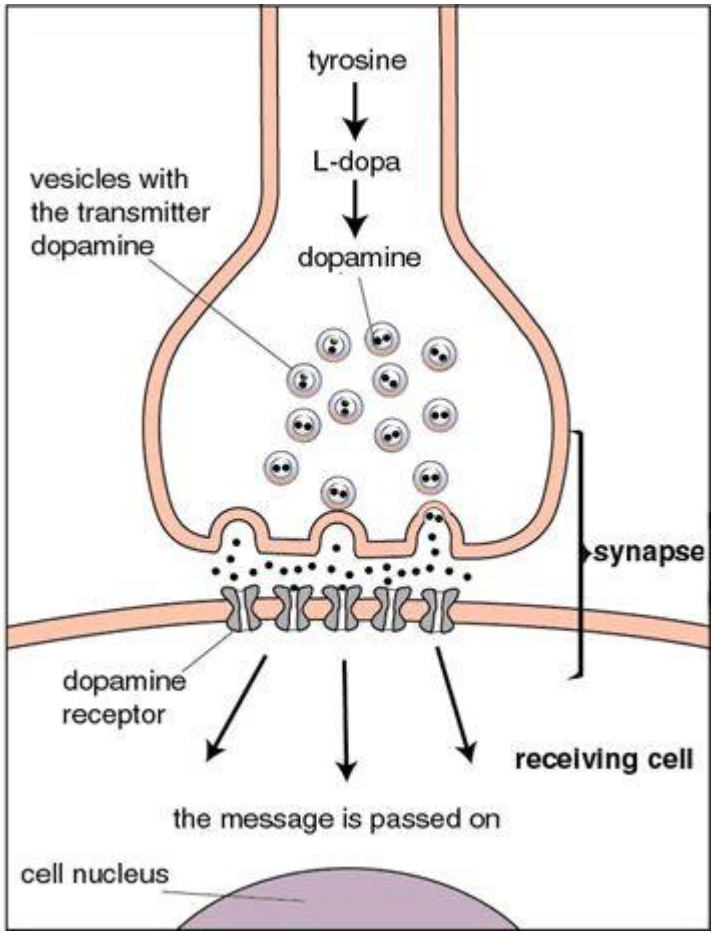


Fig. 3: Dopaminergic synapsis.

A message from one nerve cell to another is transmitted with the help of different chemical transmitters. This occurs at specific points of contract, synapses, between the nerve cells. The chemical transmitter dopamine is formed from the precursors tyrosine and L-dopa and is stored in vesicles in the nerve endings. When a nerve impulse causes the vesicle to empty, dopamine receptors in the membrane of the receiving cell are influenced such that the message is carried further into thecell. (http://nobelprize.org/nobel_prizes/medicine/laureates/2000/press.html)

DA is inactivated either by reuptake via enzymatic breakdown by catechol-O-methyl transferase (COMT) or monoamine oxidase B (MAO-B) to homovanillic acid (HVA) (Fig. 4).

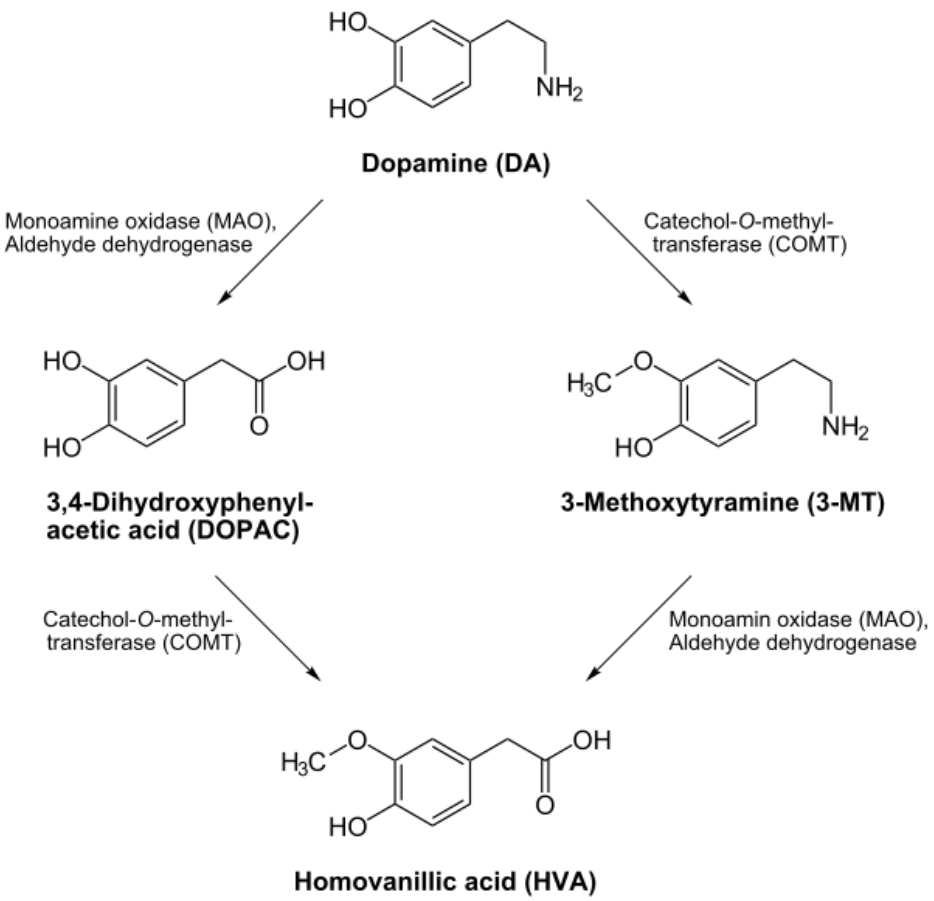


Fig. 4: Dopamine degration.

Dopamine is inactivated by reuptake of the dopamine transporter, then enzymatic breakdown by catechol-O-methytransferase (COMT) and monoamine oxidase (MAO). Dopamine that is not broken down by enzymes is repackaged into vesicles for reuse.
(http://en.wikipedia.org/wiki/Image:Dopamine_degradation.svg)

2.2. DOPAMINERGIC GENES

Dopamine transporter 1

Pharmacological agents, notably MPH, appear to exert therapeutic effects in ADHD by increasing the functional availability of extracellular DA through inhibition of the DA transporter (*DAT1/SLC6A3*) (Thapar, Langley et al. 2007). The membrane-spanning gene, encoding 620 amino acids (aa), comprises 15 exons that span more than 52 kb of genomic DNA on the human chromosome 5p15.33. *DAT1* limits the duration of synaptic activity and diffusion by reuptaking dopamine into neurons (Madras, Miller et al. 2005). It is expressed selectively in all dopaminergic neurons in the substantia nigra and the ventral tegmental area.

Most of the published association studies focus on a 40bp variable number tandem repeat (VNTR) in the 3´-UTR (untranslated region) of *SLC6A3*, ranging from 1 to 13 repeats. The VNTR may change *DAT1* function, since it has been suggested to regulate gene expression (Yang, Chan et al. 2007). In a recent study a positive association with the 10-repeat allele and ADHD has been found (Yang, Chan et al. 2007). In line with that, linkage studies support the *DAT1* locus in ADHD (Friedel, Saar et al. 2007). However, results published hitherto are equivocal and vary from no association (Brookes, Mill et al. 2006), a trend for association (Maher, Marazita et al. 2002; Curran, Purcell et al. 2005) to a modest but significant association (Faraone, Perlis et al. 2005).

Dopamine receptor 1

Once DA has been released, it binds to pre- and postsynaptic dopamine receptors (*DRD1-5*) (Missale, Nash et al. 1998). As they belong to the class of metabotropic, G-protein-coupled receptors, they modulate the activity of ion channels by second messenger cascades. D1-like family receptors (*DRD1* and *DRD5*) are coupled to the G-protein G_S which subsequently activates the adenylyl cyclase. *DRD2*, *DRD3* and *DRD4* belong to the D2-like family of dopamine receptors which are coupled to the G_I protein, thereby inhibiting adenylyl cyclase and activating K^+-channels (Missale, Nash et al. 1998).

DRD1, which is located at chromosome 5q35.2, is the most abundant dopamine receptor in the CNS. It regulates neural growth and development and mediates behavioral responses. Northern blot analyses and in-situ-hybridization demonstrated high expression in the striatum, nucleus accumbens, and olfactory tubercle. No detectable product was amplified from substantia nigra, kidney, heart or liver (Dearry, Gingrich et al. 1990). In a recently published family-based ADHD study, strong evidence for linkage of a *DRD1* haplotype with inattentive, but not with impulsive/hyperactive symptoms was found (Misener, Luca et al. 2004). This haplotype contains four markers which span the whole gene. Bobb and coworkers support this result: Although they could not replicate this association using a family-based approach, they found a significantly higher frequency of these risk alleles in the ADHD cases as compared to controls (Bobb, Addington et al. 2005).

Some animal models of ADHD refer to *DRD1*. The SHR rats (spontaneous hyperactive rat) are generally considered to be a suitable genetic model for ADHD since they display hyperactivity, impulsivity, poor stability of performance and poorly sustained attention (Russell 2002). Postsynaptic *D1* receptors were found to be up-regulated in the brains of five and 15-week-old SHR. The fact that both *D1* and *D2* receptors (Kirouac and Ganguly 1993) as well as *DAT* (Watanabe, Fujita et al. 1997) are increased in the striatum of prehypertensive SHR can also be taken as evidence that changes in the DA function might be involved in the pathogenesis of both the hypertension and behavioral characteristics of the SHR (Russell 2002).

Dopamine receptor 4

The dopamine receptor gene *DRD4* (chromosome 11p15.5), which spans 3 kb and comprises four exons, is located primarilly in the hippocampus (HC), the frontal lobes and the amygdala and shows a strong homology to *DRD2* and *3*. Both NE and DA are effective agonists of *DRD4*.

The distribution of DRD4 mRNA in the brain, mainly in the fronto-subcortical network, argues for a role in cognitive and emotional functions; functions implicated in the pathophysiology of ADHD (Faraone, Doyle et al. 2001). Also various mutations in *DRD4* were associated with behavior phenotypes and ADHD. Population and family-based association studies focused on a VNTR polymorphism in which alleles differ by the number of repeats of a 48 bp sequence in exon 3. Several studies found an association of the 7-repeat allele with ADHD.

(Faraone, Doyle et al. 2001; Roman, Schmitz et al. 2001; Ding, Chi et al. 2002; Grady, Chi et al. 2003; Li, Sham et al. 2006). However, it cannot be assumed if the presence of the *DRD4* 7R allele is necessary or sufficient to cause ADHD.

Dopamine receptor 5

The approximately (approx.) 2 kb large D5 receptor gene (*DRD5*) maps to chromosome 4q16.1. Expressed predominantly in the limbic system, it stimulates the G-protein coupled to adenylyl cyclase as *DRD1* does. Functionally and structurally it is similar to *DRD1*, too (Grandy, Zhang et al. 1991; Tiberi, Jarvie et al. 1991). However, *DRD5* has a 10-fold higher affinity for DA than the *DRD1* subtype and is mainly found in neurons in the HC, the amygdala, the nucleus mammilaris and the nucleus pretectalis anterior. Daly and colleagues (Daly, Hawi et al. 1999) reported a significant association between ADHD and the common 148 bp allele of a microsatellite marker located 18.5 kb 5´ of the transcription start codon. The effect was strongest in cases with negative family history. A more recent family-based study confirmed this result (Lowe, Kirley et al. 2004), but was limited to the inattentive and combined subtype of ADHD. However, there is still no evidence that this dinucleotide repeat is functional. Analyses of other markers in this gene yielded negative results (Thapar, Langley et al. 2007).

Dopamine β-hydroxylase

The human dopamine β-hydroxylase gene (DBH) (approx. 23 kb) is composed of 12 exons and maps to chromosome 9q34.2. *DBH,* which is mainly localized in the chromaffin granules of the adrenal medulla and the synaptic vesicles of noradrenergic neurons (Kim, Zabetian et al. 2002) is the primary enzyme responsible for the conversion of DA to NE.

Because alterations in the DA/NE level can result in hyperactivity, *DBH be*comes more and more interesting. Patients with ADHD showed decreased activities of *DBH* in serum and urine. Also, low DBH levels correlate indirectly with the seriousness of ADHD in children (Kopeckova, Paclt et al. 2006). Comings and colleagues reported an association between a polymorphism in intron 5 and ADHD symptom scores (Comings et al., 1996). This result was confirmed, inter alia by Daly in a family-based Irish sample (Daly, Hawi et al. 1999).

2.3. NORADRENERGIC SYSTEM

Noradrenergic drugs like despiramin and α_2-adrenoreceptor agonists are often used to relieve ADHD symptoms (Solanto 1998). Adrenaline, also known as epinephrine, is a hormone and neurotransmitter, which belongs to the family of catecholamines. Adrenaline was isolated and identified in 1895 by the Polish physiologist Napoleon Cybulski. As a “fight or flight” hormone, adrenaline plays a central role in the short-term stress reaction and mediates the rash appropriation of energy resources in emergency situations through adrenergic receptors of the adrenal glands. The neurons of this biogenic amid were only found in CNS, mainly in the medulla oblongata (Fig. 5).

Adrenaline is synthesized via methylation of the primary distal amine NE by the phenylamine N-methyltransferase (PNMT) in the cytosol of adrenal medullary cells (Fig. 6). After its release adrenaline is degraded via the enzymes MAO and COMT to metanephrine, HVA acid and methoxy-4-hydroxyphenylethylenglycol (MOPEG).

Norepinephrine is a key neurotransmitter in both central and peripheral nervous systems where it is released from noradrenergic neurons. The catecholamine regulates many essential functions, including attention, memory, emotion, and autonomic functions (Kim, Hahn et al. 2006). Also NE underlies the flight-or-fight response, it increases the heart rate, triggers the release of glucose from energy stores, and increases the blood flow to skeletal muscles via binding to adrenergic receptors. NE is synthesized from DA by *DBH* (see Fig. 6) and released from the adrenal medulla into the blood as a hormone. Before the final β-oxidation it is transported into synaptic vesicles. Its inactivation occurs either enzymaticly through the metabolites MAO and COMT or by a cellular reuptake into the presynapic cell.

Also, both catecholamines have no evident psychoactive effect in the brain. They are consistently linked to ADHD, mainly due to its G-protein coupled adrenoreceptors, which are expressed in different cell types.

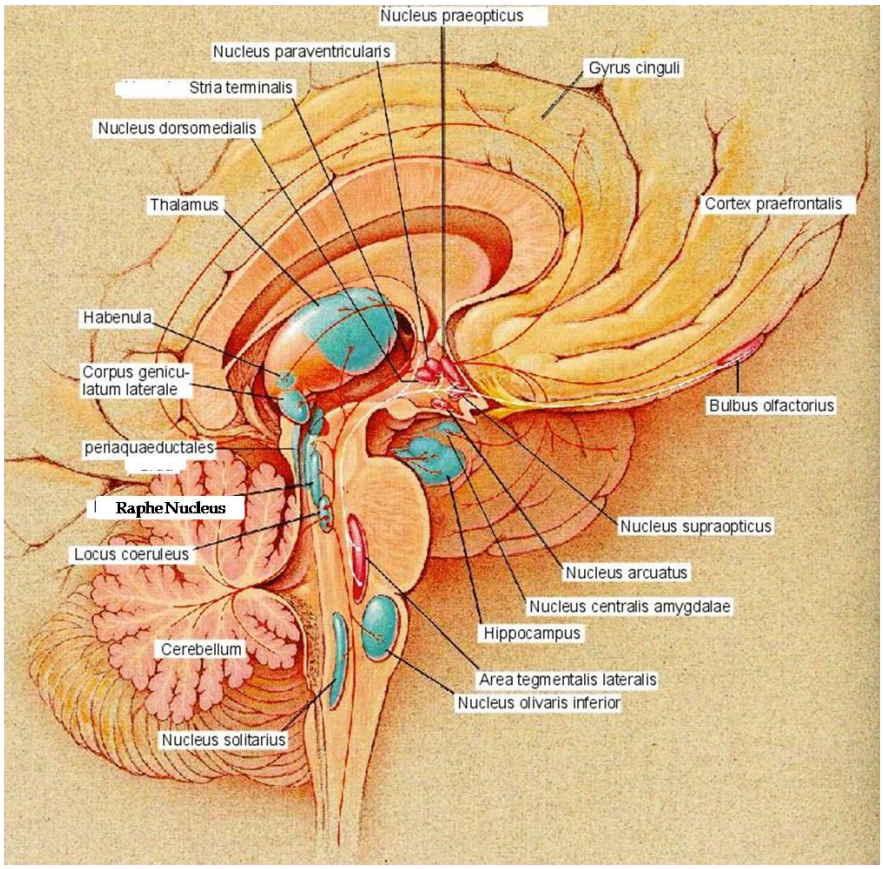


Fig. 5: Noradrenergic system.

The cell bodies of most (nor-) adrenergic neurons lay in the locus coeruleus. Approximately 3000 neurons of the locus coeruleus are connected by axons which pervade all parts of the brain (red lines) with billions of other neurons. Therefore (nor-) adrenergic neurons take simultaneous parts in different brain functions and play an integral part. Besides the locus coeruleus the area tegmentalis also harbors (nor-) adrenergic nerve tracts (white lines).
(S.H. Snyder, Chemie der Psyche, Spektrum Verlag Heidelberg (1988)

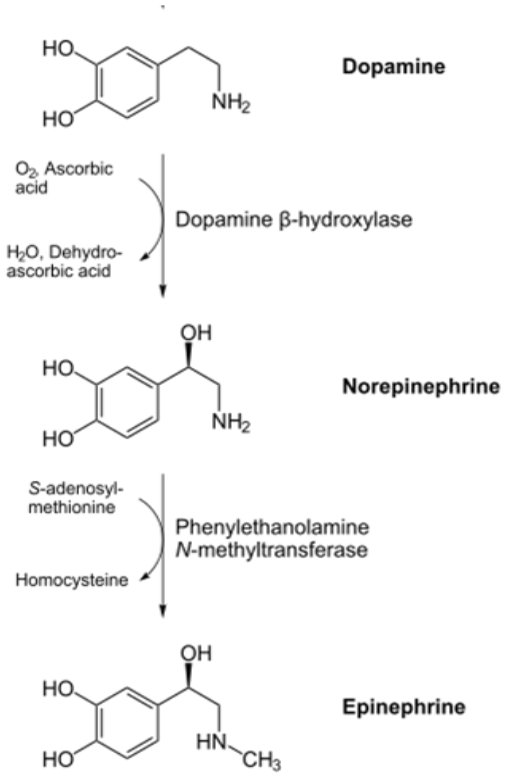


Fig. 6: (Nor-) Epinephrine biosynthesis.

Epinephrine is synthesized from norepinephrine via methylation of the primary distal amine of norepinephrine by phenylethanolamine N-methyltransferase (PNMT) in the cytosol of adrenergic neurons and cells of the adrenal medulla. PNMT uses S-adenosylmethionine as a cofactor to donate the methyl group to norepinephrine, creating epinephrine. (http://www.worldofmolecules.com/drugs/adrenaline.htm)

Norepinephrine transporter

The NE transporter (*NET*, *SLC6A2*) is a regulator of the NE homeostasis and primarily responsible for the reuptake of NE into presynaptic nerve terminals (Kim, Hahn et al. 2006). The human transporter, which spans approx. 45 kb and maps to chromosome 16q12.2, is mainly expressed in the brainstem and adrenal glands and is sensitive against *NET* inhibitors. These seem to be efficient in ADHD treatment (Biederman and Spencer 2000). SNP and haplotype analyses in families with affected adults showed no association to ADHD (Barr, Kroft et al. 2002; McEvoy, Hawi et al. 2002; Faraone, Perlis et al. 2005). Otherwise, Kim and colleagues observed a significant association between the 3081 (A/T) polymorphism and ADHD, suggesting that anomalous transcription factor-based repression of *SLC6A2* may increase the risk for the development of ADHD and other neuropsychiatric disorders (Kim, Hahn et al. 2006).

Adrenergic receptor 2A

The G-protein coupled adrenergic receptors (*ADR*) specifically bind the endogenous catecholamines adrenaline and NE. Due to their pharmacological and molecularbiological nature they are divided into two classes: α_1- and α_2- adrenergic receptors are found in pre- and postsynaptic neurons of the vegetative and central nervous system, where they inhibit the transmitter release. β-adrenergic receptors, which are found in heart, smooth muscle and fat tissue, are responsible for the regulation of the heart rate and smooth muscle relaxation.

The postsynaptic α_2- adrenergic receptors (*ADRA2*) A, B and C are known to have a critical role in regulating neurotransmitter release from adrenergic neurons as well as from sympathetic nerves. To find out more about their function the neurotransmitter release in mice in which the genes encoding the α_2- adrenergic receptor subtype were disrupted, was analyzed (Hein, Altman et al. 1999). Both *ADRA1A* and *ADRA2C* are determining factors for the presynaptic neurotransmitter release of sympathetic and central noradrenergic neurons.

ADRA2A, a 3650 bp gene, which is located at chromosome 10q25.2, has no introns in translated or in untranslated regions. The role of the noradrenergic system in ADHD is still underlined. Researches in nonhuman primates demonstrated that NE can enhance the cognitive functioning of the PFC through actions at α_2-adrenergic receptors postjunctional to

noradrenergic terminals (Arnsten, Steere et al. 1996). Also in family-based and case-control studies a strong association of the MspI polymorphism (1291 C → G SNP) in the promoter region of *ADRA2A* was found with the inattentive and combined subtype of ADHD (Halperin, Newcorn et al. 1997; Comings, Gade-Andavolu et al. 1999). Schmitz (Schmitz, Denardin et al. 2006) supported this thesis by demonstrating that homozygous subjects for the G allele have an elevated risk for the inattentive subtype. Additional evidence for an involvement of the noradrenergic system is that methylphenidate treatment improves the inattentive symptoms in children and adolescents with ADHD (Polanczyk, Zeni et al. 2007; da Silva, Pianca et al. 2008).

2.4. SEROTONERGIC SYSTEM

Because of the strong interaction between the dopaminergic and serotonergic neurosystem as well as the therapeutic effects of serotonin reuptake inhibitors (SSRI), the serotonergic system came to the focus of the researchers.

The neurotransmitter serotonin (5-HT), detected in 1948 by Irving Page, plays an important role in the modulation of anger, aggression, sexuality, psychological processes and metabolism. During stress 5-HT causes several changes in different brain areas (Fig. 7): While the 5-HT level is increased in the cerebral cortex, its release is diminished in the brainstem and diencephalon. Although it is not clarified if 5-HT deficiency in the brain causes depression, bipolar or anxiety disorders, an enhancement of the 5-HT level leads to an abatement of the symptoms. MAO-I and SSRIs enhance the 5-HT concentration in the brain, which turns them to pharmacological useful antidepressants.

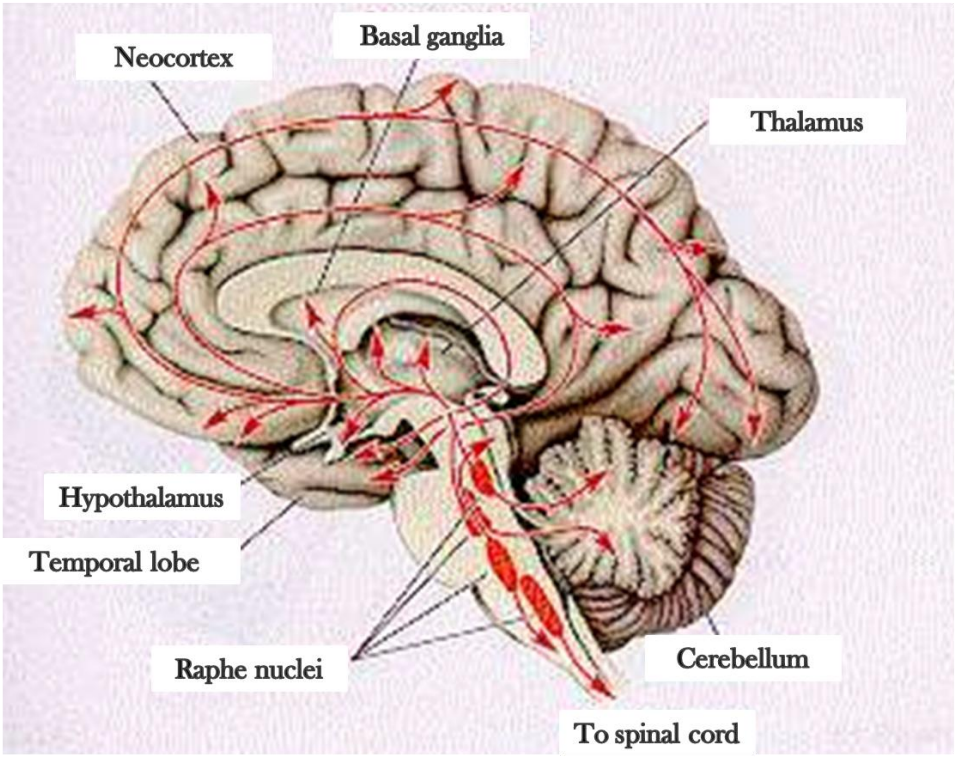


Fig. 7: The serotonergic system.

The serotonergic diffuse modulatory systems arise from the raphe nuclei. The raphe nuclei are clustered along the midline of the brain stem and project extensively to all levels of the CNS.
(http://aids.hallym.ac.kr/d/kns/tutor/medical/sero.html)

In the neuronal cytoplasm of liver, spleen and enterochromaffin cells of the intestinal mucosa, 5-HT is synthesized from the amino acid L-tryptophan by a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase (TPH) and 5-HTP decarboxylase (DDC). Because the indolamine cannot cross the blood-brain barrier, tryptophan and its metabolite 5-hydroxytryptophan (5-HTP), the direct precursor of 5-HT, attain the barrier by carrier mediated transport or diffusion. Unbounded 5-HT is abolished by *MAO-A* and aldehydhydrogenase to 5-hydroxyindoleacetic acid (5-HIAA), which is excreted in the urine. An overview about synthesis and degradation is shown in Fig. 8.

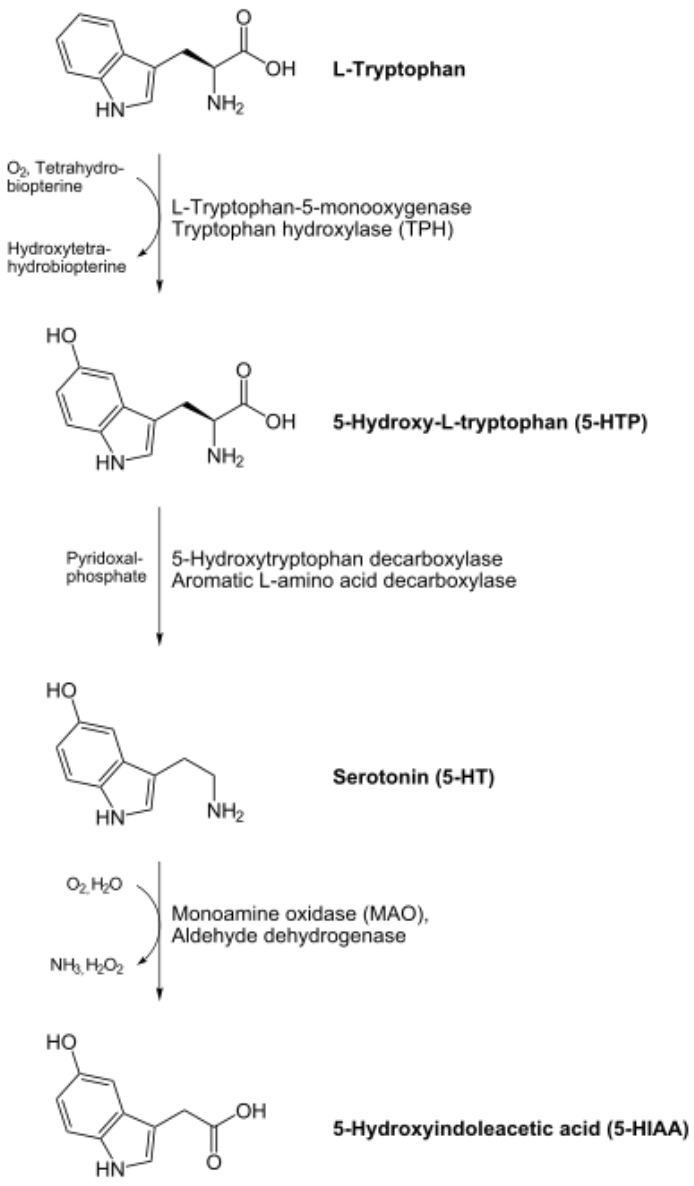


Fig. 8: Pathway for the synthesis of serotonin from tryptophan.

Serotonin is synthesized from the amino acid L-tryptophan by the tryptophan hydroxylase (TPH) and the amino acid decarboxylase (DDC). The TPH-mediated reaction is the rate-limiting step in the pathway.
(http://en.wikipedia.org/wiki/Serotonin)

2.5. SEROTONERGIC GENES

Serotonin transporter

The human serotonin transporter gene *SLC6A4,* also known as *SERT* or *5-HTT*, is mapped to chromosome 12p11.1 - q12 and consists of 14 exons which span about 35 kb. *SERT* seems to be one of the most analyzed genes in the psychiatric genetic with association to many disorders and diagnosis. In the brain it arranges as an integral membrane protein the reuptake of the released 5-HT from the synaptic cleft in neurons platelets and enterochromaffin cells and determines the magnitude and duration of postsynaptic receptor-mediated signaling (Lesch 1997). Furthermore *SERT* is the initial target for several antidepressant and neurotoxins like ecstasy. The association between ADHD and *SERT* exists mainly in the 44 bp insertion-/deletion polymorphism 5-HTTLPR in the 5´-flanking promoter region (Seeger, Schloss et al. 2001) which consists of 14 (short "s"-) or 16 (long "l"-form) repeats and builds the basis of many genetic association studies. The short version of this allele results in decreased transporter expression (Lesch, Bengel et al. 1996). Analysis of combined studies showed that ADHD children hold the l-allele and the L/L-genotype above-average in comparison to healthy controls (Fisher, Francks et al. 2002; Kent, Doerry et al. 2002; Retz, Thome et al. 2002).

Serotonin receptor 1B

The serotonin receptor 1B (*HTR1B*) encodes for the 5HT1B-receptor and maps to chromosome 6q13. Specific evidences for a connection to ADHD were found in mice which miss this receptor and show motor hyperactivity (Brunner, Buhot et al. 1999) and are increasingly aggressive (Bouwknecht, Hijzen et al. 2001). Preclinical and clinical studies also prove that serotonergic inputs may moderate DA´s effects on attention and hyperactivity/impulsivity while *HTR1B* regulates DA release in the striatum, midbrain and PFC (Smoller, Biederman et al. 2006). Further studies in and around the HTR1B-locus refer to an association between this gene and ADHD (Hawi, Dring et al. 2002; Quist, Barr et al. 2003; Faraone, Perlis et al. 2005). Smoller (Smoller, Biederman et al. 2006) genotyped 21 SNPs in and around *HTR1B* in 12 multigenerational pedigrees with regard to ADHD. Only three SNPs were nominally associated with the inattentive subtype.

Tryptophan hydroxylase 2

Primary it was assumed that the tryptophan hydroxylase gene (*TPH*) is widely distributed, but then a second isoform, *TPH2*, was identified. This isoform is only expressed in the brain, especially in serotonergic neurons of the raphe nuclei and formation reticularis. *TPH2*, mapped to chromosome 12q21.1, is the rate-limiting enzyme in 5-HT synthesis. It catalyzes, together with oxygen and tetrahydrobioptren as cosubstrates and iron as cofactor, the hydroxylation from tryptophan to 5-hydroxytryptophan. Ko-mice showed a reduced HT-production in brain and behavior abnormalities which are in accordance with human depression or anxiety disorders (Beaulieu, Zhang et al. 2008). Furthermore, *TPH2* is also in humans the purpose of numerous phenotype studies in psychiatric disorders like ADHD. In 2005 Walitza and colleagues analyzed the effects of polymorphic variations in the *TPH2* gene in 225 ADHD children out of 103 families. Two SNPs (rs4570625 and rs11178997) revealed a trend towards an association to ADHD in a haplotype analysis (Walitza, Renner et al. 2005). Sheehan established a significant association between diverse markers and HKS (Sheehan, Lowe et al. 2005). Thus different polymorphisms of this gene, in the promoter region and in introns are connected to ADHD.

2.6. NEUROPEPTIDES

Neuropeptides are released as second messengers by different neurons and affect either the endocrine as neurosecretatory peptide hormones or paracrine as co-transmitters. They depolarize or hyperpolarize other neurotransmitters not by binding to ion channels at the postsynaptic membrane, but over receptors.

Neuropeptide Y

Neuropeptide Y (*NPY*) is a tyrosine-rich, highly conserved, 36 aa neuromodulatoring peptide that has high structural similarity to peptide YY and pancreatic polypeptide. Since its discovery in 1982 by Tatemoto (Tatemoto 1982) is has been characterized as one of the most abundantly expressed peptides throughout the mammalian peripheral and central nervous system mainly in the cortex, hippocampus, hypothalamus and metencephalon

(Chronwall, DiMaggio et al. 1985). Initial research discovered *NPY´s* effects on a large number of neuroendocrine functions, circadian rhythms, stress response, central autonomic functions, eating and drinking behaviors, and sexual and motor behavior (Wahlestedt, Ekman et al. 1989; Westwood and Hanson 1999). Even behaviors related to neuropsychiatric disorders (i.e. depression and schizophrenia) seem to be modified by *NPY*. The potent neurotransmitter exerts its biological effects through at least five G-protein coupled receptors termed Y_1, Y_2, Y_4, Y_5 and Y_6 (Karl and Herzog 2007) and were frequently analyzed for connection to neurological diseases including ADHD. In addition, *NPY* has been shown to interact with neurotransmitter systems such as DA, γ-aminobutyric acid (GABA) and NE, and co-localizes with several other neurotransmitters (Westwood and Hanson 1999). Because the NPY-system is altered in many DA-associated psychotic diseases and moreover DA plays a role (see chap. 2.2.), a connection to ADHD is most likely. Indeed, *NPY* was implicated in ADHD only in one study: Oades detected that an elevated level of circulating *NPY* as well as a decreased electrolyte excretion exists in ADHD children that may reflect a common disturbance in metabolic homeostasis (Oades, Daniels et al. 1998). While it has widely been investigated in the context of energy balance and body weight regulation, NPY has recently not only been implicated in behavioral traits, particularly negative emotionality and aggression (Raveh, Grunwald et al. 1993), but also in several neuropsychiatric disorders including depression, panic disorder, bipolar disorder, and schizophrenia (Koetzner and Woods 2002). A functional polymorphism in the human *NPY* (Leu7Pro) resulting in increased NPY release from sympathetic nerves is associated with characteristics of metabolic syndrome and it has been suggested that the Pro7 allele is associated with an increased risk for alcohol dependence, a common co-morbid disorder of ADHD (Manoharan, Kuznetsova et al. 2007).

Latrophilin 3

Currently three different isoforms of the latrophilin family are known, latrophilin (LPHN) 1, 2 and 3. The name came from its binding to α-latrotoxin (LTX), a potent presynaptic neurotoxin from the venom of black widow spiders, which induces neurotransmitter and hormone release by way of extracellular Ca^{2+}-influx and cellular signal transduction pathways (Erdogan, Chen et al. 2006). All isoforms are brain-specific chimeras of G-protein coupled, Ca^{2+}-independent receptors (GPCR) of the secretin/calitonin family and of cell adhesion

molecules (CAM) (Matsushita, Lelianova et al. 1999). Also latrophilins play an important role both with cell adhesion and signal transduction. In a genome-wide linkage analysis was shown that the region 4q13.1-13.2 (Arcos-Burgos, Castellanos et al. 2004) is connected with ADHD and obsessive-compulsive disorder (OCD) (Jain, Palacio et al. 2007). Within this 40 Mb large region the gene *LPHN3* was found to be associated with ADHD. Furthermore, subsequent haplotype analyses identified a susceptibility locus inside exon 7 - 9 (413 kb) of *LPHN3* ((Arcos-Burgos, Jain et al.). The approx. 6 Mb large *LPHN3*, which consists of 24 exons, encodes for a 1249 aa protein. Unfortunately, the endogenous ligands are still unknown for all three homologues.

2.7. OTHER CANDIDATE GENES

Monoamine oxidase isoenzyme A

Two monoamine oxidase isoenzymes *MAO-A* and *MAO-B*, lying in antipodal direction on the X-chromosome, are mainly expressed in the outer membrane of mitochondria of neurons and astroglia. Both oxidases catalyze the oxidative deamination of neurotransmitters and monoamines. Man-made drugs which block MAOs, so-called monoamine oxidase inhibitors (MAO-I), are applied more and more frequently as antidepressants.

Mutations in *MAO-A*, which exists of 15 exons and spans approx. 90 Mb, or a low *MAO-A* activity were still associated with impulsive and criminal behavior (Chen, Holschneider et al. 2004). Based on different evidences of MAO-systems in the etiology and the course of ADHD, Li and colleagues analyzed two polymorphisms in *MAO-A* and three in *MAO-B* (Li, Kang et al. 2007). The results showed a significant association between both *MAO-A* polymorphisms and ADHD in adolescents as well as between those and the hyperactive/impulsive subtype.

Synaptosomal associated protein 25

The synaptosomal associated protein (*SNAP-25*), mapped to chromosome 20p11.2, regulates membrane trafficking and is involved in the release of neurotransmitters as well as the translocation of proteins to the cell membrane. Altered expression will have diffuse

effects on neuronal function. Interest in this gene has come from animal research. The *SNAP-25* deficient mouse mutant coloboma (CM/+) displays spontaneous motor hyperactivity that is alleviated by stimulant medication (Barr, Feng et al. 2000; Mill, Curran et al. 2002; Russell, Sagvolden et al. 2005; Thapar, Langley et al. 2007). The ko-mouse shows therefore no hyperactivity (Washbourne, Thompson et al. 2002). In humans, evidence for an association between *SNAP-25* and ADHD is still not evident (Kustanovich, Merriman et al. 2003) because only a low accordance is denoted between numerous SNP analyses.

3. MEGALOENCEPHALIC LEUKOENCEPHALOPATHY WITH SUBCORTICAL CYSTS

3.1. CLINICAL FEATURE

Megaloencephalic leucoencephalopathy with subcortical cysts (MLC) is characterized by diffuse swelling of the white matter, large subcortical cysts, and megaencephaly with infantile onset. As the disease progresses, the white matter swelling decreases and cerebral atrophy ensues, while the subcortical cysts generally increase in size and number. The appearance of subcortical cysts in the anterior-temporal region and often also in the frontoparietal region is typical for this disease. This neurologic disorder shows an autosomal-recessive mode of inheritance. MLC has a wide heterogeneity both within and between families and it is speculated that this might be related to specific genetic determinants (Montagna, Teijido et al. 2006). Also, its clinical heterogeneity indicates that unknown environmental or genetic factors may impact the severity of the disease.

3.2. FINDINGS

MLC seems to be caused by mutations in the *MLC1* (Leegwater, Yuan et al. 2001), a ~26.1 kb gene, also known as *WKL1* or *KIAA0027* and maps to chromosome 22q13.3.

Chromosome $22q_{tel}$ is known to harbor several genes involved in severe neurodegenerative disorders, like myoneurogastrointestinal encephalopathy or metachromatic leukodystrophy (Rubie, Lichtner et al. 2003). *MLC1* encodes the protein MLC1 which is mainly expressed in distal astrocytes, Bergman glia and subependymal cells, and in leukocytes, but not in oligodentrocytes or microglia (Teijido, Martinez et al. 2004). Its biochemical properties and its function are still unknown, although there are many assumptions, i. e. a transporter function as a cation-channel, ABC-2 type transporter or sodium/galactoside transporter (Leegwater, Yuan et al. 2001). Most of all, the presence of eight putative transmembrane domains and its localization suggest a transporter function across the blood-brain and brain-cerebrospinal fluid barrier (Boor, de Groot et al. 2005).

Since the first report, 50 mutations in this gene have been found, which include all different types: eleven splice-site, one nonsense, 24 missense mutations and 14 deletions and insertions. All of these mutations can lead to frame-sifts or loss-of-function (Boor, de Groot et al. 2005), and still novel mutations are discovered. But almost nothing is known about the pathogenic mechanism of these mutations, but recent heterologous expression studies proposed that gene mutations impair protein folding (Teijido, Martinez et al. 2004). A different approach is to study the expression of the gene in specific brain regions known to be involved in MLC. Because MLC1 is highly conserved between vertebrates, the murine *Mlc1* can likely give a better insight of *MLC1* involvement in the pathogenesis of MLC and catatonic schizophrenia. Mlc1 expression seems to be developmentally regulated in a region- and cell type-specific manner and may be important in the development of the brain, mainly for initial events of myelination (Schmitt, Gofferje et al. 2003). Some mutations are quite frequent in certain populations, indicating a founder effect. Imaging studies have described a disorder very similar to MLC among the Agarwals, a discrete, genetic isolated ethnic group found in India (Gorospe, Singhal et al. 2004). The Agarwals are known to be an enterprising business group whose members have migrated to widespread regions of India and different parts of the world. But in about 20% of the patients with MLC no mutations in *MLC1* are found, so likely a second gene accounts for a smaller subset of MLC patients.

In addition, linkage analysis and positional cloning reveals that haplo-insufficiency in *MLC1* (amino acid change Leu309Met) is associated in a dominant manner with a periodic subtype of catatonic schizophrenia in a large pedigree (Meyer, Huberth et al. 2001). Recent studies have brought forward compelling arguments that genetic variants of *MLC1* are not associated with schizophrenia (Ewald and Lundorf 2002; Kaganovich, Peretz et al. 2004). Rubie and coworkers (Rubie, Lichtner et al. 2003) also provided evidence of allelic

heterogeneity in MLC and ruled out the possibility that MLC and schizophrenia are allelic disorders.

Identification of sequence variations in all 13 exons and flanking intronic sequences of *MLC1* revealed eight SNPs which seem to be associated with schizophrenia and bipolar affective disorder and could therefore increase the susceptibility to these disorders (Verma, Mukerji et al. 2005).

A generation of a transgenic mouse model would provide a useful tool to elucidate both, function and disease pathomechanisms as well as behavior and possible motor impairment.

II. MATERIAL AND METHODS

1. MATERIAL

1.1. ENZYMES

Name	**Manufacturer**
Hind III (inclusive Buffer 2)	New England BioLabs, Frankfurt, Germany
Xho I (inclusive Buffer 2)	New England BioLabs, Frankfurt, Germany
DNase I	Fermentas, St. Leon-Roth, Germany
RNase A	Roche, Mannheim, Germany

Tab. 1a: Restriction enzymes.

Name	**Manufacturer**
Taq-DNA polymerase	Fermentas, St. Leon-Roth, Germany
Sp6 polymerase (inclusive 5x transcription buffer)	Fermentas, St. Leon-Roth, Germany
T7 polymerase (inclusive 5x transcription buffer)	Fermentas, St. Leon-Roth, Germany

Tab. 1b: Polymerases.

1.2. ANTIBODIES

Antibody	Name	Manufacturer
Secondary	Ovine anti-digoxigenin (DIG) Fab-fragments linked to alkaline phosphatase (aP)	Roche, Mannheim, Germany

Tab. 2a: Secondary antibodies.

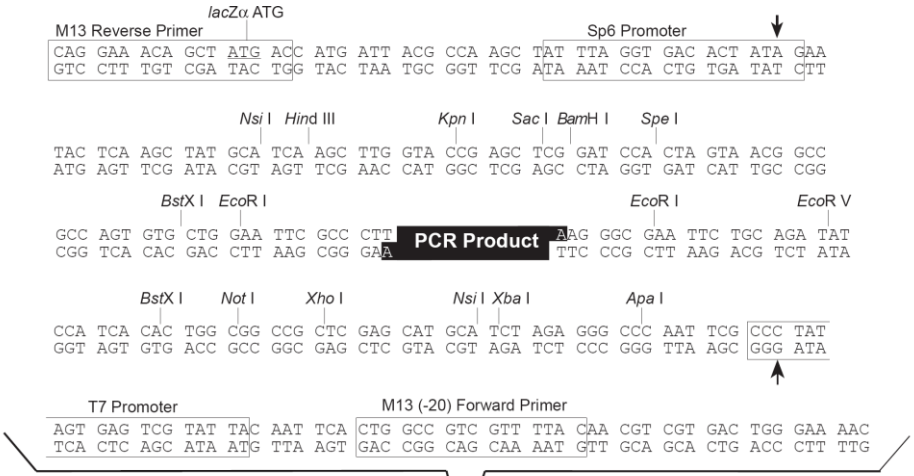
Name	Manufacturer
Normal goat serum (NGS)	VectorLaboratories, Burlingame, CA, USA
Bovine serum albumin (BSA)	Sigma, Deisenhofen, Germany

Tab. 2b: Further proteins.

1.3. PLASMIDS

pCR®II-TOPO®-TA cloning vector Invitrogene, Carlsbad, CA, USA

A



B

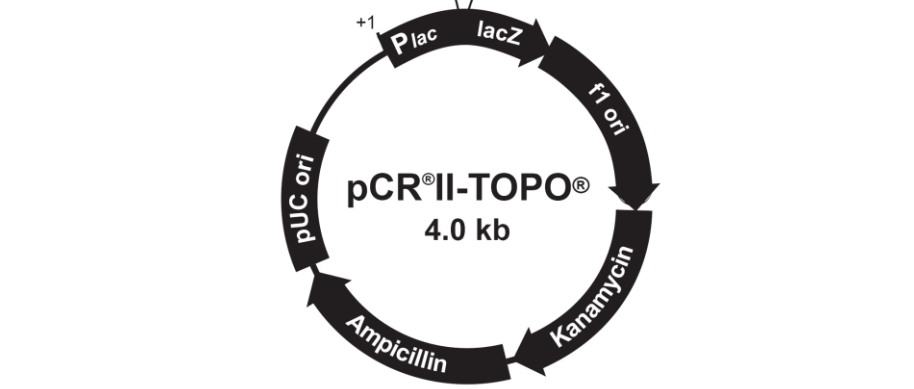


Fig. 9: pCR®II vector map (modified by Invitrogen).

A: *Sequence of the Multiple Cloning Site (MCS).* Shown are the forward and reverse priming sites (M13), the promoter sequences of RNA polymerases SP6 and T7, the start codon of LacZα gene, 3´-thymidinoverhangs with schematic integrated PCR product as well as the recognition sites for restriction endonucleases.

B: *View of the pCR®II-TOPO® vector.* Amplicillin and Kanamycin: antibiotic resistance genes; pUC ori: plasmid; f1 ori: single strand replication origin; P*lac*: lac promoter; lacZ: β-galactosidase gene.

1.4. DESOXYRIBONUCLEIDS

Name	QuantiTect® Primer Assay (Qiagen)
Latrophilin 3 (LPHN3)	Hs_LPHN3_1_SG

Tab. 3a: Human primer for RT-PCR.

Primer	Orientation (forw/rev)	Sequence (5´→3´)	Location	Product size [bp]	Melting temperature [Tm; °C]
SA Mlc1 for TK	forw	GGACGACAGCAGAGGTAAGC	Exon 1	1546	57
Neo 340 rev TK	rev	ATACTTTCTCGGCAGGAGCA	Neo		57
Mlc1 integ ex f	forw	AGGGTGCCAATGTCTCCA	Exon 1	735	56
Mlc1(Neo) nested SA rev	rev	CTCGTCCTGCAGTTCATTCA	Neo		57
Mlc1 ex1 nest f	forw	CCAATGTCTCCAGGCAAATG	Exon 1	1879	61
Mlc1 int nest r	rev	CTGTTGTGCCCAGTCATAGC	Neo		59

Tab. 3b: Used primer for searching of the integrated pMlc1-ko plasmid vector.

Forw / f: forward; rev / r: reverse; neo: neomycin-cassette.

Name	Manufacturer
100bp DNA ladder	Fermentas, St. Leon-Roth, Germany
1kb DNA ladder	Fermentas, St. Leon-Roth, Germany

Tab. 3c: DNA gene ladders.

1.5. REACTION KITS

Name	Manufacturer
DIG RNA Labeling Kit (Sp6/T7)	Roche, Mannheim, Germany
iScript™ cDNA Synthesis Kit	Bio-Rad, Munich, Germany
RNeasy Mini Kit	QIAGEN, Hilden, Germany
PeqGOLD RNAPure™-System	QIAGEN, Hilden, Germany

Tab. 4: Used reaction kits.

1.6. BUFFER

All used buffers are in-house productions.

Buffer	Contents
Goldstar PCR buffer (10x)	750mM Tris-HCl, pH 9.0 200mM ammoniumsulfate 0.1% Tween-20
TAE buffer	1mM EDTA, pH 8.0 40mM Tris-acetat
TE buffer (1x)	pH 8.0
Sodium saline citrate (SSC, 20x)	0.3 sodium citrate, pH 7.0 3M NaCl
Phosphate buffered saline (PBS, 10x)	1.3 NaCl 70mM Na_2HPO_4 30mM NaH_2PO_4

Tab. 5a: General buffers.

Buffer	**Contents**
Acetylation buffer	0.1M triethanolamine, pH 8.0 0.25% acetic acid anhydride
Hybridization buffer (sterile filtered)	50% deionisated formamide 4x SSC 10% dextransulfate 1x Denhardt´s solution, RNase free 250µg/ml denatured salmon sperm DNA, RNase free
RNase buffer	10mM Tris-HCl, pH 8.0 500mM NaCl 1mM EDTA
DIG 1 buffer	100mM Tris-HCl, pH 7.5 150mM NaCl
DIG 3 buffer (detection buffer)	100mM Tris-HCl, pH 9.5 100mM NaCl 50mM $MgCl_2$
Blocking buffer	DIG 1 buffer with 0.5% Blocking reagent

Buffer	Contents
Antibody incubation buffer	DIG 1 buffer with 0.25% Blocking reagent 0.15% TritonX-100
aP reaction medium	DIG 3 buffer with 0.4mM BCIP 0.4mM NBT
Blocking solution 1	TBS with 5% NGS 2% BSA 0.25% Triton X-100
Blocking solution II (BSA/Goat serum)	TBS with 2% NGS 2% BSA 0.25% Triton X-100

Tab. 5b: Buffers for *in situ* hybridization and immunohistochemistry.

1.7. SOLVENTS AND SOLUTIONS

Name	Manufacturer
A. bidest	Merck, Darmstadt, Germany
Chloroform	Sigma, Deisenhofen, Germany
Ethanol, absolute	J.B. Baker, Phillipsburg, NJ, USA
Formamide (deionisated)	AppliChem, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Phenol (waterlogged, stabilized)	AppliChem, Darmstadt, Germany
Roti-phenol (TE-buffer logged)	Roth, Karlsruhe, Germany
Xylol	Merck, Darmstadt, Germany

Tab. 6a: Solvents.

Name	Manufacturer
1x Denthardt´s solution, RNase free	Sigma, Deisenhofen, Germany
Ethidium bromide solution (10mg/ml)	Sigma, Deisenhofen, Germany

Tab. 6b: Solutions.

1.8. CHEMICAL COMPOUNDS

Name	Manufacturer
Agarose (Seq Kem LE)	Biozym, Oldendorf, Germany
Blocking reagent	Roche, Mannheim, Germany
BSA (bovine serum albumin)	J.B. Baker, Phillipsburg, NJ, USA
Desoxynucleotides (dATP, dCTP, dGTP, dTTP)	Promega, Madison, USA
DIG RNA marker mix	Roche, Mannheim, Germany
Acetic acid	Merck, Darmstadt, Germany
Acetic acid anhydride	Sigma, Deisenhofen, Germany
Salmon testis DNA	Sigma, Deisenhofen, Germany
NGS (normal goat serum)	Sigma, Deisenhofen, Germany
t-RNA	Sigma, Deisenhofen, Germany

Tab. 7a: Biochemicals.

Name	Manufacturer
BICP (5-bromo-4-chloro-3-indolyl-phosphate)	Sigma, Deisenhofen, Germany
DAB (3,3-diaminobenzidine)	Roche, Mannheim, Germany
DEPC (diethylpyrocarbonat)	Sigma, Deisenhofen, Germany

Name	**Manufacturer**
Dextran sulfate	Sigma, Deisenhofen, Germany
DTT (dithioreitol)	Sigma, Deisenhofen, Germany
EDTA (ethylendiamintetraacetic acid)	AppliChem, Darmstadt, Germany
Fluorescin	Bio-Rad, Munich, Germany
Hydrochlorid acid (5M)	Merck, Darmstadt, Germany
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt, Germany
Phosphate buffered saline (PBS)	Bio, Whittaker, Charles City, USA
Potassium chloride	AppliChem, Darmstadt, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Protease inhibitor cocktail	Sigma, Deisenhofen, Germany
RNase inhibitor	Fermentas, St. Leon-Roth, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Triethanolamine (TAE)	Merck, Darmstadt, Germany
Tris(hydroxymethyl)aminomethane (Tris)	Merck, Darmstadt, Germany
Triton X-100	Sigma, Deisenhofen, Germany
Tween-20	Sigma, Deisenhofen, Germany

Tab. 7b: Further chemical compounds.

1.9. FURTHER MATERIALS

Name	Manufacturer
10 ml single-use injection	Braun. Melsungen, Germany
Aquatex	Merck, Darmstadt, Germany
Cultureslides, poly-D-lysins and laminine coated	BD Bioscience, Heidelberg, Germany
Coverslips (24 x 50mm)	Marienfeld, Lauda-Königshofen, Germany
Filter pipet tips	Eppendorf, Hamburg, Germany
Filter units (FP30/0.2 CA-S; for ISH)	Schleicher&Schuell, Dassel, Germany
Superfrost Plus glass slides	Menzel, Braunschweig, Germany
Tissue-Tec	Sakura

Tab. 8: Further materials.

1.10. APPARATUS

Name	Manufacturer
Autoclave 3850 ELV	Systec GmbH, Nuremberg, Germany
Biofuge Fresco (table centrifuge)	Heraeus Instruments, Hanau, Germany
Hybridization oven	Heraeus Instruments, Hanau, Germany
Cycler iQ™Real Time Detection System	Bio-Rad, Munich, Germany

Name	Manufacturer
Cryostat Microm HM 500 O	Microm GmbH, Neuss, Germany
Leica TCS SP2 confocal microscope	Leica, Wetzlar, Germany
NanoDrop®ND-1000 fluorospectrometer	Peqlab, Erlangen, Germany
PCR gradient thermocycler	Biometra, Goettingen, Germany
Chemi-Doc (gel documentation system)	Bio-Rad, Munich, Germany
Nanodrop	NanoDrop, Wilmington, DE, USA
Axon 4000B scanner	Axon instruments, Burlingame, CA, USA

Tab. 9: Apparatus.

1.11. COMPUTER SYSTEMS

Name	Manufacturer
iCycler iQ 3.1	Bio-Rad, Munich, Germany
Leica Confocal Software 2.61	Leica, Wetzlar, Germany
Genepix 5.0	Axon Instruments, Union City, Calif., USA
CGHPRO	(Chen, Holschneider et al. 2004)
CGH Analytics	Agilent, Santa Clara, USA

Tab. 10a: General computer systems.

Software	Version
Assay Design	3.0.0.
Services	2.0.8.
Assay Editor	3.1.4.
Plate Editor	3.1.4.
TYPER Analyzer	3.3.0.
Acquire	3.3.1.
Caller	3.3.0.

Tab. 10b: MassARRAY workstation version 3.3. and software components.

2. METHODS

2.1. BASAL MOLECULAR GENETIC METHODS

Polymerase chain reaction

Polymerase chain reaction (PCR) is a widely used method for the *in vitro* replication of DNA by a DNA polymerase. It is based on three partial steps, which are repeatedly multiplied: Denaturation consists of heating the reaction to 94 - 98°C. It causes melting of the DNA template and primers by disrupting the hydrogen bonds between the complementary bases of the DNA strands, yielding single strands of DNA. During the annealing step the reaction temperature is lowered to 50 - 65°C allowing annealing of the primers to the DNA template. Typically, the annealing temperature is 3 - 5°C below the Tm of the used primers. The heat stable polymerase binds to the primer-template hybrid and begins the DNA synthesis. The

temperature at the extension step depends on the used DNA polymerase. At this step the polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs.

Reagent	**Volume**
10x Goldstar buffer	2.5 µl
$MgCl_2$ (25mM)	1.0 µl
dNTPs (2.5 mM each)	2.0 µl
Primer forward (10 pmol/µl)	1.0 µl
Primer reverse (10 pmol/µl)	1.0 µl
Genomic DNA (40 - 60 ng)	2.0 µl
Taq DNA polymerase (5 U/µl)	0.3 µl
a. d. (Merck)	17.2 µl
Final volume	25.0 µl

Tab. 11a: PCR components protocol.

Temperature	**Time**	**Cycles**
95°C (denaturation)	3 min	1x
95°C (denaturation)	45 sec	35 - 45x
54-65°C (annealing) (Primer-specific temperature)	45 sec	35 - 45x
72°C (elongation)	45 sec	
72°C (final elongation)	3 min	1x
4°C	∞	

Tab. 11b: PCR cycle protocol.

For mass spectrometry (see chap. 2.4) composition and cycles of the PCR reactions are modified.

The used primers are oligonucleotides, allowing the DNA polymerase to extend the nucleotides and to replicate the complementary strand. Typically, synthesized oligonucleotides are single-stranded DNA molecules around 17 - 30 bases in length and with cysteine or guanine at their 3´ end. Whereas polymerases synthesize DNA in 5´ to 3´ direction, chemical DNA synthesis is done backwards in 3´ to 5´ reaction. The G/C content of the selected DNA sequence averaged 50 - 60%. Diverse software programs (Oligo 4.0; FastPCR 3.6.97) choose the primers automatically depending on the selected conditions.

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR), established by Powell and colleagues (Powell, Wallis et al. 1987), is the most sensitive technique for mRNA detection and quantification, based on the properties of the conventional PCR. After producing a DNA copy of cDNA of each mRNA molecule, the gene expression levels were further amplified from the cDNA mixture together with a housekeeping gene as internal control. DNA amplification was visualized with a fluorescent dye. RT-PCR machines can detect the amount of fluorescent DNA and thus the amplification progress which is given in a curve with an initial flat-phase followed by an exponential phase. Here we used the sequence independent fluorescent dye SYBR-Green I (Qiagen, Hilden, Germany).

Reagent	Volume
2x QuantiTect SYBR Green master mix	12.50 µl
10x QuantiTect Primer Assay (with specific primers)	2.50 µl
Fluorescein	0.25 µl
cDNA	1.00 µl
a. d.	8.75 µl
Final volume	25.00 µl

Tab. 12a: RT-PCR components protocol.

The used master mix already contains the required DNA-polymerase as well as free dNTPs.

All required reagents except for the cDNA are components of “QuantiTec TM SYBR Green PCR Kits” (Qiagen, Hilden, Germany). The analysis was carried out by an “iCycles iQ Realtime-PCR Detection System” with a corresponding evaluation program “iCycler, Version 3.1” (both Bio-Rad, Munich, Germany).

Temperature	Time	Cycles
95°C	15 min	1x
95°C	15 sec	35 - 45x
55°C	30 sec	
72°C	30 sec	
95°C	30 sec	1x
72°C – 94°C (in 0.5°C measures)	each 15 sec	50x
15°C	∞	

Tab. 12b: RT-PCR cycle protocol.

Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA molecules by size and to appraise concentration. This is achieved by moving negatively charged nucleic acids through an agarose matrix (1 - 1.5% in 1x TAE buffer) with an electric field (90 - 120 volt). Shorter molecules move faster and migrate farther than longer ones. The most common dye used to make DNA or RNA bands visible is ethidium bromide (EtBr). It fluoresces under UV light (λ = 302 nm) when intercalated with DNA/RNA. As loading buffer 1x TAE is used; it has a low buffering capacity but provides a good resolution for large DNA/RNA. Also a DNA ladder (199 bp or 1 kb) is laid on the gel for valuation of the size of the nucleic acids.

DNA precipitation

Ethanol precipitation is a most facile and rapid method to purify and/or concentrate nucleic acids and polysaccharides. DNA is precipitated by adding 1/10 volume of sodium acetate (3 M, pH 5.5). Then, 2.5 volumes of 100% ethanol were admitted and the DNA was stored at -20°C over night. During incubation DNA and some salts precipitated from the solution, the precipitate itself was sedimented by centrifugation in a microcentrifuge tube at high speed (14,000 rpm, 4°C; 30 min). Time and speed of centrifugation have the biggest effect on DNA recovery rates. During centrifugation the precipitated DNA has moved due to the ethanol solution to the bottom of the tube, the supernatant solution was removed afterwards, leaving a pellet of crude DNA. One volume 70% ethanol was added to the pellet, it was gently mixed to break the pellet loose and to wash it. This step removes some of the salts present in the leftover supernatant and binds to the DNA pellet making the DNA cleaner. The suspension was centrifuged once again for 15 min. Finally, the pellet was air-dried and the DNA was resuspended in a. d. or another desired buffer.

DNA cleaving by restriction endonucleases

The used Type II restriction endonucleases are bacterial enzymes, which recognitions sites are usually undivided, palindromic sequences. There, they recognize and cleave to the DNA at this site by hydrolyzation of the phosphodiester bond. For cutting a DNA fragment out of a plasmid, two different restriction enzymes are necessary, for linearization just one enzyme with only one cutting site is sufficient. Cleaving is affected by recommendation of the

manufacturer. 1 - 5 units of the particular endonuclease were used per µg DNA. Each approach had the volume of 25 - 50 µl.

2.2. *IN SITU* HYBRIDIZATION

In situ hybridization (ISH) represents a powerful and sensitive method for examining gene expression in individual cells and to characterize the phenotype of cells expressing neurotransmitter or specific neuroreceptors. Via a marked probe both RNA and DNA can be detected. The basic requirement is that the tissue is native or fixed by paraformaldehyde (PFA).

In this research we used single stranded, DIG marked cRNA probes for a non-radioactive ISH.

Establishing of DIG-labeled cDNA

RNA probes have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNases. In vitro transcription of linearized plasmid DNA with RNA polymerase was used to produce two RNA probes, a “sense” and an “antisense” one. The first named corresponds with the base sequence of the cellular mRNA and provides a specificity control. The last named is complementary to the mRNA and shows a specific signal after hybridization. The used plasmid, the pCR®II vector, contained the polymerase from the bacteriophages *T7* (antisense) and *SP6* (sense). The plasmid was linearized with *Hind* III, when producing an antisense sensor, and alternatively with *Xho* I for a sense one. After DNA precipitation the marked cRNA probes were produced in the following reaction batch:

Reagent	Volume
1,500 ng linearized plasmid	17.5 µl
DIG RNA labeling mix (Roche)	3.0 µl
5x Transkription buffer (Fermentas)	6.0 µl
RNase inhibitor [40U/µl] (Fermentas)	0.5 µl
T7 or *Sp6* RNA polymerase (Fermentas)	3.0 µl
Final volume	30.0 µl

Tab. 13: Reaction batch for *in vitro*-transcription.

After incubation at 37°C for 2 h, addition of 2 µl DNase and a new incubation for 15 min, the probe was again precipitated and dissolved in 40 µl DEPC-treated ddH_2O. RNA concentration was measured at a Nanodrop.

Preparation of the sections

The ISH was carried out on 16 µm sections of native, untreated and alternatively perfused mouse brains done at the crytostst *HM 500 O*. These were lifted on prefrosted Superfrost Plus glass slides and stored at -80°C until use.

Pretreatment

The thawing sections were incubated in 4% PFA (solved in 1x PBS) for 5 min and rehydrogenated in a downward alcohol line (100%, 95%, 80%, 70% ethanol). After 2x washing in 2x SSC for 10 min and 5 min incubation in 0,02N HCl for arousing the tissue permeability, positive amino groups were acetylated in 0.25% acetic acid anhydride in 0.1 M triethanolamine to avoid unspecific binding with the negative cRNA probes.

Hybridization

To avoid unspecific bindings the sections were coated for 1 h with a 100 µl prehybridization buffer at 58°C. Afterwards each section was overlaid with a 100 µl hybridization buffer containing 10 – 15 ng of DIG-labeled RNA probe which was prelinearized at 84°C for 5 min. The samples were covered with a hydrophobic plastic coverslip and incubated overnight at 42°C in a humid chamber. The contained formamide removes hydrogen bonds and therefore secondary structures.

Posthybridization

After washing two times in 2x SSC for 10 min at room temperature, two times in 2x SSC / 50% formamide buffer for 30 min at 58°C and again in 2x SSC, the sections were incubated in RNase buffer containing 40 µg/ml RNase A to digest any single-stranded unbound RNA probes. In a shaking, 58°C water bath the reaction was stopped with RNase buffer without RNase A.

Using a shaking platform again, the sections were washed 5 min in DIG1 buffer and 30 min in blocking buffer at room temperature to block unspecific antibody binding sites. They were covered 1 h with 100 µl buffer containing 0.3% Triton X-100, 1% normal goat serum, and a 1:500 dilution of anti-DIG-alkaline phosphatase (Fab fragments). Afterwards the sections were again washed two times in DIG1 for 5 min.

The immunological detection resulted from a DIG3-color solution containing 0.4 mM BCIP in the dark. When the color development was optimal, the reaction was stopped by incubating the slides in 1x PBS buffer.

2.3. IMMUNOHISTCHEMISTRY

Immunohistochemistry (IHC) refers to processes of localizing proteins in cells of a tissue section and exploiting the principle of antibody binding specifically to antigens. The indirect, but specific detection of proteins in tissues by unlabeled prime antibodies (1st layer) and labeled secondary antibodies (2nd layer) is called the avidin-biotin-complex (ABC-) method.

This method is used mainly for double-labeling after ISH with cRNA probes. The glycoprotein avidin, which is produced by *Streptomyces avidinii*, is a tetramer and can therefore bind physically with each subunit of one molecule biotin. A biotinylated secondary antibody, which is coupled with streptavidin-horseradish peroxidase, is reacted with 3,3´-Diaminobenzidine (DAB) to produce a brown staining (see Fig. 10).

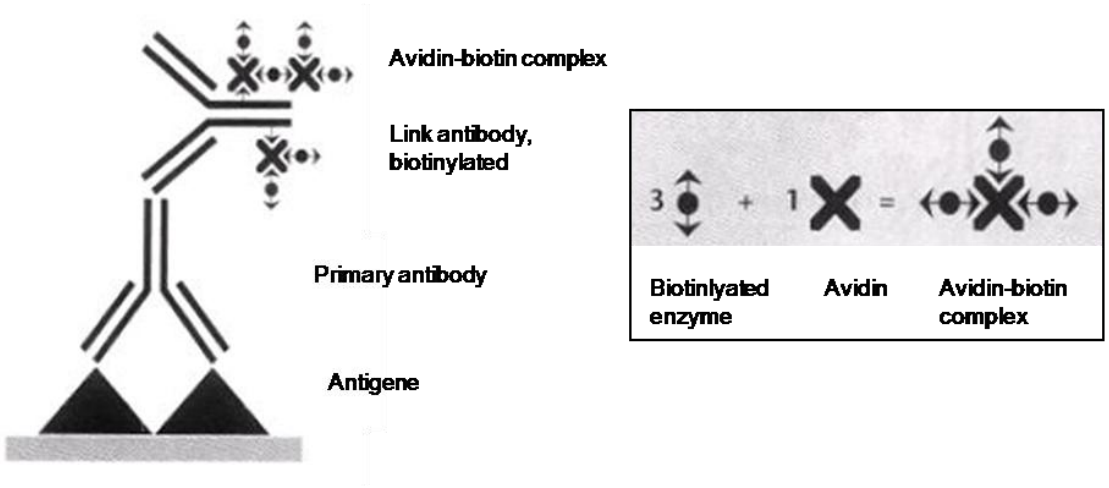


Fig. 10: Detection of the primary antibody via secondary antibody and avidin-biotin-peroxidase complex.
(Strept-)Avidin is a basic glycoprotein and has four high affinity binding sites for the small water soluble vitamin biotin. It forms together with the biotinylated enzyme peroxidase the avidin-biotin-enzyme complex. The detection of the primary antibody results from the simultaneous binding of the biotinylated secondary antibody and the biotinylated peroxidase to avidin.

Directly after ISH the reaction is stopped in 1x TBS-buffer. To remove unspecific protein bindings and to retrieve antigens, the sections were incubated in 2% BSA / 5% normal goat serum for 1 h. The LPHN3 primary antibody is produced by a rabbit; 100 µl of a 1:200 antibody-dilution in blocking buffer were applied to each section and incubated over night at 4°C in a humid chamber. The unbounded antibodies were rinsed for 3x 5 min washing in

1x TBS, before the sections were incubated in the polyclonal secondary antibody (100 µl each section; 1:200 dilution in blocking buffer) for 90 min at room temperature. Subsequently the already compounded AB-complex was applied for 90 min. The visualization of the peroxidase and therefore the antigen localization resulted from a 5-10 min incubation – depending on the color intensity – in 1:10 DAB buffer:1x PBS. The reaction was stopped again with TBS buffer and the glass slides were coversliped.

The documentation of this double staining as well as after ISH occurred via the confocal microscope *Leica TCS SP2*.

2.4. ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

Identification of chromosomal imbalances and variations in DNA copy-number is essential to our understanding of disease mechanisms and pathogenesis, because DNA sequence copy-number changes have been shown to play an important role in the etiology of many disorders including trisomy 21 or cancer. Newly developed microarray technologies enable simultaneous measurements of copy numbers of 1000s of sides in a genome.

In the used Array Comparative Genomic Hybridization (array CGH), differentially labeled total genomic “test” and “reference” DNAs are cohybridized onto arrays of genomic BAC clones. An aberration in the genome of the patient is indicated from spots showing aberrant signal intensity ratios. Fig. 11 shows an overview of array CGH.

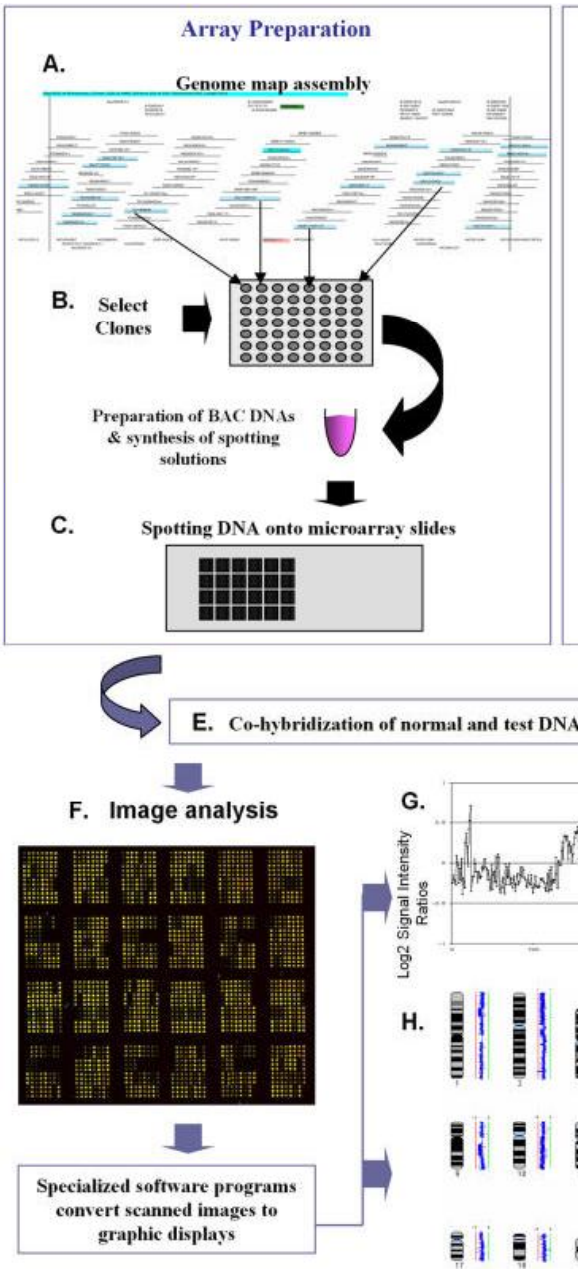


Fig. 11: Principle of array CGH.

(A) BAC clones are selected from a physical map of the genome. (B) DNA samples are extracted from selected BAC clones and their identity is confirmed by DNA fingerprinting or sequence analysis. (C) A multi-step amplification process generates sufficient material from each clone for array spotting. (D) Reference and test DNA are differentially labeled with cyanine 3 and 5 respectively. (E) The two labeled products are combined and hybridized onto the spotted slide. (F) Images from hybridized slides

are obtained by scanning in two channels. Signal intensity ratios from individual spots can be displayed as a simple plot (G) or by using more complex software which can display copy number variations throughout the whole genome (H).
(Garnis et al., 2004)

Samples

A cohort of children and adolescents with ADHD (n = 99; 78 male, 21 female) were included in the CNV scan. Sixty-seven patients were from nuclear families with at least two members affected with ADHD, eight patients were from extended multigenerational families with high density of ADHD and 24 patients had sporadic ADHD. Patients and their families were recruited and phenotypically characterized by a team of experienced psychiatrists in the outpatient units of the Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy and the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Germany, according to DSM-IV criteria (APA 2000). As reference DNA for the aCGH experiments, we used a sex-matched of unscreened blood donors (n = 100, 50 females) of European ancestry and originating from the same catchment area as the patients. All individuals agreed to participate in the study and written informed consent was obtained from either the participants themselves or the appropriate legal guardian. The study was approved by the Ethics Committee of the University of Würzburg.

Nuclear families, if they had one or more children affected with ADHD, were recruited to perform family-based segregation and association studies. The index patient was required to be older than eight years and to fulfill DSM-IV criteria for ADHD combined subtype, other affected siblings in a family had to be older than six years. The lower limit was chosen in order to ensure relative persistence of ADHD symptoms and to exclude children who may show phenocopies of the disorder during preschool age but lack diagnostic criteria for ADHD during subsequent developmental stages (Shelton, Barkley et al. 2000; Barkley, Shelton et al. 2002). Exclusion criteria were: a) general IQ ≤ 80, b) potentially confounding psychiatric diagnoses such as schizophrenia, any pervasive developmental disorder, Tourette´s disorder, and primary affective or anxiety disorder, c) neurological disorders such as epilepsy, d) history of any acquired brain damage or evidence of the fetal alcohol syndrome, e) premature deliveries, and/or f) maternal reports of severe prenatal, perinatal or postnatal

complications. Psychiatric classification was based on the Schedule for Affective Disorders and Schizophrenia for School-Age Children Present and Lifetime version (K-SADS-PL). Mothers completed: 1) the unstructured Introductory Interview, 2) the Diagnostic Screening Interview, and 3) the Supplement Completion Checklist and upon fulfillment of screening criteria the appropriate Diagnostic Supplements. Children were interviewed with the screening interview of the K-SADS and in the case of positive screening for affective or anxiety disorders with the respective supplements of the K-SADS-PL. In addition, we employed the Child Behavior Checklist and a German Teachers' Report on ADHD symptoms according to DSM-IV.

When parents reported individuals with presumable or definite ADHD symptomatology in the extended family, pedigrees were established to determine family size and structure. Reported ADHD symptoms in more than two generations resulted in intensified recruitment of additional family members. Bi-linearity was not an exclusion criterion for recruitment, since it was presumably present in most recruited families due to assortative mating, intra-familiar heterogeneity and cannot be completely ruled out in complex traits such as ADHD. All members of extended pedigrees were assessed by at least two clinicians experienced in diagnosis of childhood and adult ADHD. Due to a tendency toward severe obesity with evidence for co-segregation of this trait with ADHD in an extended family, additional data on body mass index (BMI) and endocrine functions was obtained for further analysis.

Sonification

For a fast cell disruption without detergents or enzymes we used ultrasound with high amplitude.

10 μg test and reference DNA (counterpart) in a total volume of 200 μl were sonificated to a fragment length of 100 bp - 2 kb. Due to the redundancy of heat the probes were continuously held on ice. For control the sonificated DNAs were applied on a 1% agarose gel.

Protein contaminations were removed by use of QIAquick PCR Purification Kit (Quiagen, Hilden, Germany) according to the manufacturer's recommendations. Finally the DNA was eluted in 80 μl a. d..

Labeling and DNA hybridization

Test and reference DNA (1 µg of DNA in a total volume of 21 µl a. d. to each of two tubes) were labeled using an array CGH Genomic Random Prime Labeling System (Invitrogen, Carlsbad, Calif., USA). Briefly, 20 µl of 2.5x Random Primer Solution was added to each tube. After denaturing of the DNA for 5 min at 95°C and cooling down for 5 min on ice, 5 µl 10x dUTP Nucleotide Mix, 3 µl 1 mM Cy3-dUTP (test DNA) or Cy5-dUTP (reference DNA) (Amersham/ GE Healthcare, Munich, Germany) and 1 µl Klenow Fragment supplied in the kit were added on ice to produce a final reaction volume of 50 µl. The reaction was incubated at 37°C for 2 h and stopped by adding 5 µl Stop Solution (Kit). Unincorporated nucleotides were removed by use of the Array CGH Purification Module (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's recommendations. Finally, probes were eluted with 50 µl a. d. and the same probes were pooled to 100 µl final volume.

Test and reference DNA (100 µl each) were combined, precipitated together with 500 µg human Cot1 DNA (competitor DNA), 30 µl sodium acetate (3 M, pH 5.5) and 825 µl at -20°C overnight. During the incubation Cot1 DNA binds to the repetitive sequences of the human DNA and thus diminishes the risk of false positive results.

After ethanol precipitation the DNA pellet was dissolved in 4 µl tRNA (100µg/µl; Invitrogen), 8 µl 10% SDS and 30 µl FDST (formamide dextran sulfat). The added formamide influences the denaturizing of nucleic acids, i.e. by unrequested hairpins. Finally, the DNA was denatured by heating it up to 70°C for 15 min and incubated for 2 h at 42°C for preannealing. Afterwards, the probes were coated with prehybridzed glass slides (see next subitem) and hybridized under a coverslip for 20 - 24 h at 42°C using a Slide Booster (Advalytix, Munich, Germany) (3:7 mixing/pausing).

Prehybridization of the slides

For the array CGH a submegabase resolution tiling path BAC array was used, comprising the human 32 k Re-Array set (http://bacpac.chori.org/pHumanMinSet.htm; clones and DNA provided by Pieter de Jong) (Osoegawa, de Jong et al. 2001), the 1 Mb Sander set (clones provided by Nigel Carter, Wellcome Trust Sanger Centre) (Fiegler, Carr et al. 2003), and a set of 390 subtelomeric clones (assembled by members of the COST B19 initiative: Molecular Cytogenetics of Solid Tumors). BAC DNA was amplified using linker-adaption

ligation PCR, ethanol precipitated, dissolved in 3x SSC, 1.5 M betaine and spotted on epoxy-coated slides (NUNC, Wiesbaden, Germany).

Arrays were prehybridized as follows: 0.3 g BSA, 60 ml PreHyb solution (composition see Material & Methods) and 200 μl of herring sperm DNA (10 mg/ml; Sigma) was prepared and warmed up to 42°C. The used glass slides were incubated in the same solution for 1 h, washed in a. d. and stored in an opaque box until use.

Washing

Slides were immersed into 2x SSC and the coverslips were carefully removed. Then they were washed in a prewarmed wash solution for 15 min at 42°C, shortly immersed in PN buffer (room temperature), again in a second coplin jar with fresh PN buffer and put on a rocking table for 10 min at room temperature. The slides were washed in PBS for 30 sec at room temperature and immersed for a few seconds in a.d., before being dried by spinning in a centrifuge for 5 min at 150 g and stored until scanning.

Data analysis

The high-stringency washed slides were scanned using an Axon 4000B scanner (Axon instruments, Burlingame, CA, USA) and images were analyzed using Genepix 5.0 (Axon Instruments, Union City, Calif., USA). For the analysis and visualization of array CGH data the especially designed software package CGHPRO (Chen, Holschneider et al. 2004) was employed. No background substraction was applied, and the raw data were normalized by “Subgrid LOWESS” and manually adjusted where necessary. Fluorescence intensities of all spots were then calculated after the subtraction of local background. For identifying potentially disease-related DNA copy number gains and losses, we initially called those genomic variants that were composed of three or more consecutive clones with log2 signal intensity ratios beyond 0.3 and -0.3, respectively. In order to increase sensitivity of the read-out, we then the selection criteria to enable the identification of CNVs in which as few as two consecutive clones scored above threshold. As this approach entails the risk of an increased false positive rate, only selected CNVs with highest quality scores (defined by the coefficient of median average deviation and ratio shift) were added to the list obtained using the previous, more stringent selection criteria. CNVs were then prioritized and categorized by

mirroring them against two CNV datasets derived from individuals not affected by clinically relevant ADHD. One dataset was composed of CNVs from 700 healthy individuals and patients suffering from diseases other than ADHD. These samples have been analyzed in our laboratory using the same BAC array platform and data interpretation parameters as those for the ADHD samples in this study.

The second dataset, which had also been employed to assess potential disease association in a recent SNP-based CNV study of ADHD patients (Iafrate, Feuk et al. 2004), was obtained from the Database of Genomic Variants (DoGV). The DoGV is a public domain depository for CNVs identified in the healthy population (http://projects.tcag.ca/variation/, release Aug 2009) (Iafrate, Feuk et al. 2004). It includes all CNVs that were identified in a cohort of 2026 clinically well characterized individuals free of serious medical disorder, including but not limited to neurodevelopmental disorders (including severe ADHD), cancer, chromosomal abnormalities, and known metabolic or genetic disorders (Shaikh, Gai et al. 2009). Based on this comparison, we first identified those CNVs that were not present in either of these two reference datasets and refer to them as those above the high stringency thresholds. Given the fact that, compared to SNP data, BAC array data are known to exaggerate the real size of a CNV, in an inter-platform comparison, CNVs were considered identical if the size differed no more than 100kb at both ends or, for CNVs smaller than 300kb, if they shared at least 50% of the genomic sequence. In a separate, less stringent category we have summarized the CNVs that have been previously reported in the healthy population but are rare, or where independent evidence exists that genes within these intervals could be associated with ADHD. All CNVs discussed here were either verified by confirmation of inheritance using the same method, or by CGH on 244K oligo arrays, performed according to the protocol provided by the manufacturer and analyzed using the company's software CGH Analytics (Agilent, Santa Clara, CA).

Plasma neuropeptide Y

NPY plasma concentrations were determined in 12 individuals of the extended multigenerational family 3. Plasma was immediately separated from venous blood samples by centrifugation, kept on dry ice during transportation, and stored at -80°C until processing. For measurement of plasma NPY a commercial radioimmunoassay (IBL Hamburg,

http://www.ibl-hamburg.com) was performed according to protocol provided by the manufacturer.

Functional magnetic resonance imaging analysis of 7p15 duplication carriers

The impact of the 7p15 duplication and associated increase in NPY plasma concentrations on brain function was explored by functional magnetic resonance imaging (fMRI). Imaging was performed during two paradigms: Both were modified versions of the Monetary Incentive Delay (MID) Task which has been shown to reliably elicit neural responses related to the anticipation of rewards and losses, respectively (Knutson, Adams et al. 2001). Data were pre-processed and analyzed using Statistical Parametric Mapping software (SPM5, Wellcome Department of Cognitive Neurology, UK) as described previously (Hahn, Dresler et al. 2009). To show potential alterations in reward- and loss-related neural responses, we compared four ADHD patients carrying the 7p15 duplication (F2-1, F2-4, F2-6, F2-8; Fig. 2A and B) to an age-matched sample of healthy control subjects (n = 21; mean = 42.0, SD = 6.7; all within 10 years of the median of the patient group) using a voxel-wise non-parametric procedure. p values represent the probability of the median neural activation during the anticipation of rewards/losses of the patient group to be smaller than the median distribution obtained from all possible sets of four subjects (k = 4) that can be drawn from the control sample (5985 combinations). Subsequent statistical analyses focused on the ventral striatum and the posterior insula as defined by voxel masks from a publication-based probabilistic MNI atlas at a probability threshold of 0.9 35. Correction for multiple comparisons was realized using AlphaSim (provided with AFNI software) with a single voxel p-value of 0.05. With this procedure, we assured an overall corrected alpha threshold of $p < 0.05$.

Statistics

The family-based association test (FBAT; http://biosun1.harvard.edu/~fbat/fbat.htm) (Laird, Horvath et al. 2000; Rabinowitz and Laird 2000) was used to investigate whether the 7p15.2-15.3 duplication is associated with ADHD, sex, BMI (kg/m^2), binge eating (no/yes), and NPY plasma concentrations (pmol/ml) within a multigenerational pedigree comprising 20 individuals. By means of 10,000 simulations empirical two-sided p values were obtained, which are more reliable than the respective asymptotic p values in the case of small sample

size. The offset parameter was set to null for residuals of BMI and NPY which were adjusted for sex and age, whereas for binge eating an offset minimizing variance of the test statistic was chosen. The reported p values are nominal, *i. e.* not adjusted for multiple testing, at the significance level of 0.05.

2.5. HIGH THROUPUT SNP GENOTYPING USING MALDI-TOF MASS SPECTROMETRY

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) system is a relatively novel technique in which a co-precipitate of an UV-light (λ = 337 nm) absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. The ionized biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. This method enables the analysis of SNPs directly from human genomic DNA without the need for initial target amplification by PCR.

SNPs are the most abundant type of variation found in the human genome (~$10x10^6$), approximately 10 million are registered in public databases (http://www.ncbi.nlm.nih.gov/SNP/index.html; dbSNP BUILD 122) and seems to play an important role in the development of many diseases (depression, anxiety disorders) (Strobel, Gutknecht et al. 2003; Pearson, Huentelman et al. 2007).

For the whole course we used the iPlex™ Assay protocols and apparatus of Sequenom®GmbH, Hamburg, Germany as well as reagents of Quiagen, Hilden, Germany.

Samples

All patients were diagnosed with ADHD as described in 2.3. Samples. In total, 437 in- and outpatients were recruited at the Department of Psychiatry, University of Würzburg. The control sample consisted of 540 subjects who were either health blood donors of Caucasian origin, not screened for psychiatric disorders (n = 273) or screened and psychiatrically healthy un-related individuals from the same ascertainment area as the recruited patients. The study was approved by the local Ethics Committee of the University of Würzburg. In

addition, 453 patients were ascertained from all over Norway, as described by Johansson (Johansson, Halleland et al. 2008) and Halleland (Halleland, Lundervold et al. 2009). Here the control group (n = 548) was comprised of 137 university students, 251 randomly selected people (in the age-range of 18 to 40 years) from the general population and 198 healthy blood donors (Franke, Neale et al. 2009).

Selection of adequate SNPs

Both, the 5´ and 3´ region of the analyzing genes were determined exactly by HapMap Genome Browser B35 (http://www.hapmap.org), whereas a putative promoter region (about 10.000 bp upstream exon 1) and an end region (about 3.000bp downstream the last exon) were included. By use of Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview) markers were selected automatically depending on adjustments (p-value cutoff = 0.001; minimum minor allele frequency = 0.001; r^2 threshold = 0.8) and was shown tabular and figurative (LD plot). Additional synonymous and non-synonymous SNPs with high population diversity found by genome-wide association studies (GWAS) were also included (http://www.ncbi.nlm.nih.gov).

PCR amplification

SNPs were investigated by the Sequenom iPlex® method (Sequenom, San Diego, CA). The principles of PCR were described before (see Material & Methods). Admittedly, both the configuration of the reaction batch and the cycles were modified in mass spectrometry. The used primers were created due to the selected SNPs by RealSNP™ Assay Database (http://www.realsnp.com) (Sequenom®GmbH, Hamburg, Germany). All primer sequences were available on request and were ordered by Metabion, Martinsried, Germany.

The PCR was performed in a 384 well plate following amplification in a Biometra thermocycler (Biometra, Goettingen, Germany):

Reagent	Volume
10x PCR buffer	0.625 µl
$MgCl_2$ (1.625 mM)	0.325 µl
dNTPs (500 µM each)	0.100 µl
Primer forward (500 nM)	1.000 µl
Primer reverse (500 nM)	1.000 µl
Genomic DNA (5 ng/µl)	2.000 µl
Hotstar Taq® (0.5 U/µl)	0.100 µl
H_2O	1.850 µl
Final volume	5.000 µl

Tab. 14a: PCR cocktail mix.

Temperature	Time	Cycles
94°C	15 min	1x
94°C	20 sec	45x
56°C	30 sec	
72°C	1 min	
72°C	3 min	1x
4°C	∞	

Tab. 14b: PCR cycles.

Each plate contained intern controls as well as DNA of two colleagues (Dr. Andreas Reif, Theresia Töpner).

SAP treatment

After PCR, unincorporated dNTPs were dephosphorylated via the enzyme shrimp alkaline phosphatase (SAP) and were therefore inactivated. Otherwise, needless nucleotides could extend in the primer extension reaction and cause contaminant peaks that greatly complicate data interpretation.

2 µl of a solution containing 0.17 µl 10x SAP buffer and 0.3 U SAP enzyme (Sequenom, San Diego, CA, USA) were added to each PCR reaction and incubated at 37°C for 20 min, followed by 5 min at 85°C to inactivate enzyme activity.

The SAP treated PCR reaction was incubated as follows in a standard thermocycler.

Temperature	Time	Cycles
37°C	20 min	1x
85°C	5 min	1x
4°C	∞	

Tab. 15: Incubation of SAP treatment.

Adjusting extension primers

When conducting multiplexing experiments, the concentration of oligos to equilibrate signal-to-noise ratios has to be adjusted. As masses increase, signal-to-noise ratios tend to decrease. A general method to adjust extension primers is to divide the primers into a low mass and a high mass group. All primers in the high mass group are doubled in concentration in contrast to the low mass group. Via special developed computer programs (http://www.realsnp.com/default.asp) extension primers were adjusted according to Sequenom iPLEX protocol to a final concentration of 0.625 µM for low mass primers and 1.25 µM for high mass primers in a reaction volume of 2 µl.

The iPLEX primer extension reaction was performed by a mix of three didesoxynucleotides and one desoxynucleotide. The first named cannot elongate after their integration by the enzyme thermosequenase due to the stop reaction according to Sanger.

Reagent	Volume
10x iPlex buffer	0.200 µl
iPlex termination mix	0.200 µl
dNTPs (2.5 mM each)	2.000 µl
Primer mix	0.804 µl
iPlex enzyme	0.041 µl
H_2O	0.755 µl
Final volume	2.000 µl

Tab. 16a: iPLEX cocktail mix.

The iPLEX reaction was cycled using a 2-step 200 short cycles program on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Forster City, CA, USA) with the following conditions:

Temperature	Time	Cycles
94°C	30 sec	1x
94°C	5 sec	5x / 40x
52°C	5 sec	
80°C	5 sec	
72°C	3 min	1x
4°C	∞	

Tab. 16b: iPLEX cycles.

To optimize mass spectrometric analysis the iPLEX reaction products were desalted. Via a nanodispenser the probes were transferred to a 286 dimple plate containing 6mg clean resin in each well. After dilution in 16 µl a.d., the plate was rotated manually for 20 min and afterwards spun down for 3 min at 3,000 rpm.

Dispensing to SpectroCHIP® Bioarray

By the use of the Sequenom Mass ARRAY Nanodispenser the reaction products were dispensed onto a 384-element SpectroCHIP bioarray. Further instructions can be seen in chapter 4 “High Troughput Dispensing” in the “MassARRAY Nanodispenser User´s Guide”.

MALDI-TOF MS analysis

Mass spectrometric analysis was carried out on a Bruker Autoflex time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA, USA). To process bioarrays, the MassARRAY workstation version 3.3. software was used. Software components and their respective versions are found in Material & Methods chap. 1.11. Computer systems.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was assessed for all available samples as well as chi-square-tests for frequency differences between cases and controls. The reported p-values are nominal, i. e. not adjusted for multiple testing, at the significance level of 0.05.

The procedure for the analysis of the GLUT3 and GLUT6 SNPs was divided into two steps. First, the SNPs were studied in pairs in regard to their common genotype distribution, using Fishers extract tests. An interaction existed if the p-value (FisherPx) was less than the GLUT3 or GLUT6 value. Relevant for further investigation are mainly these results where FisherPx is smaller than 0.05 or when FisherPx is relatively small and deviates sharply of the GLUT3 and GLUT6 value. These results were shown in graphics. The second part is the logistic regression including the interaction to explain the interaction out of step one.

2.6. TARGETING VECTOR CONSTRUCTION FOR KNOCKOUT MICE

Homologous recombination with exogenous DNA constructs is used to capture two genomic fragments into a compatible vector and is therefore the most powerful technique available for analyses and fundamental insights into mammalian gene functions. To circumvent the

embryonic lethality problem and to investigate gene function temporally *in vivo* and spatially, conditional knockout (cko) approaches have been developed over the past several years. The current cko strategy takes advantage of the bacteriophage-derived Cre-*lox*P site specific recombination system that functions well in mouse cells. In a typical cko allele, the critical exon(s) of a gene is flanked by two *lox*P sites that can be deleted by spatial and temporal *Cre* expression. So, gene targeting involves the inactivation of a given gene in the genome of totipotent embryonic stem (ES) cells. Transfer of mutant ES cells into early mouse embryos allows the transmission of the mutation in question into the mouse germline.

Ligation

Oppositely orientated mutant *lox*P sites (floxed exons 1 and 2 for the short arm (SA) rather 4 and 5 for the long arm (LA)) were synthesized by cutting out *Mlc1* via the restriction enzymes *Not* I and *Xho* I for SA and alternatively Bam I and EcoR I for LA and subcloned via ligation into the pPNT vector (see Fig. 12) containing a neomycin resistance gene which is under the control of the mouse phosphoglycerate kinase 1 gene (*Pgk-1*). We used a 1:2 molar ratio of vector:insert DNA when cloning the fragments consecutively into the plasmid vector. According to the recommendations of the manufacturer (Promega, Madison, USA) 100 ng vector DNA, 33 ng insert DNA, 5 µl 2x Rapid Ligation Buffer and 3 U T4 DNA Ligase were filled up with nuclease-free water to a total volume of 10 µl and incubated 5 min for cohesive-ended ligations.

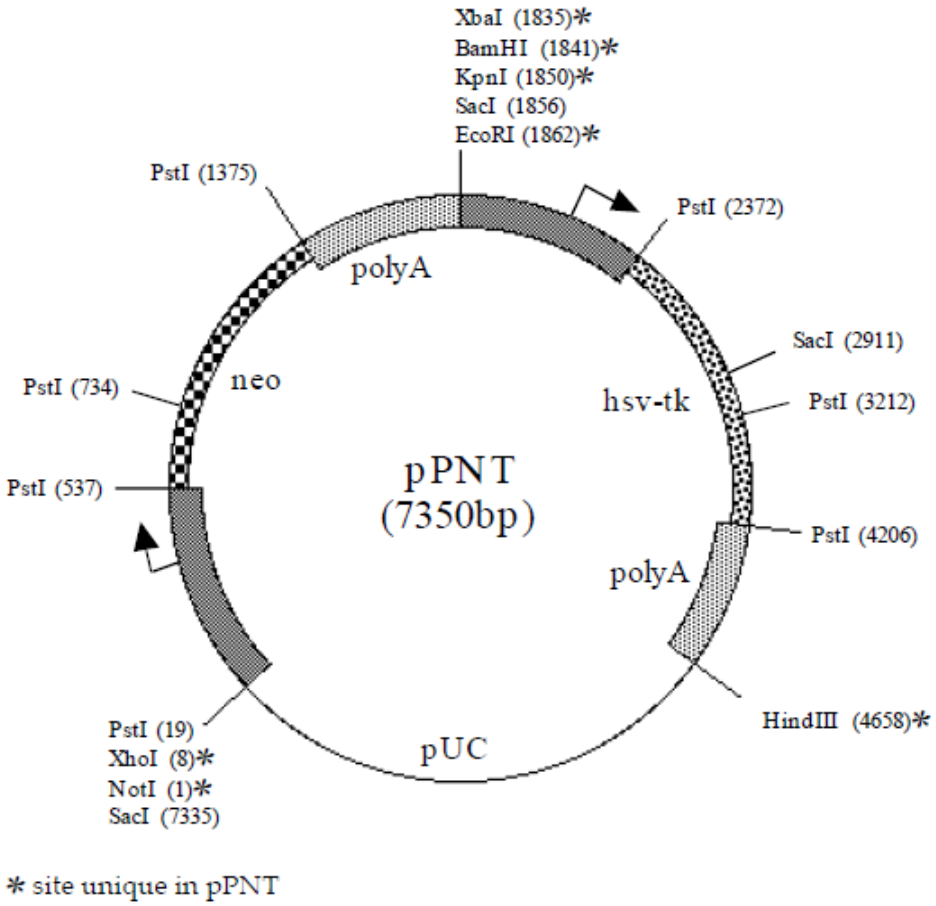


Fig. 12: pPNT vector map.

This gene targeting vector is based on the pUC/Bluescript vector. Elements are: PGK promoter, neomycin resistence gene (PGKneo cassette), PGK polyA site, hsv-tk gene and the unique *Not* I site for linearization. (Tybulewicz, Crawford et al. 1991)

Transformation

DH5α-FT™ competent cells (Invitrogen, Carlsbad, CA, USA) were thawed on wet ice. For DNA from ligation reaction, 1-10 ng of DNA was added to 100 µl competent cells, tapped to mix and incubated on ice for 30 min, followed by a heat-shock for 45 sec at 42°C and again 2 min on ice. 900 µl room temperature S.O.C. medium (Invitrogen, Carlsbad, CA, USA) was added and shaken at 225 rpm (37°C) for 1 h.

Selection of positive clones via colony screening

To find positive clones the transformation was spread on IPTG/X-gal LB-plates for blue/white selection and incubated overnight at 37°C. In the Prime-a-Gene® Labeling System (Promega, Madison, USA) a mixture of random hexadeoxyribonucleotides was used to prime the DNA synthesis *in vitro* from any double-stranded DNA template. The radioactive labeled DNA probe was produced by following protocol:

Reagent	Volume
Labeling 5x Buffer	10 µl
Mixture of unlabeled dNTPs	2 µl
Denatured DNA template	25 ng
Nuclease-free BSA	2 µl
[α-^{32}P]dCTP, 50 µCi, 3,000 Ci/mmol	5 µl
DNA Polymerase I, Klenow Fragment	5 units
Nuclease-free water to achieve a	
Final volume	50 µl

Tab. 17: Radioactive DNA labeling protocol.

The tube was incubated at room temperature for 60 min. Then the reaction was terminated by heating at 95-100°C for 2 min with subsequent chilling in an ice bath. 20 mM EDTA was added to use it directly for a hybridization reaction or to store at -20°C for later use.

Unincorporated, labeled nucleotides were removed by size exclusion chromatography using Sephadex® G-50 spin columns following the instructions of the manufacturer (Amersham Bioscience, Freiburg, Germany).

Colony/Plaque Screen™ are circles of a supported, positively charged nylon membrane. These dry membrane discs were placed carefully onto the agar plates. After 2 - 3 min the disc with colony side up was laid two times into a pool of 0.75 ml 0.5 N NaOH on a plastic

wrap for 2 min. On a new sheet of plastic wrap 0.75 ml 1.0 M Tris-Hcl, pH 7.5 was pipetted, the disc was then placed in the same direction as before and repeated.

Before prehybridization the ExpressHyb Hybridization Solution (Clontech Laboratories, Saint-Germain-en-Laye, France) was warmed up to 60°C. The dried membranes were put in a heat sealable bag with about 5 ml ExpressHyb solution and heated with continuous shaking at 60°C for 30 min.

Meanwhile, the radioactive labeled carrier DNA was denatured for 2 - 5 min at 95 - 100°C and chilled on ice for at least 15 min before adding 5 ml fresh prehybridization buffer to the bag. The membranes were agitated overnight at 60°C.

The next day the membranes were rinsed repeatedly in wash solution 1 (2x SSC, 0.05% SDS) for 30 min at room temperature replacing the wash solution several times to remove non-specifically bound probes. After each wash the blots were monitored for background. Then the plots were washed again under continuous shaking for 40 min at 50°C with wash solution 2 (2x SSC, 0.1% SDS). After the final rinse, the damp membranes were wrapped securely in plastic wrap. Finally the blots were exposed to x-ray film at -80°C with two intensifying screens.

Electroporation

Positive clones were picked and incubated in LB medium containing 100 µg/ml ampicillin under permanent shaking at 37°C overnight. Via Wizard® *Plus* SV Minipreps DNA Purifiction System using a vacuum (Promega, Madison, USA) the plasmids were isolated and linearized by the 1-cut endonuclease *Not* I.

The targeting vector was electroporated into ES cells kindly supported by the Institute for Clinical Neurobiology, University Würzburg. Finally, the ES clones with correct targeting events should be identified by PCR (used primers see Material & Methods chap. 1.4. Desoxyribonuceotides).

III. RESULTS

1. GENOMIC COPY NUMBER VARIATIONS IN ADHD

1.1. ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

Using sub-megabase resolution BAC array CGH, a cohort of 99 children and adolescents diagnosed with ADHD were screened for the presence of non-polymorphic copy number variations (CNV). Approximately 75% of the patients (14 children) were characterized by a family history of ADHD. Using stringent criteria for data analysis (see Material & Methods 2.3.), a total of eleven duplications and two deletions were identified. These aberrations are likely disease-associated, based on the fact that they are not documented reference datasets. One of these variations was confirmed to be *de novo*, while seven were inherited from an affected parent. These variations are summarized and the affected genes are listed in Tab. 18a. Except for one patient in whom inheritance could not be determined, the remaining variations falling into this category were inherited from an unaffected parent (Tab.18b). The tables include the CNV boundaries, and implicated genes are listed. For additional comparison, we indicate the number of times a similar CNV has been described in the DoGV.

Patient	**Family history**	**Var.**	**Chr.**	**Inheritance**	**Physical position (Mb)***	**Shaikh et al./DoGV****	**Genes**
991, m	-	Del	3q26.1	De novo	166.944967 - 168.896272	0/0	***BCHE**, ZBBX, SERPINI2, WDR49, PCD10*
1421, m	+	Dup	4q12	Parental (affected)	53.18-53.91	0/0	*USP46, KIAA0114, **RASL11B**, SCFD2*

Patient	Family history	Var.	Chr.	Inheritance	Physical position (Mb)*	Shaikh et al./DoGV**	Genes
201, m	+	Dup	5q11.2	Maternal (affected)	58.263673-58.339293	0/0	***PDE4D***
241, m	+	Del	5q12.1	Maternal (affected)	60.069539-60.353244	0/0	*ELOVL7, ERCC8,* ***NDUFA12L***
21, m	+	Dup	5q13.3	Parental (affected)	75.590060-75.782477	0/0	***SV2C****, IQGAP2*
1671, f	+	Dup	7p15.2-15.3	Paternal (affected)	23.01-26.07	0/0	*NUPL2, GPNMB, IGF2BP3, RPS2P32, TRA2A, CLK2P, CCDC126,C7orf46, STK31,* ***NPY****, MPP6, DFNA5, OSBPL3, CYCS, C7orf31, NPVF, NFE2L3, HNRPA2B1, CBX3*
51, m	+	Dup	11q13.4	Maternal (affected, also in affected sibling)	72.90-73.40	0/0	*FAM168A,* ***PLEKHB1****,* ***RAB6A****, MRPL48, CHCHD8, WDR71, DNAJB13, UCP2, UCP3* (none in DoGV)
701, f	+	Dup	17q25.1	Maternal (affected)	69.25-70.18	0/4	C17orf54, RPL38, TTYH2, DNAI2, KIF19, LOC388419, GPR142, GPRC5C, CD300A, CD300LB, CD300C, C17orf77, CD300E

Tab. 18a: *De novo* and co-segregating CNVs not present in the reference dataset.

Bold = potential candidate genes.

Patient	Family history	Var.	Chr.	Inheritance	Physical position (Mb)*	Shaikh et al./DoGV **	Genes
1191, m	-	Dup	2p25.3	Maternal (healthy)	0.66-0.94	0/4	*TMEM18, SNTG2*
441, f	-	Dup	4p14	Maternal (healthy)	36.31-37.03	0/0	No genes
461, m	-	Dup	4q26	Maternal (healthy)	114.833251 - 115.343086	0/0	***CAMK2D**, ARSJ*
431, m	-	Dup	Xq12	Not determined	65.4-65.6	0/1	*EDA2R*
471, m	-	Dup	Xq21.33	Maternal (healthy)	93.81-94.64	0/0	No genes

Tab. 18b: Other variations not observed in the reference datasets.

Bold = potential candidate genes.

*Chromosome coordinates according to HG18 (NCBI36) based on BAC clone or oligo hybridisation results; for BAC clone hybridisation results, coordinates were rounded appropriately in order to reflect inherent limitations in determining precise CNV boundaries.

**Number of corresponding CNVs in 2026 healthy individuals published by Shaikh et al. (Shaikh, Gai et al. 2009)/ number of corresponding CNVs in the Database of Genomic Variants (DoGV, including those from Shaikh et al. (Shaikh, Gai et al. 2009) as of Aug 21, 2009 (Iafrate, Feuk et al. 2004).

Finally, we detected an additional two duplications and two deletions that we consider potentially syndrome-associated despite the fact that they did not meet the high stringency threshold scores because they were also observed at low frequency in one or both of the reference datasets (Tab. 19). All aberrations were definite as all have been verified either directly by oligo array or indirectly by analysis of parental DNA. None were observed at high frequencies in other patient cohorts, suggesting that they may indeed be risk factors for

ADHD. One of these CNVs was *de novo*, two were inherited from affected parents, and one was inherited from a health person.

Patient	Family history	Var.	Chr.	Inheritance	Physical position (Mb)*	Shaikh et al./DoGV **	Genes
1761, m	-	Del	6q16.1	*De novo*	95.447226-95.664033	1/4	No genes
131, m	-	Dup	8q11.1	Parental (healthy)	47.61-47.98	1/1	BEYLA
1141, f	+	Del	9p21.3	Parental (affected)	25.217246-25.336971	1/4	No genes
211, m	+	Dup	12p13.31	Maternal (affected)	7.894681-8.009303	11/37	*SLC2A14,* ***SLC2A3***

Tab. 19: CNVs present in healthy controls at low frequency or affecting genes with independent support for disease association.

Bold = potential candidate genes.

*Chromosome coordinates according to HG18 (NCBI36) based on BAC clone or oligo hybridisation results; for BAC clone hybridisation results, coordinates were rounded appropriately in order to reflect inherent limitations in determining precise CNV boundaries.

**Number of corresponding CNVs in 2026 healthy individuals published by Shaikh et al. (Shaikh, Gai et al. 2009) / number of corresponding CNVs in the Database of Genomic Variants (DoGV, including those from Shaikh et al.(Shaikh, Gai et al. 2009) as of Aug 21, 2009 (Iafrate, Feuk et al. 2004).

Among apparent candidates is the gene encoding neuropeptide Y (*NPY*) contained in a duplication on chromosome 7p15.2-15.3 (described in detail in the next section). Further candidates included genes expressing acetylcholine-metabolising butyrylcholinesterase (*BCHE*) involved in a *de novo* chromosome 3q26.1 deletion in an individual severely affected

with ADHD, and a brain-specific pleckstrin homology domain-containing protein (*PLEKHB1*), with an established function in primary sensory neurons, in two siblings with severe ADHD carrying a 11q13.4 duplication inherited from their affected mother. Other potentially disorder-causing genes involved in confirmed aberrations and inherited from affected parents include the genes for the mitochondrial NADH dehydrogenase 1 alpha subcomplex, assembly factor 2 (*NDUFAF2*), the brain-specific phosphodiesterase 4D isoform 6 (*PDE4D6*) (Fig. 13), and the neuronal glucose transporter 3 (*SLC2A3*).

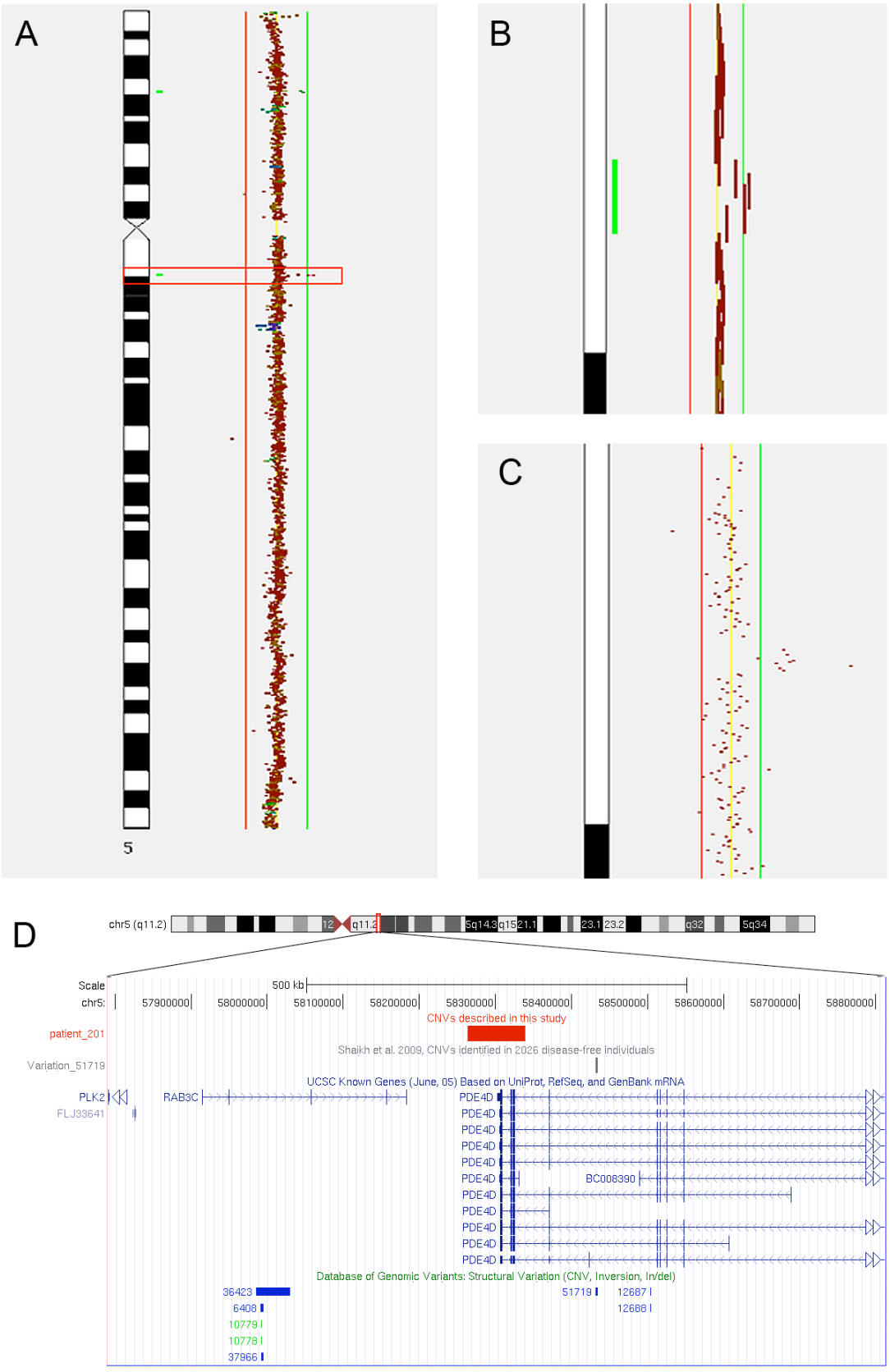

A
B
C
5
D
chr5 (q11.2)
12
q11.2
5q14.3
q15
21.1
23.1
23.2
q32
5q34
Scale
500 kb
chr5:
57900000
58000000
58100000
58200000
58300000
58400000
58500000
58600000
58700000
58800000
CNVs described in this study
patient_201
Shaikh et al. 2009, CNVs identified in 2026 disease-free individuals
Variation_51719
UCSC Known Genes (June, 05) Based on UniProt, RefSeq, and GenBank mRNA
PLK2
RAB3C
FLJ33641
PDE4D
BC008390
Database of Genomic Variants: Structural Variation (CNV, Inversion, In/del)
36423
6408
10779
10778
37966
51719
12687
12688

Fig. 13: Duplication of 5q11.2 in patient 201.

A, schematic view of chromosome 5, with mapped genomic clones depicted to the right. For each BAC clone, Cy3/Cy5 signal intensity ratios are plotted alongside the chromosome. Red and green lines correspond to log2 ratios -0.3 (loss) and 0.3 gain), respectively. The region encompassing the aberration is highlighted by a red rectangular. **B**, closer view of the relevant region. **C**, ratio plot of the corresponding verification experiment using a 244k oligonucleotide array. **D**, UCSC screenshot epicting genomic region chr5:57,791,039-58,811,926 (HG17). The red bar indicates the location of the duplication identified in patient 201. Grey bars in the custom track below represent CNVs detected in 2026 control individuals by Shaikh et al. The specific identifying number is given on the left. Genes and their positions are indicated below these. Finally, all variations observed in the Database of Genomic Variants (DoGV) are included at the bottom of each panel for reference. These variations are colour-coded according to DoGV convention to reflect gain (red), loss (blue), or gain/loss (green). Noteworthy, the CNV identified in patient 201 includes the complete brain-specific PDE4D6 isoform described by Wang et al., while all other CNVs are located within intronic regions.

1.2. PHENOTYPE OF THE 7q15 DUPLICATION IN A MULTIGENERATIONAL PEDIGREE

Based on the findings in the initial patient cohort resulting in the identification of a ~3 Mb duplication located on chromosome 7q15.2-15.3 (Fig. 14), we ascertained the extended multigenerational pedigree (displaying a high density of ADHD) of the index patient to further investigate the phenotypical consequences of an additional copy of the NPY gene.

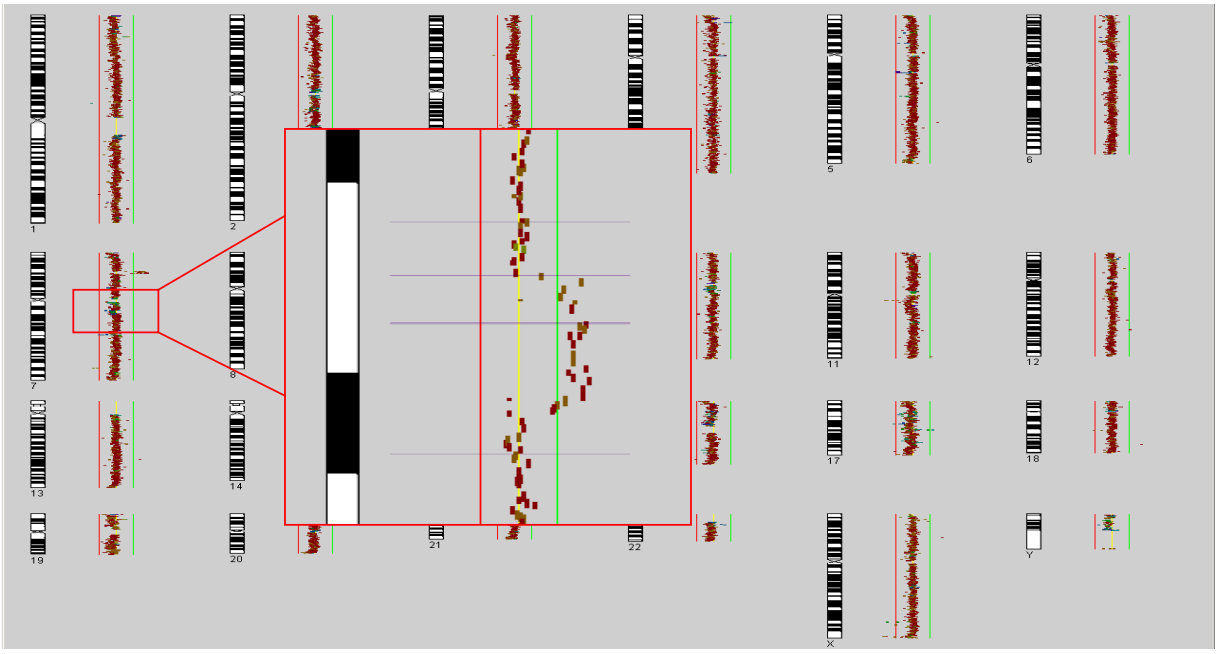


Fig. 14: Array CGH result for patient F3-4 using BAC-Array.

Data analysis and visualization was performed by CGHPRO. Cy3 and Cy5 signal intensity ratios are given for each BAC clone. Red and green lines correspond to log2 rations -0.3 (loss) and 0.3 (gain). Insert: closer view of the duplication of 7q15.3.

Using array CGH, the described duplication was detected in several additional family members throughout three generations (Fig. 15). It is inherited from individual F1-2 of the first generation and 8 out of 12 affected family members of the F2 and F3 generation are also carriers. All individuals carrying the duplication are affected by ADHD, whereas in four affected descendants of the F1 generation no chromosomal rearrangement was detected at 7q15 suggesting a bilineal transmission of the syndrome in this family, as F1/1 also suffered from ADHD. Assuming that the 7q15 duplication may influence the development of ADHD and further phenotypes such as BMI, binge eating, and NPY plasma concentration, we additionally conducted FBAT for these phenotypes.

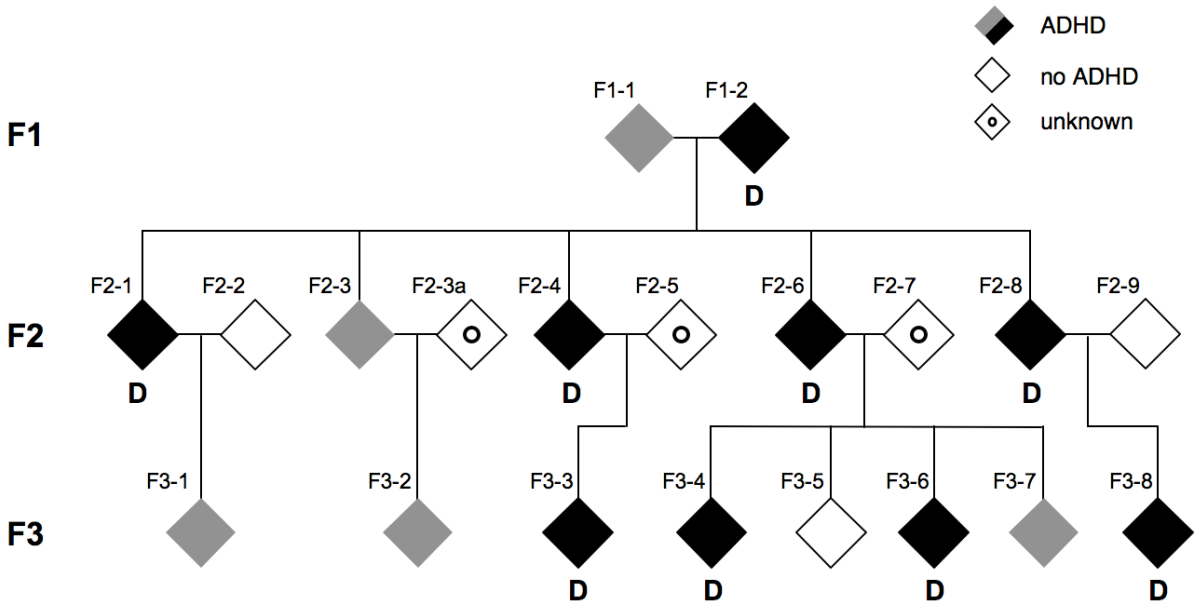


Fig. 15: Segregation of the chromosome 7p15.2-15.3 duplication (D) in a multigenerational family with diagnosed ADHD.

Affected members are symbolized by solid black symbols when the duplication is present, and by solid grey when absent; unaffected members are identified by open symbols. Unknown clinical status is indicated by a circle. DNA of individuals F2-2, F2-3a and F2-5 was not available for analyzes.

Tab. 20a displays the clinical phenotype in carriers and non-carriers with respect to ADHD, food intake and obesity-related parameters as well as NPY plasma concentrations. Tab. 20b describes these phenotypes in relation to the transmission pattern of the 7p15 duplication. NPY plasma concentrations were significantly higher in offsprings having inherited the 7p15 duplication than in non-carriers (empirical FBAT, p = 0.023; median NPY level 78.5 versus 46.6 pmol/L; Tab. 20b, Fig. 16). There was a trend towards a preferable transmission of the 7p15 duplication to affected family members (empirical FBAT, p = 0.138, 8 transmissions versus 3 nontransmissions) and binge eating (empirical FBAT, p = 0.117, 6 transmissions versus 1 non-transmissions). However, these results did not reach an overall significance level if corrected by Bonferroni's approach. Finally, the empirical FBAT for BMI indicated no association with this trait (p = 0.192).

Phenotypes	Duplication	
	Carriers	Non-carriers
ADHD		
affected	9	5
non-affected	0	3
unknown	0	3
Binge eating disorder		
yes	7	1
no	2	7
unknown	0	3
BMI (n; median; range)	9; 29.8; 20.8-42.7	10; 24.4; 17.4-36.6
NPY (n; median; range)	9; 73.9; 53.9-136.5	10; 46.6; 30.5-69.9

Tab. 20a: Distribution of relevant phenotypes in family members with or without the 7p15.2-15.3 duplication.

Phenotypes	Duplication		Nominal empirical[1] FBAT p-value
	transmitted	non-transmitted	
ADHD			
affected	8	3	0.138
not affected	0	1	
Binge eating behavior			
yes	6	1	0.117
no	2	3	
BMI (n; median; range)	8; 28.4; 8-42.7	4; 21.3; 17.4-36.3	0.192
NPY (n; median; range)	8; 78.5; 53.9-136.5	4; 46.6; 43.3-50.3	0.023

Tab. 20b: Investigation of association between relevant phenotypes and the 7p15.2-15.3 duplication.

[1] Test based on 10.000 simulations.

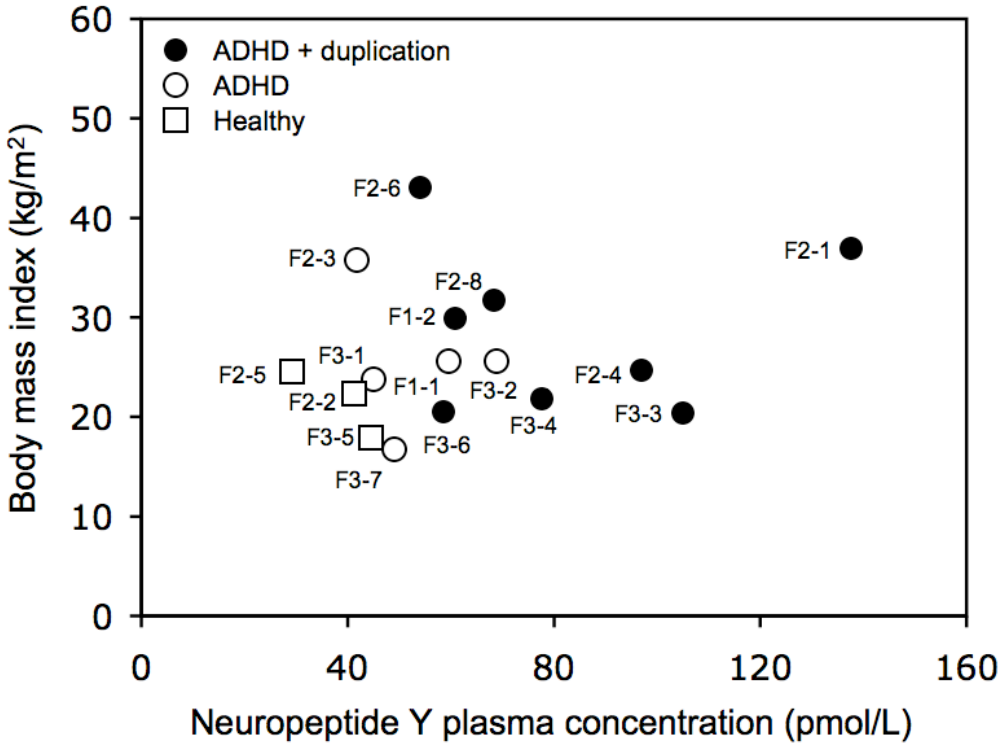


Fig. 16: Neuropeptide Y (NPY) plasma concentrations blotted against the body mass index (BMI) in 7p15.2-15.3 duplication carriers with ADHD, non-carriers with ADHD, and healthy family members.

F numbers allow allocation to the pedigree.

The effect of the 7p15 duplication and gene dose-dependent increase in NPY plasma concentrations on brain function was explored by fMRI in four carriers with ADHD compared to healthy controls. Region of interest analyses revealed a significantly lower activation of the left ventral striatum during the anticipation of large rewards for duplication carriers than for controls ($p < 0.05$, corrected; Fig. 17, upper panels). A significantly lower activation of the left posterior insula during the anticipation of large losses was also observed in carriers compared to controls ($p < 0.05$, corrected; Fig. 17, lower panels). In none of the two regions, a significant difference between carriers and controls was observed for no or small rewards or losses. Furthermore, activation for the carriers never exceeded the controls' responses in those two structures.

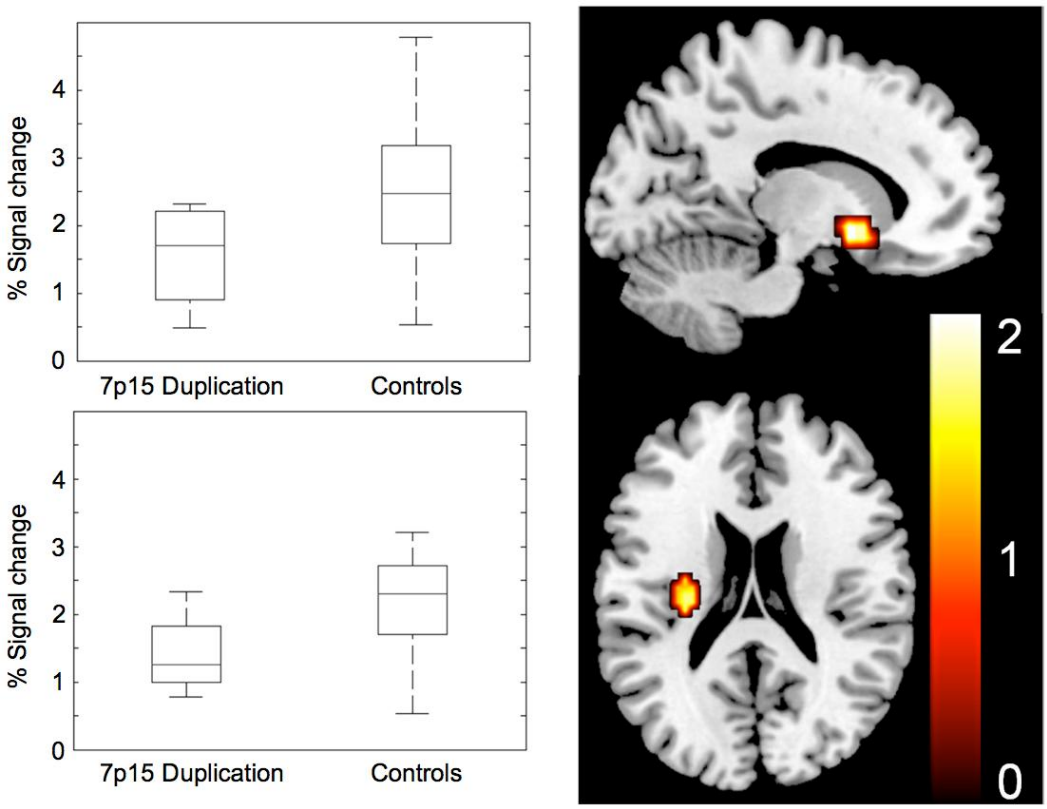


Fig. 17: Neural activation in the ventral striatum during the anticipation of large rewards (upper panel) and in the posterior insula during the anticipation of large losses (lower panel) for 7p15.2-15.3 duplication carriers with ADHD (n = 4) and healthy controls (n = 21).

Brain maps show significant –log10-transformed p values ($p < .05$, corrected) in the left ventral striatum (upper right panel) and in the left Posterior insula (lower right panel). Boxplots show medians, 25th and 75th percentiles and most extreme signal changes (whiskers extend to the most extreme subject values) corresponding to the brain maps of the ventral striatum (upper left panel) and the posterior insula (lower left panel).

2. LINKAGE ANALYSIS

2.1. GLUCOSETRANSPORTER 3 AND 6

Also by applying array CGH to the same cohort of 110 ADHD patients a noticeable duplication was found on chromosome 12q13.31. This locus contains the gene coding for the glucose transporter 3 (*GLUT3, SLC1A3*) know to facilitate the neural glucose transport. Interestingly, we could identify an isoform of *GLUT3*, namely *GLUT6*, as a relevant candidate gene in a GWAS adult ADHD (Lesch, Timmesfeld et al. 2008). In order to further examine the association between *GLUT3*, *GLUT6* and ADHD in greater detail we performed a fine-mapping of polymorphisms in the human *GLUT3* and *GLUT6* genes including their 5´ and 3´ regions by conducting a case-control association analysis in adult ADHD as well as a TDT analysis in a family based ADHD sample.
For *GLUT3*, five SNPs, for *GLUT6* 10 SNPs were chosen by Haploview (www.broad.mit.edu/mpg/haploview/*; version 3.32*) (Tab. 21 / 22).

Gene	Chr.	SNP	Chromosome localization*	Allele
GLUT3	12	rs12842	8072008	C/T
GLUT3	12	rs741361	8075685	A/G
GLUT3	12	rs2244822	8088227	C/T
GLUT3	12	rs933552	8090703	G/T
GLUT3	12	rs7309332	8090839	C/T

Tab. 21: Used *GLUT3* markers in ADHD.
SNPs were chosen by Haploview version 3.32. The underlined alleles are ancestral.
* University of California, Santa Cruz (UCSC) May 2004 National Center for Biotechnology Information (NCBI) Build 35.

Gene	Chr.	SNP	Chromosome localization*	Allele
GLUT6	9	rs4962153	136323754	A/G
GLUT6	9	rs739467	136326054	G/T
GLUT6	9	rs756820	136326857	A/G
GLUT6	9	rs3124765	136328657	A/G
GLUT6	9	rs2073935	13634200	A/C
GLUT6	9	rs968471	136344613	C/T
GLUT6	9	rs3124758	136344853	C/T
GLUT6	9	rs736417	136359085	C/T
GLUT6	9	rs17810852	13636575	A/G
GLUT6	9	rs9331726	136368685	G/T

Tab. 22: Used *GLUT6* markers in ADHD.

SNPs were chosen by Haploview version 3.32. The underlined alleles are ancestral.

* University of California, Santa Cruz (UCSC) May 2004 National Center for Biotechnology Information (NCBI) Build 35.

The distribution equilibrium of these two genes was shown in a LD-block. The LD-block of *GLUT3* consists of two blocks; block1 comprises the marker rs663303, rs741361 and rs2244822, whereas block 2contains rs933552 and 7309332 (Fig.18a).

On the contrary, the LD-block of *GLUT6* revealed three blocks. Here, block 1 contains rs4962153 to rs2073935, block 2 rs9368471 and rs3124758, and block 3 rs736417 to rs9331726 (Fig. 18b).

All SNPs in one block are transmitted mostly together.

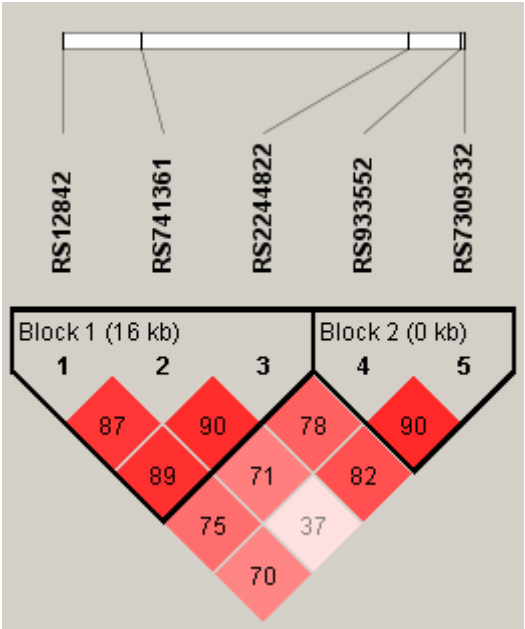


Fig. 18a: Linkage disequilibrium map for *GLUT3*.
LD map of SNP Markers created using HAPLOVIEW version 3.32. Only markers selected by HAPLOVIEW are shown. The dark squares represent higher r^2 values; triangles surrounding markers represent haplotype blocks under the 4 gamete rule.

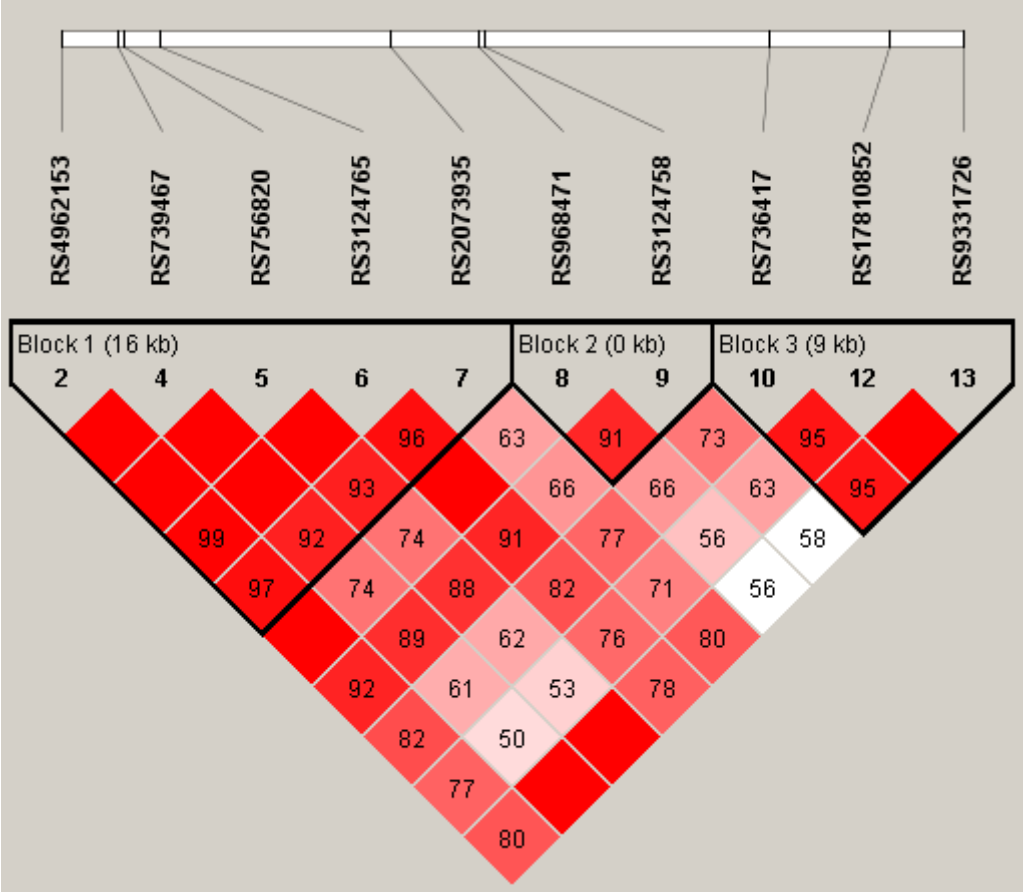


Fig. 18b: Linkage disequilibrium map for *GLUT6*.

LD map of SNP Markers created using HAPLOVIEW version 3.32. Only markers selected by HAPLOVIEW are shown. The dark squares represent higher r^2 values; triangles surrounding markers represent haplotype blocks under the 4 gamete rule.

2.2. GENOTYPING OF *PLEKHB1*, *RAB6A* AND *PDE4D*

For examination of an association between the candidate genes *RAB6A*, *PLEKHB1* and alternatively *PDE4D*, found by array CGH, and ADHD several SNPs (four *RAB6A*SNPs, eight *PLEKHB1SNPs* and ten *PDE4D* SNPs) were analyzed in a case-control study. We genotyped these variants in a sample of 450 HKS probands, 200 families with almost one

child affected by ADHD according to DSM-IV criteria and 90 controls. For all probands all SNPs were ascertained.

PLEKHB1 and *RAB6A,* analyzed together due to their localization side by side on chromosome 11, were located in three haplotypes blocks, whereas *PDE4D* markers were in two haplotype blocks (not shown). The degree of LD varied among the SNPs examined. This most likely is a factor of the wide distribution of the SNPs, the large genomic size of the analyzed genes and the complex linkage disequilibrium structure. All analyzed marker weres in the Hardy-Weinberg equilibrium (HWE).

PLEKHB1 and RAB6A

In total, 59 *PLEKHB1/RAB6A* markers span 134.978 bp (including 10.000 bp upstream and downstream). 11 SNPs were chosen by Haploview (www.broad.mit.edu/mpg/haploview/*;* version 3.32), three others ones were included because of their occurrence in the population (Tab. 23). The distribution equilibrium of these two genes has shown three LD-blocks (Fig. 19). Block 1 comprises the marker rs663303 to rs591804, block 2 rs6592527 and rs940828, block 3 rs10736793 to rs7127066. All SNPs in one block are transmitted mostly together. Only rs3741147 and rs12274970 are transmitted separately and are not linked to the others.

Gene	Chr.	SNP	Localization	Chromosome localization*	Allele
	11	rs663303	5´UTR	73029494	C/T
PLEKHB1	11	rs4944850	5´UTR	73033744	A/C
PLEKHB1	11	rs11538627	Intron 4	73040539	A/T
PLEKHB1	11	rs591804	Intron 4	73040858	A/G
PLEKHB1	11	rs6592527	Intron 5	73042016	C/G
PLEKHB1	11	rs940828	Intron 5	73043807	G/T
PLEKHB1	11	rs3741147	Intron 5	73044399	G/T
	11	rs12274970		73055790	C/T
RAB6A	11	rs3182788	3´UTR	73066620	A/C
RAB6A	11	rs10736793	Exon 8	73080224	A/C
RAB6A	11	rs3203705	Intron 6	73107544	C/T
RAB6A	11	rs11235876	Intron 3	73108249	A/G
RAB6A	11	rs11235880	Intron 2	73112944	A/C
RAB6A	11	rs7127066	Intron 1	73136522	C/G

Tab. 23: Used *PLEKHB1* and *RAB6A* markers in ADHD.

SNPs were chosen by Haploview version 3.32, underlined SNPs were added afterwards. The underlined alleles are ancestral.

* University of California, Santa Cruz (UCSC) May 2004 National Center for Biotechnology Information (NCBI) Build 35. UTR, untranslated region.

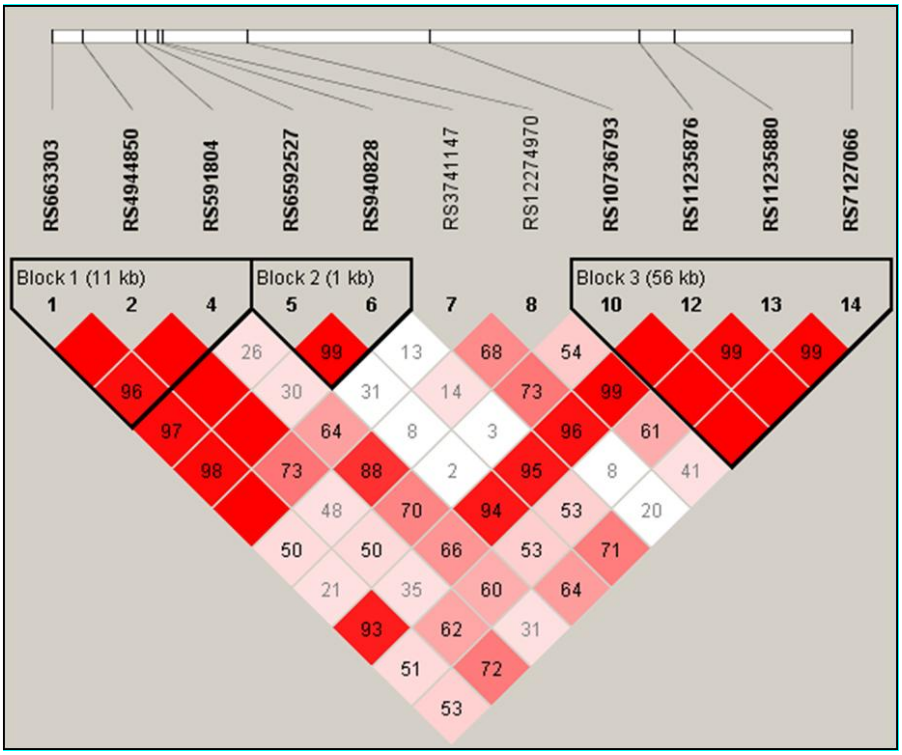


Fig. 19: Linkage disequilibrium map for *PLEKHB1* and *RAB6A*.
LD map of SNP Markers created using HAPLOVIEW version 3.32. Only markers selected by HAPLOVIEW are shown. The dark squares represent higher r^2 values; triangles surrounding markers represent haplotype blocks under the 4 gamete rule.

Two markers of *PLEKHB1* (rs6592527 and rs940828) within intron 5 and one marker of *RAB6A* (rs3182788), located in exon 8, archived a statistical significance in Fisher´s exact test ($p < 0.1$) (Tab. 24). Notable, rs3182788 also showed a strong significant HWE (case, $p = 0.0$ vs. control, $p = 0.0577$; data not shown) as well as in the χ^2 test (Tab. 24). The genomic distribution of the other polymorphisms did not deviate significantly from HWE in both patient and controls ($p < 0.05$).

Gene	SNP	HWE_P Control	HWE_P Case	HWE_P total	χ2 Control	χ2 Case	χ2 total	P value	P value combSubt
	rs663303	0.025	0.254	0.242	0.875	0.614	0.623	0.459	0.231
PLEKHB1	rs4944850	0.568	0.343	0.013	0.452	0.558	0.911	0.919	0.572
PLEKHB1	rs11538627	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
PLEKHB1	rs591804	0.025	0.077	0.102	0.875	0.781	0.750	0.478	0.385
PLEKHB1	rs6592527	0.923	0.298	0.984	0.334	0.585	0.321	0.075	0.100
PLEKHB1	rs940828	0.788	0.159	0.694	0.375	0.691	0.405	0.078	0.118
PLEKHB1	rs3741147	0.024	0.580	0.446	0.877	0.446	0.504	0.357	0.181
	rs12274970	0.232	0.058	0.268	0.670	0.810	0.612	0.784	0.655
RAB6A	rs3182788	18.406	3.603	15.364	0.0	0.058	0.0	0.000	0.000
RAB6A	rs10736793	0.170	0.001	0.102	0.680	0.970	0.749	0.665	0.705
RAB6A	rs3203705	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
RAB6A	rs11235876	1.667	0.928	2.513	0.197	0.335	0.113	0.975	0.753
RAB6A	rs11235880	0.310	0.052	0.053	0.578	0.820	0.817	0.945	0.639
RAB6A	rs7127066	0.336	3.123	2.922	0.562	0.073	0.087	0.793	0.729

Tab. 24: Hardy-Weinberg equilibrium, chi-square-tests for frequency differences between cases and controls and P value of the *PLEKHB1* and *RAB6A* markers in ADHD.

NaN: Not a Number; red: significant results

PDE4D

12 *PDE4D* markers out of a calculable possible 66 SNPs (chosen by Haploview, www.broad.mit.edu/mpg/haploview/; version 3.32), spanning 49.910 bp (including 10.000bp upstream and downstream) on chromosome 5, were tested (Tab. 25). Rs17291089 to rs12656462 in block 1 as well as rs1005662 to rs7714708 in block 2 were transmitted mostly together. The LD-plot of this gene is shown in Fig. 20, the genomic localization is listed in Tab. 25.

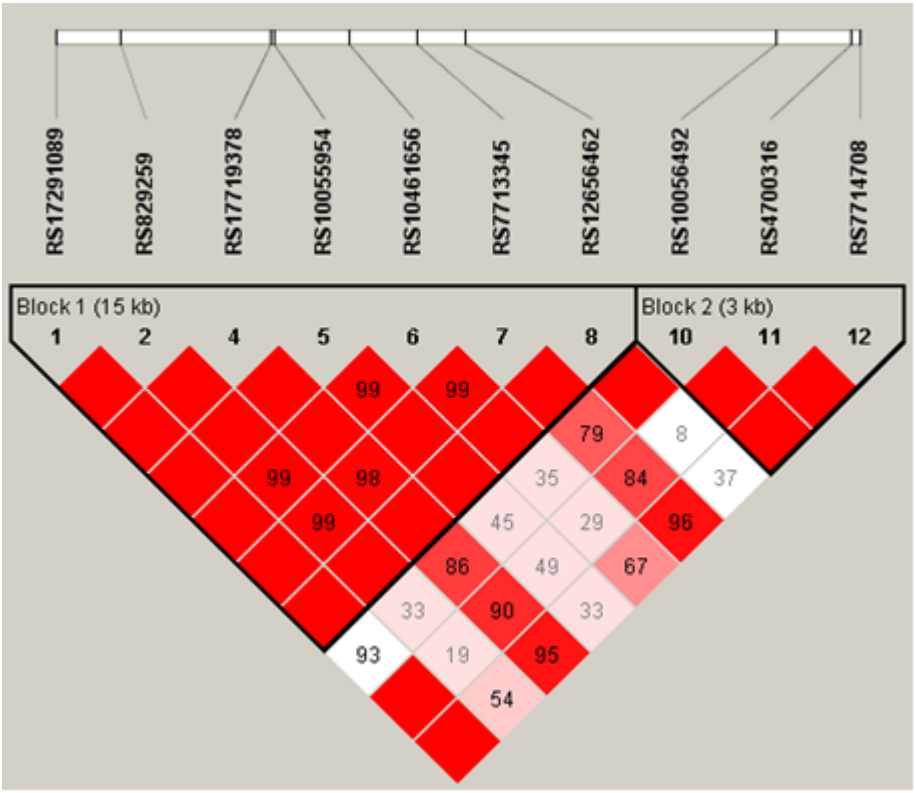


Fig. 20: Linkage disequilibrium map for PDE4D.
LD map of SNP markers created using HAPLOVIEW. Only 10 selected markers are shown. The dark squares represent higher r^2 values; triangles surrounding markers represent haplotype blocks under the 4 gamete rule.

. Gene	Chr.	SNP	Chromosome localization*	Allele
PDE4D	5	rs17291089	58301362	G/T
PDE4D	5	rs829259	58303733	A/T
PDE4D	5	rs1058458	58306373	C/T
PDE4D	5	rs17719378	58309205	C/T
PDE4D	5	rs10055954	58309342	C/G
PDE4D	5	rs10461656	58312107	C/T
PDE4D	5	rs7713345	58314588	C/G
PDE4D	5	rs12656462	58316381	A/T
PDE4D	5	rs17853590	58320100	A/G
PDE4D	5	rs10056492	58327740	A/G
PDE4D	5	rs4700316	58330448	C/G
PDE4D	5	rs7714708	58330771	A/G

Tab. 25: Used *PDE4D* markers in ADHD.

SNPs were chosen by Haploview version 3.32. The underlined alleles are ancestral.

* University of California, Santa Cruz (UCSC) May 2004 National Center for Biotechnology Information (NCBI) Build 35.

As expected, no marker has a statistical significance in HWE. Also no marker shows a significant P value ($p < 0.05$) (Tab. 26). In summary, there were no differences in genotype or allele frequencies between cases and controls.

Gene	SNP	HWE_P Control	HWE_P Case	HWE_P total	χ2 Control	χ2 Case	χ2 total	P value	P value combSubt
PDE4D	rs17291089	1.700	0.393	0.144	0.192	0.531	0.704	0.554	0.387
PDE4D	rs829259	0.817	0.083	0.672	366	0.774	0.412	0.403	0.602
PDE4D	rs1058458	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
PDE4D	rs17719378	0.897	0.025	0.273	0.344	0.874	0.602	0.602	0.414
PDE4D	rs10055954	0.246	0.561	0.038	0.620	0.454	0.846	0.077	0.066
PDE4D	rs10461656	0.293	0.874	1.038	0.588	0.350	0.308	0.164	0.184
PDE4D	rs7713345	0.0518	0.504	0.456	0.820	0.478	0.500	0.956	0.771
PDE4D	rs12656462	0.149	0.836	0.859	0.699	0.360	0.354	0.217	0.139
PDE4D	rs17853590	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
PDE4D	rs10056492	0.225	0.087	0.296	0.635	0.768	0.586	0.793	0.815
PDE4D	rs4700316	0.309	0.266	0.559	0.579	0.606	0.455	0.411	0.299
PDE4D	rs7714708	1.263	0.725	0.022	0.261	0.395	0.882	0.359	0.415

Tab. 26: Hardy-Weinberg equilibrium, chi-square-tests for frequency differences between cases and controls and P value ($p < 0.05$) of the *PDE4D* markers in ADHD.

NaN: Not a Number

2.3. THE SYNAPTIC VESICLE PROTEIN 2C

Three SNPs (rs1862519, rs30196 and rs30198) in the promoter region of *SV2C* (Fig. 21) which may be associated with ADHD were chosen for an association linkage analysis by Haploview (www.broad.mit.edu/mpg/haploview/; version 3.32).

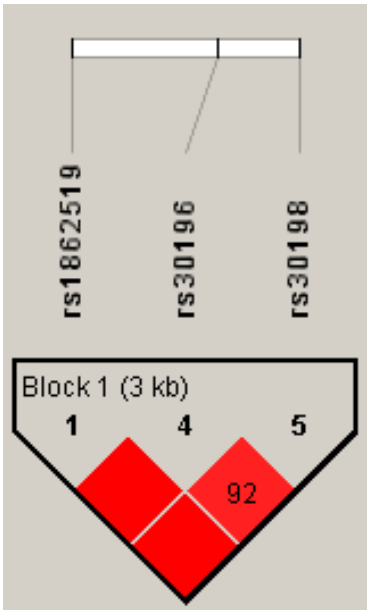


Fig. 21: Linkage disequilibrium map for the promoter region of *SV2C*.
LD plot was created using HAPLOVIEW.
The dark squares represent higher r^2 values; triangles surrounding markers represent haplotype blocks under the 4 gamete rule.

A haplotype analysis using 200 nuclear families, identified through a proband child with ADHD according to DSM-IV criteria derived from the Department of Child and Adolescent Psychiatry and Psychotherapy and the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, was tested for associations with ADHD. Allele frequencies for all markers showed a significant deviation according to Hardy-Weinberg

equilibrium (HWE) (Tab. 27) in the case of the parents which argue for a genotypical failure. A revision exhibited the same results again.

	p-value	
SNP	**HWE_P**	**HWE_C**
rs1862519	0.9884	0.174
rs30196	0.0442*	0.2357
rs31098	0.0025*	0.2691

Tab. 27: Hardy-Weinberg equilibrium (HWE) in parents (P) and children (C).
* significant; P = permutation based p value with $p < 0.05$ considered significant

Transmission disequilibrium test (TDT) analyses of haplotypes were performed on the total sample (p-value = 0.8764). Three haplotypes were not used for TDT analyses because of their rare appearance. As shown in Tab. 28, neither haplotype was significantly associated with the disease.

Marker						
rs1862519	rs30196	rs31098	Frequency (%)	T	NT	OR
G	G	G	58.897	79,951	70	1,142
C	T	A	18.047	37,952	37	1,026
G	G	A	0.248			
G	T	A	20.049	48,047	53	0,907
C	T	G	0.248			
C	G	G	0.509			
G	T	G	2.003	6,001	6	1

Tab. 28: Haplotype distribution in *SV2C*.

T: transmitted; NT: not transmitted; OR: odds ratio.

The pedigree disequilibrium test (PDT), was applied to search for evidence of allelic association in general pedigrees, but showed no significant linkage (Tab. 29).

SNP	Allele	P
rs1862519	G	0.917411
rs1862519	C	0.917411
rs30196	G	0.796253
rs30196	T	0.796253
rs31098	G	0.680051
rs31098	A	0.680051

Tab. 29: Pedigree disequilibrium test with nominal significance level 0.05 on the basis of 200 nuclear families.

P = permutation based p value with $p < 0.05$ considered significant.

3. IMMUNOHISTOCHEMICAL ANALYSIS OF *LPHN3*

3.1. REGIONAL DISTRIBUTION OF LPHN1-mRNA IN THE MURINE BRAIN USING ISH

The regional distribution of Lphn3 transcripts was accomplished by ISH in wildtype (C57BL/6J) murine brain sections. To verify the specificity of the used Lphn3-mRNA antisense probe a DIG-marked sense probe was used.

Lphn3 mRNA was widely distributed in the murine brain. The striatum, cortex, hippocampus and cerebellum were analyzed in greater detail. Whereas all laminae in the cortex contained LPHN3-mRNA, but with less expression in laminae IV (Fig. 22a and b), no noticeable expression pattern was found in the striatum in comparison to the environmental cerebral regions. Also the corpus callosum showed no Lphn3 expression at all (Fig. 22c and d). The hippocampus revealed a distinct expression. Mainly the CA1 region showed a strong Lphn3 expression, but declined abruptly to CA2 and CA3 (Fig. 22e). In the striatum granulosum of the gyrus dentatus some cells were clearly colored and different in size in contrast to others (Fig. 22f). An inhomogeneous distribution of Lphn3-mRNA also appeared in the brain stem. Here, the expression was especially strong in the Erdinger-Westphal nucleus, the dorsal raphe and the central raphe nucleus (Fig. 22g and h). In the purkinje layer of the cerebellum a very strong expression has been revealed (Fig. 22i), followed by the striatum granulosum and the molecular layer. The clear colored cells in the two last named layers could be basket or stellate cells (Fig. 22k). These results were also found by Mario Kreutzfeldt (Diploma thesis).

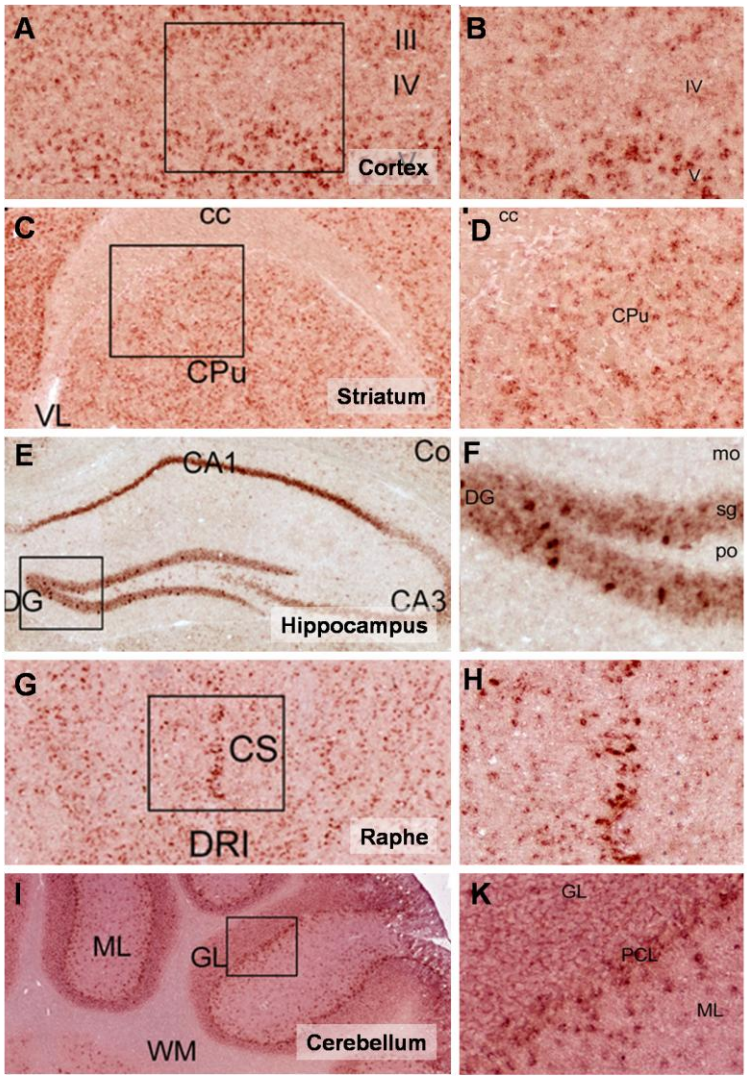


Fig. 22: Overview of the Lphn3-mRNA distribution in the murine brain.

Different regions such as the cortex (A,B), striatum (C,D), Hippocampus (E,F), raphe (G,H) and cerebellum (I,K) are shown. Right column displays higher power images of boxed inserts from the left column. (Picture: Mario Kreutzfeldt).

Aq (aqueduct), CA1/3 (cornu ammonis regions), cc (corpus callosum), Co (cerebral cortex), CPu (caudate putamen), CS (central raphe nucleus), DG (gyrus dentatus), DRI (inferior dorsal raphe), DRD (dorsolateral raphe), ML/mo (molecular layer), PCL (purkinje cell layer), po (polyform layer), sg/GL (striatum granulosum), VL (lateral ventricle), WM (white matter).

3.2. CELLULAR AND REGIONAL DISTRIBUTION PATTERN OF LPHN3 PROTEIN IN HUMAN AND MURINE BRAIN SECTIONS

Immunohistochemistry in the human hippocampus revealed Lphn3 protein in both the striatum granulosum of the gyrus dentatus (Fig. 23a) and the pyramidal cells of CA1 - CA4. The stronger coloring in region CA3 could be explained by its higher cell density. The stratum oriens and the stratum radiatum showed very little Lphn3 expression. The individual laminae in the human cortex were distinguishable due to their Lphn3 distribution pattern. Basically all laminae were strained, but the strongest one could be found in the pyramidal cells of laminae V, less straining in contrast in laminae I and VI (Fig. 23b). Whereas the striatum granulosum and the molecular layer of the cerebellum seemed to be homogenously strained, the purkinje cells became strata surface (Fig. 23c). The strongest Lphn3 expression was found in their somas and also involved the dentrites, but not the nuclei. However, the expression intensity decreased in direction to the soma. Lphn3 immunoreactive regions were rarely found, but this doesn`t speak against a principal protein localization in axons.

Due to the high interspecific LPHN3 homology between mouse and human and also because of the missing offers for murine polyclonal LPHN3 antibodies, the human ones were also used in murine tissue sections.

Hippocampus, cortex and cerebellum were dyed via the ABC-method. Clearly noticeable were some cells in the gyrus dentatus of the hippocampus, mainly in the CA3 (stratum lucidium) (Fig. 23b). In the cortex the individual laminae were distinguished clearly, especially Laminae II and V (Fig. 23d). In contrast, in the cerebellum both stratum granulosum and the molecular layer showed some homogenous colored cells. Indeed, the strongest staining was found in the purkinje cell layer; its distribution pattern referred to immunoreactive purkinje cells (Fig. 23f).

All results were again also found in the Diploma thesis of Mario Kreutzfeldt.

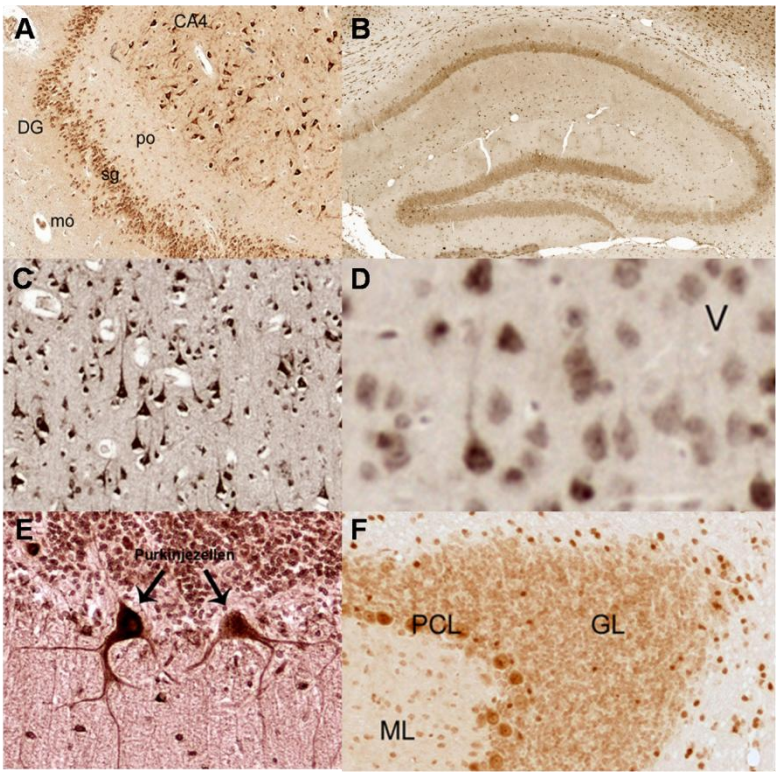


Fig. 23: Immunohistochemical detection of LPHN3 on human (A,C,E) and murine (B,D,F) paraffined brain sections.
For staining the ABC-method was used. Indicated are representative images of the dentate gyrus (hippocampus, A,B), of the laminae VI / V of the cortex (C,D) and the purkinje cells in the cerebellum (E,F). (Picture: Mario Kreutzfeldt).
CA1-4 (cornu ammonis regions), DG (dentate gyrus), mo/ml (molecular layer), PCL (purkinje cell layer), po (polyform layer), sg/gl (stratum granulosum).

4. RESEARCHES IN MLC1

4.1. GENOTYPING OF MLC1 POLYMORPHISMS FOR ASSOCIATION WITH PERIODIC CATATONIA

SNP1 (rs235349) and SNP2 (rs2076137), previously found to be associated with Schizophrenia (SCZ) (Verma, Mukerji et al. 2005), were chosen for an initial association screen. They were significant in LD (D´ = 0.95; p ≥ 0.001; Tab. 30).

Marker	TCR1	SNP1	SNP2
TCR1	0.091	0.99	0.92
TCR2		0.96	0.95
SNP1			0.95

Tab. 30: 2-Locus Linkage Disequilibria between *MLC1* markers.
D´, all p < 0.001. TCR: transcriptional control region; SNP: single nucleotide polymorphism.

First it was tested for an association with Periodic Catatonia (PC); second, in an exploratory analysis, it was investigated to see if the SNPs were associated with all cases combined, SZC alone, or Bipolar Affective Disorder (BPD) alone. As shown in Tab. 31, both were significantly associated with PC. However, no association was found with the combined patient sample, SCZ, BPD, or type A or type B schizophrenia (Reif, Fritzen et al. 2006) (all p > 0.05). Thus, both named SNPs were specifically associated with PC. Therefore, we restricted further analyses incorporating transcriptional control region (TCR) variants to PC cases. These variants also showed a significant LD (Tab. 30). In Tab. 31, TCR 1 and 2 showed association with PC, which slightly missed the conventional significance level when comparing carriers of the rare alleles to subjects homozygous for the frequent variant (TCR1: p = 0.061; TCR2: p = 0.051).

Marker		Total	Controls	PC	Controls vs. PC
TCR1	G/G	185 (0.54)	160 (0.57)	25 (0.43)	
	G/T	129 (0.38)	103 (0.36)	26 (0.45)	χ^2_{CC} = 4.03, P = 0.134
	T/T	27 (0.08)	20 (0.07)	7 (0.12)	
		χ^2_{HWE} = 0.46, P = 0.500	χ^2_{HWE} = 0.37, P = 0.542	χ^2_{HWE} = 0.00, P = 0.952	
	T-	185 (0.54)	160 (0.57)	25 (0.43)	χ^2_{CC} = 3.50, P = 0.061
	T+	156 (0.46)	123 (0.43)	33 (0.57)	
TCR2	C/C	202 (0.59)	161 (0.57)	41 (0.71)	
	C/G	118 (0.35)	104 (0.37)	14 (0.24)	χ^2_{CC} = 3.87, P = 0.144
	G/G	21 (0.06)	18 (0.06)	3 (0.05)	
		χ^2_{HWE} = 0.45, P = 0.500	χ^2_{HWE} = 0.05, P = 0.827	χ^2_{HWE} = 1.38, P = 0.240	
	G-	202 (0.59)	161 (0.57)	41 (0.71)	χ^2_{CC} = 3.80, P = 0.051
	G+	139 (0.41)	122 (0.43)	17 (0.29)	
SNP1	T/T	200 (0.59)	160 (0.57)	40 (0.73)	
	C/T	118 (0.35)	106 (0.37)	12 (0.22)	χ^2_{CC} = 5.29, P = 0.071
	C/C	20 (0.06)	17 (0.06)	3 (0.05)	
		χ^2_{HWE} = 0.22, P = 0.641	χ^2_{HWE} = 0.01, P = 0.920	χ^2_{HWE} = 2.26, P = 0.132[1]	
	C-	200 (0.59)	160 (0.57)	40 (0.73)	χ^2_{CC} = 4.50, P = 0.025
	C+	138 (0.41)	123 (0.43)	15 (0.27)	

Marker		Total	Controls	PC	Controls vs. PC
SNP2	C/C	241 (0.72)	195 (0.69)	46 (0.84)	χ^2_{CC} = 5.94, P = 0.051
	C/T	87 (0.26)	80 (0.28)	7 (0.13)	
	T/T	9 (0.03)	7 (0.02)	2 (0.04)	
		χ^2_{HWE} = 0.12, P = 0.734	χ^2_{HWE} = 0.13, P = 0.721	χ^2_{HWE} = 4.72, P = 0.030[2]	
	T-	241 (0.72)	195 (0.69)	46 (0.84)	χ^2_{CC} = 4.74, P = 0.029
	T+	96 (0.28)	87 (0.31)	9 (0.16)	

Tab. 31: Genotype frequencies of *MLC1* markers.

χ^2_{HWE} = chi-square-tests for deviation from Hardy-Weinberg-equilibrium, df = 1; deviations observed for [1] SNP1 C/C genotype slightly overrepresented, and [2] SNP2 T/T genotype overrepresented; χ^2_{CC} = chi-square-tests for frequency differences between cases and controls, df = 2 for full genotype tests, df = 1 for dichotomous genotype tests.

Haplotype analyses included all SNPs. A test for global haplotype association with PC did not show a significant result (p = 0.35). However, on the level of specific haplotypes, the T-C-T-C haplotype was significantly more common in PC (p = 0.025, Tab. 32). Similar to the 4-marker test, analyses using 3-marker and 2-marker haplotypes yielded insignificant results (all p > 0.05).

TCR1	TCR2	SNP1	SNP2	Controls	PC	Perm. P
G	C	C	C	0.010	0.00	0.190
G	C	C	T	0.000015	0.00	0.583
G	C	T	C	0.494	0.491	0.686
G	G	C	C	0.077	0.064	0.470
G	G	C	T	0.158	0.100	0.116
G	G	T	T	0.004	0.00	0.111
G	G	T	T	0.002	0.00	0.167
G	G	C	C	0.002	0.00	0.167
T	**C**	**T**	**C**	**0.242**	**0.345**	**0.025**
T	G	C	C	0.006	0.00	0.024
T	G	C	C	0.000062	0.000045	0.819
T	G	C	T	0.000003	0.000020	0.636
G	C	C	C	0.636	0.00	0.183

Tab. 32: Estimated *MLC1* haplotype frequency differences between control subjects and patients suffering from Periodic Catatonia using GENECOUNTING.

Bold = significant and meaningful haplotype association: in the PC patient group, the estimated T-C-T-C haplotype is overrepresented. PC = periodic catatonia, Perm. P = permutation based p value with $p<0.05$ considered significant.

4.2. MLC1 KNOCKOUT PLASMID VECTOR

The Mlc1-targeting vector was constructed by inserting a 1,2 kb Pst I-fragment containing complete 492 bp exon 1 (untranslated) and 154 bp of the 217 bp exon 2 (“left arm”), which contains the start codon ATG, into a Not I/Xho I site and additionally a 4,8 kb Sac I-fragment containing complete exon 4 (54 bp) and 5 (102 bp) (“right arm”) into a BamH I/EcoR I site of pPXT.

Both arms were inserted by *in vitro* integration and are bordered by *loxP* sites. Fig. 24 shows a schematic representation of the Mlc1 ko plasmid vector (pMlc1).

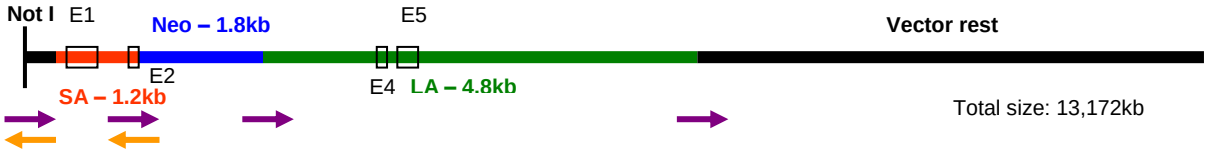


Fig. 24 Schematic representation of the linearized pMlc1 ko vector.
Left and right arm are inserted by ligation. Both are generated between two LoxP sites. SA: short arm; LA: long arm; Neo: Neo cassette; E: exon; →: used forward primers; ←: used reverse primers.

After electroporation the ES cells were analyzed for integration of this pMlc1 ko vector plasmid by PCR (Fig. 25). The used primers and their localization are summarized in Material & Methods chapter 1.4. Desoxyribonucleotides.

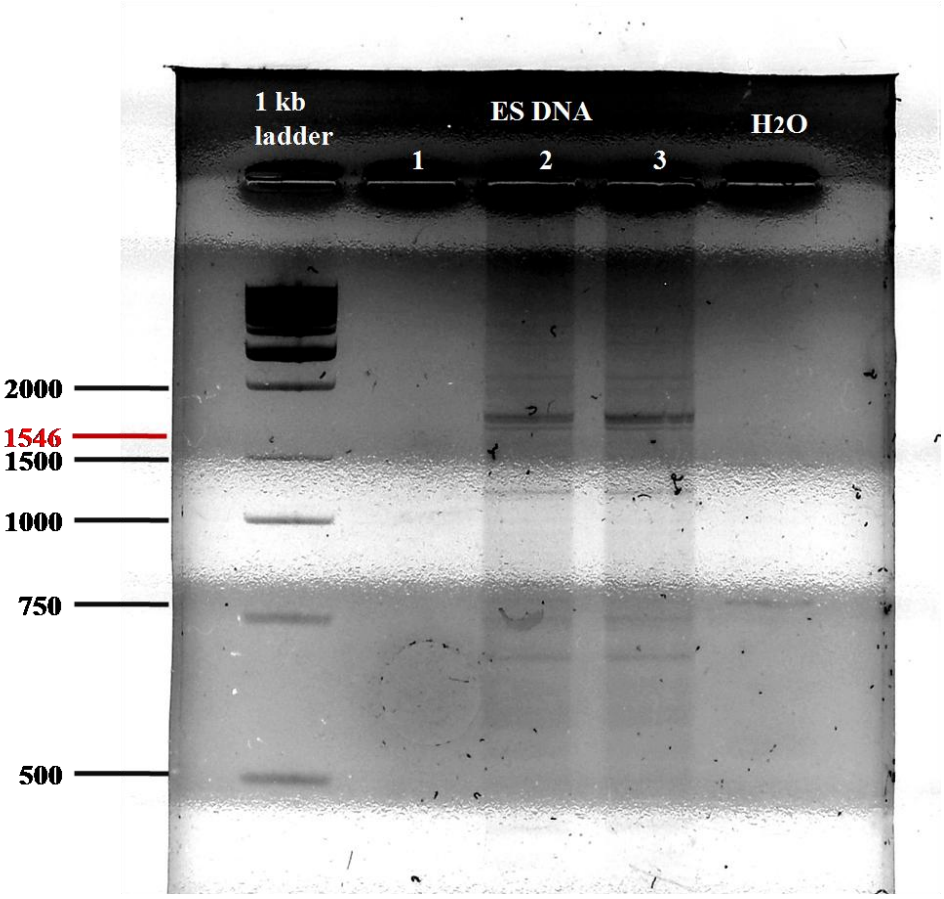


Fig. 25: PCR amplification for integration of the pMlc1 knockout vector plasmid into human embryonal stem cells (ES) and the PCR products were analyzed by agarose gel electrophoresis. 1st lane: 1 kb plus DNA marker; lane 1-3: human ES cells: lanes 2/3 show the expected size (1546 bp) of the knockout vector plasmid; last lane: H_2O as negative control.

IV. DISCUSSION

1. NEW ADHD CANDIDATE GENES BY ARRAY CGH

Sub-megabase resolution array CGH identified a total of 17 potentially disease-associated CNVs in a cohort of 99 children and adolescents severely affected with ADHD. The aberrations comprise five deletions and 13 duplications with approx. sizes between 130 kb and 3 Mb. Two CTVs occurred *de novo* and eight were inherited from a parent with ADHD, whereas five were transmitted by an unaffected parent. For one case, inheritance was not determined. These CNVs showed no overlap between individual patients, i. e. they are not recurrent, but several of the genes involved may be integrated into behaviorally relevant functional pathways, including neurodevelopment, neurotransmission, and synaptic plasticity.

1.1. NEUROPEPTIDE Y

Given the remarkable heritability of ADHD, polymorphisms, inherited from an affected parent, are likely to contain risk genes. Among the most apparent identified candidate genes, an approx. 3 Mb duplication, occurring in two affected cousins, includes the gene encoding for NPY on chromosome 7q15.2-15.3. This co-segregates with a unique syndrome comprising severe ADHD and obesity. The subsequent investigation of the extended multigenerational family with high density of ADHD patients revealed evidence for an association of this duplication with ADHD, increased BMI as well as binge eating, suggesting that the aberration contributes to the syndrome in this family. Admittedly, in four descendants of the F1 generation affected with ADHD no chromosomal rearrangement was detected. Due to the often appearing assortative mating which is common in ADHD, bilinear transmission of at least two causative gene variants, including the *NPY*-containing duplication, passed by two affected F1 founders can be assumed.

The additional copy of *NPY* within the investigated extended family was associated with an almost 2-fold increase of plasma NPY concentrations in peripheral blood. This provides indirect evidence for NPY overexpression. Enhanced NPY receptor subtype-dependent signaling in the brain with consequences on learning/memory, cognition, and emotion

regulation are likely to be altered in duplication carriers. Although increased plasma NPY concentrations were previously observed in children with ADHD (Oades, Daniels et al. 1998), the general role of this neuropeptide in the pathophysiology of ADHD remains to be determined.

The potential link between ADHD, metabolic dysregulation, and NPY is underscored by studies revealing that ADHD is highly prevalent among obese patients and highest in those with extreme obesity. NPY is an orexigenic key regulator in the brain of mammalians and non-mammalians. The neuropeptide increases food intake especially carbohydrates (Beck 2006). Also elevated levels of NPY cause an increase of food intake and reciprocally (Williams, McKibbin et al. 1991; Widdowson, Upton et al. 1997; Ishihara, Tanaka et al. 1998). In the analyzed family both ADHD and obesity are observed. The potential link between ADHD, food intake, metabolic dysregulation, and NPY is also underscored by studies revealing that ADHD is highly prevalent among obese patients and highest in those with extreme obesity (Agranat-Meged, Deitcher et al. 2005; Curtin, Bandini et al. 2005; Fleming, Levy et al. 2005). Mechanisms for this co-morbidity are unknown, but may involve brain dopamine function, glucose utilization, and insulin receptor activity (Agranat-Meged, Deitcher et al. 2005). Alterations in the brain dopamine system affect a wide range of behavioral phenotypes ranging from ADHD-associated behavior to food intake and from an evolutionary perspective, gene variations selected to increase cognitive and behavioral flexibility may presently be associated with attention deficits and increased food consumption in an obesogenic environment. However, both ADHD and adiposity are of multigenetic origin and the consideration of a monogenetic cause is obsolete. This is again in line with the relatively small effects detected by the statistical analysis.

Despite *NPY* being widely investigated in the context of body weight regulation and energy balance, it has recently not been implicated in behavioral traits, including aggression and negative emotionality, but also in several neuropsychiatric disorders like schizophrenia, panic disorder, bipolar disorder and depression (Karl and Herzog 2007). A recent study revealed that the functional Leu7Pro polymorphism in the human *NPY* resulting in increased NPY released from sympathetic nerves is associated with traits of the metabolic syndrome (Ruohonen, Pesonen et al. 2008). Moreover, diverse studies suggested that the Pro7 allele is associated with an increased risk for alcohol dependence (Lappalainen, Kranzler et al. 2002; Zhu, Pollak et al. 2003) a common comorbid disorder in ADHD.

In the rodent model central administration or viral vector-induced overexpression of NPY produces a profound increase in food intake, whereas a NPY reduction leads to a decrease

(Primeaux, York et al. 2006; Thorsell, Repunte-Canonigo et al. 2007). Food deprivation upregulates NPY in the arcuate nucleus of the hypothalamus (Beck 2006), and repeated administration of NPY induces obesity (Stanley, Anderson et al. 1989; Ruohonen, Pesonen et al. 2008). Transgenic mice overexpressing NPY in noradrenergic neurons were reported to display disturbances in glucose and lipid metabolism, key components of the cluster of abnormalities characterizing the metabolic syndrome (Ruohonen, Pesonen et al. 2008). Bannon revealed that NPY-deficient mice show reduced food intake in response to fasting and an anxiety-like phenotype with increased startle response (Bannon, Seda et al. 2000). Several receptors (Y1, Y2, Y4-Y6) mediate the physiological effects of NPY (Chamorro, Della-Zuana et al. 2002; Karl and Herzog 2007) and data suggest that the energy balance effects of NPY are mediated by both the NPY Y1 and the Y5 receptor (Chamorro, Della-Zuana et al. 2002). NPY Y4 receptor knockout display increased locomotor activity, less anxiety-like behavior and behavioral despair, whereas behavioral characterization of NPY Y2 knockout mice revealed reduced attention and increased impulsivity (Greco and Carli 2006; Painsipp, Wultsch et al. 2008).

Finally, it is noteworthy that the NPY level is also related to the DA system, especially to *DRD1* (Sunahara, Guan et al. 1991). There is evidence that the NPY level is regulated through *DRD1*. For example, a *DRD1* antagonist could block the inhibitory effects of the psychotomimetic drug methamphetamine on NPY levels, especially in nucleus accumbens and caudate (Westwood and Hanson 1999). In addition, NPY expression in the PFC (Caberlotto and Hurd 1999) supports the assumption that the NPY level has a stake in the etiology of ADHD. The dysfunction of the PFC in this neurodevelopmental disorder is suggested in several functional as well as morphological studies (Hynd, Semrud-Clikeman et al. 1990; Filipek, Semrud-Clikeman et al. 1997; Rubia, Overmeyer et al. 2000; Langleben, Austin et al. 2001; Mostofsky, Cooper et al. 2002). Alike, drugs used for the treatment of ADHD often interfere with the NE system by inhibiting the reuptake of DA and NE. This raises the question if the concentration of NPY is also affected by such medication because of its co-expression with NA (Karl and Herzog 2007) and as well in which way.

Since we observed increased plasma NPY concentrations in the presence of an additional copy of NPY within the investigated extended family as a peripheral biomarker, receptor subtype-dependent signaling in the brain with consequences on the regulation of metabolic homeostasis as well as cognition, learning/memory, and emotion regulation are likely to be altered in duplication carriers.

In support of an impact of gene dosage-dependent increases in *NPY* expression on brain function, fMRI of reward and emotion processing detected lower activation of the left ventral striatum and left posterior insula during anticipation of large rewards/losses in duplication carriers, respectively. As left ventral striatal hyporesponsiveness during reward anticipation has repeatedly been shown in patients with adult ADHD (Scheres, Milham et al. 2007; Strohle, Stoy et al. 2008) *NPY* overexpression may result in deviant rewardrelated neural processing in duplication carriers. Moreover, the relative hypoactivity within the left posterior insula during the anticipation of monetary loss in carriers could reflect anxiolytic effects of NPY (Bannon, Seda et al. 2000; Greco and Carli 2006; Painsipp, Wultsch et al. 2008). Higher genotype-driven *NPY* expression has recently been shown to be associated with reduced pain/stress-induced activations of endogenous opioid neurotransmission and accounted for 37% variance in left posterior insular cortex activation (Zhou, Zhu et al. 2008). Hence, our fMRI findings replicate previously reported NPY-related alterations in the processing of aversive stimuli while extending evidence for an interaction of NPY with reward circuits. Taken together, our findings provide evidence that increased *NPY* dosage is not only reflected by the peripheral biomarker of increased NPY plasma concentration but also by fMRI elicited alteration in brain function related to reward and emotion processing.

In summary, there is substantial evidence supporting a role for NPY in the ADHD-related behavioral phenotype and dysregulation of energy balance in carriers of the 7p15.2-15.3 duplication, especially in this specific family, but its role for the general population is relativized by the interaction and modulation with other genes and environmental factors. While presumably increased NPY concentrations in the brain are likely to play a causative role in the ADHD and obesity-related phenotype of NPY duplication carriers, it should be noted that the duplication is large and also harbors other brain-expressed genes that may influence behavior. This kind of interaction is suggested especially for complex psychiatric diseases with a clinical phenotype being an extreme variant of a personality trait.

1.2. GLUCOSETRANSPORTER 3 AND 6

Another duplication, which was bequeathed by an affected mother to her child, was found via the same method on chromosome 12p13.31 and led to subsequent investigations of *GLUT3*,

a glucose transporter mainly expressed in the brain. A whole-genome association examination using an Affymetrix 500k chip set revealed, amongst others, a promising peak at chromosome 9q34.2 (data not published). This region includes *GLUT6*, which could also be involved in ADHD. This finding needed to be confirmed by SNP genotyping in ADHD patients and controls using mass spectrometry. In order to examine the possible associations between *GLUT3* and alternatively *GLUT6* in greater detail than has been done so far, we performed a direct genetic analysis of polymorphisms in these genes including regions both 5` and 3´ to the coding sequence to cover flanking regulatory elements.

Glucose is the main energy source for the mammalian brain and plays a central role in cellular homeostasis and metabolism. A family of facilitative transmembrane glucose transporter proteins, the GLUT (glucose transporter), also known as SLC2A (solute carrier) family, allows the transport of glucose across the plasma membrane into or out of cells. The 12 family members encode for integral membrane proteins, which are highly homologous (Joost and Thorens 2001). The predominant glucose transporters in the brain are *GLUT 1*, *3* and *6*. While the first named is expressed in astrocytes and the blood-tissue barrier (Flier, Mueckler et al. 1987; Walker, Donovan et al. 1988), *GLUT3 (SLC2A3)* is responsible for the glucose uptake in neurons (Walmsley 1988; Duelli and Kuschinsky 2001), whereas *GLUT6* is found in leucocytes as well as in the brain (Joost and Thorens 2001).

Immunochemical analysis revealed that *GLUT3,* first detected by Kayano and colleagues in 1988 (Kayano, Fukumoto et al. 1988), is highly expressed in tissues which show a high glucose demand such as the brain or nerves (Shepherd, Gould et al. 1992; Gould and Holman 1993; Maher, Davies-Hill et al. 1996). Here, *GLUT3* can be found in the neuronal cell bodies of the cerebellar Purkinje cell layer and in neurofilament expressing processes (Mantych, James et al. 1992). GLUT3 mRNA was also detected in regions such as the cerebellar cortex and hippocampus (Maher, Vannucci et al. 1994). The cerebellum is of increasing interest in ADHD because of its involvement in cognitive and emotional processing and in behavioral control (Schneider, Retz et al. 2006). An additional distracting effect such as a duplicated glucose transporter could cause further disturbances in this part of the brain.

GLUT3 maps to chromosome 12p13.31, which interestingly was also identified as a suggestive locus in one of the first linkage analyses on ADHD producing a peak LOD score of 2.6 between the two markers D12S352 (chromosome 12p13.33: 431652 – 631971) and D12S336 (chromosome 12p13.31: 9285296 – 9485634) (Fisher, Francks et al. 2002). This

linked region also harbors the coding sequence of *GLUT3*. Furthermore, a linkage scan from our group utilizing a 50K SNP chip detected a broad linkage peak with a maximal parametric LOD score of 2.92 on chromosome 12, also containing the *GLUT3* gene (Lesch, Timmesfeld et al. 2008; Zhou, Dempfle et al. 2008)(Romanos M.; data not published). Thus, together with our CNV analyses, three independent genome wide studies provided converging evidence for *GLUT3* being a risk gene for ADHD, which could be corroborated in this study using a candidate-gene based approach.

On the other side, not much is known about function and possible interactions of *GLUT6*. It appears to be regulated by sub-cellular redistribution, because it is targeted to intra-cellular compartments by di-leucine motifs in a dynamin dependent manner (Joost and Thorens 2001) and also seems to be involved in the glucose transmembrane transport via its sugar:hydrogen symporter activity .

Of particular interest affecting both genes is the assumption that sugar influences ADHD (Schnoll, Burshteyn et al. 2003; Cormier and Elder 2007). Several issues have been addressed in this context. Mothers and teachers who have witnessed ADHD-children before, during and after sugar consumption claimed that the kids became more hyperactive afterwards. Wender and Solanto (Wender and Solanto 1991) concluded that inattention increased only in the ADHD group following sugar consumption but not after saccharin and aspartame. According to this data, a high carbohydrate diet exacerbated inattentiveness at least in some ADHD children. In line with this, another study revealed a relationship between the consumption of soft-drinks and hyperactivity in adolescents in a cross-sectional population-based survey (Lien, Lien et al. 2006), although this study raised discussions about this methodology. Despite technical shortcomings of these epidemiological investigations, there is still further support for the notion that carbohydrates might negatively influence ADHD as animal studies demonstrated a cross-sensitivity between sugar and stimulants (Avena and Hoebel 2003; Avena and Hoebel 2003). In a series of studies Wolraich and associates reported that there is no effect even of high doses of sugar on hyperactive children neither after consumption of sugar, aspartame nor saccharine (Wolraich, Milich et al. 1985), although these children's parents claimed that sugar triggered hyperactive behavior. But also, these studies were technically flawed by methodological issues (Rojas and Chan 2005) and the small sample sizes.

1.3. CUB AND SUSHI MULTIPLE DOMAINS 1

Also, inherited by the affected mother, a duplication at chromosome 8q23 is related to CUB and Sushi multiple domains 1 (*CSMD1*), a gene which may be an important regulator of complement activation in the developing CNS (Kraus, Elliott et al. 2006). The 3,508 amino acid protein has 14 alternating CUB and sushi domains, 13 additional tandem sushi domains and a cytoplasmatic C-terminus, which contains several phosphorylation sites. Rare alternative transcripts that lack diverse exons are also identified (OMIM).

Duplications of distal 8p with and without clinical phenotypes have already been reported and seem to be often associated with an unusual degree of structural complexity. Glancy et al. ascertained a duplication of chromosome 8 in a patient with autism and his mother suffering from learning problems, which distal breakpoint interrupts *CSMD1* in 8p23.2 (Glancy, Barnicoat et al. 2009). Duplicated repressors at the 3´ end of *CSMD1*, which is directed on the minus strand, as well as a doubled phosphorylation site could inhibit the normal expression of the protein which blocks the developing CNS by a decrease in the nerve growth cone (Kraus, Elliott et al. 2006). Because learning problems belong to most common co-morbidities of ADHD an association between a disturbed gene function and the pathology is not to be dismissed, but needs further investigation.

In a recent study to improve the understanding of human methamphetamine dependence, Uhl identified several genes by association studies. Variants in these genes were likely to alter, amongst others, cell adhesion, enzymatic function, transcription, DNA/RNA/protein handling and modification (Uhl, Drgon et al. 2008). The cell adhesion genes *CSMD1* and *CDH13* displayed the largest number of clustered nominally positive SNPs.

1.4. BUTYRYLCHOLINESTERASE

Both *de novo* CNVs are deletions on chromosome 3q26 and 6q16.1. First named (patient 991) comprises an interval of 2 Mb and involves at least five genes, the butyrylcholinesterase (*BCHE*, OMIM *177400), B-box domain containing zinc finger protein (*ZBBX*), WDR49, serpin peptidase inhibitor (*SERPINI2*), and programmed cell death

protein 10 (*PDCD10*, disrupted by deletion of four exons at the 3' end). Among these, the *BCHE* gene is of particular interest, given that variations in BCHE enzyme levels have recently been associated with specific differences in cognitive functioning (Manoharan, Kuznetsova et al. 2007). BCHE is a glycoprotein enzyme within the family of serine esterases, such as acetyl choline. In the brain, BCHE is strongly expressed in cholinergic neurons of the pedunculopontine tegmentum where it regulates the interaction with dopaminergic, noradrenergic, and serotonergic networks the sleep-wake behavior and vigilance (Darvesh, Hopkins et al. 2003) suggesting, it may also directly influence locomotor activity, attention and reward-related behavior. It seems to be involved in the catalysis of endogenous choline esters, and is known to deactivate various toxic substances in the plasma (Raveh, Grunwald et al. 1993). For example, BCHE administration inhibits cocaine-induced behavioral changes in mice, apparently by catalyzing the breakdown of cocaine into non-toxic metabolites (Koetzner and Woods 2002). In addition, BCHE is expressed in glia and neurons in the brain, and in a subset of brain structures (Darvesh, Grantham et al. 1998; Darvesh, Hopkins et al. 2003) suggesting it may also directly influence behavior disorders. Variations in BCHE concentration have recently been associated with specific differences in cognitive function (Manoharan, Kuznetsova et al. 2007).

Haplotype insufficiency with reduced *BCHE* activity in the patient carrying the deletion in conjunction with as yet unknown environmental factors during brain development may possibly moderate the risk for the development of ADHD symptoms including cognitive dysfunction. It has been shown in mice that BCHE protects against cognitive deficits that arise from soman administration (Brandeis, Raveh et al. 1993).

The other genes within the 3q26 deletion are not obvious candidate genes for behavioral disorders, display no (*ZBBX, SERPINI2*) or moderate (*WDR49*) to low (*ODCD1*0) brain expression, but contribution to the phenotype cannot be excluded.

Patient 1761 carries a confirmed *de novo* deletion on chromosome 6q16.1. There are no genes in the deleted interval, but it is possible that a critical regulatory region is affected. There are confirmed examples of disease-causing chromosome aberrations affecting critical regulatory regions at considerable distance from the disease genes themselves (Kleinjan and van Heyningen 1998).

1.5. PLEKHB1, RAB6A AND PDE4D

Patient 51, who is severely affected, carries a ~500 kb deletion located at 11q13.4, inherited from his affected mother. The aberration, which was also detected in an affected brother, harbors the brain-expressed gene *PLEKHB1*, which exerts cellular functions in primary sensory neurons (Xu, Wang et al. 2004), making it an interesting candidate gene for disordered attention. *PLEKHB1* encodes for an evolutionary conserved protein that is required for normal synapse development. *PLEKHB1* postnatal expression includes regions associated with long-term changes in synaptic activity, and has been shown to inhibit adenylyl cyclase activity, suggesting an involvement in learning and memory (Scholich, Pierre et al. 2001). The expression pattern also proposes a role of *PLEKHB1* in the establishment of nerve terminal morphology and activity for multiple neural cell types in the developing nervous system (Burgess, Peterson et al. 2004; Young, Stauber et al. 2005).

Next to *PLEKHB1* on chromosome 11 maps the mammalian Ras-associated GTP-binding protein *RAB6A* which is involved in the regulation of synaptic vesicle function and secretion. *RAB6A,* which exists in two isoforms, is expressed ubiquitously but in a large part in the brain, and interacts with rabkinesin-6. It has been shown that a downregulation of RAB6A expression i.e. caused by a deletion disturbs the organization of the Golgi apparatus and delays microtubule-dependent Golgi-to-ER recycling (Young, Stauber et al. 2005). Moreover, when *RAB6A* function is altered, cells are unable to progress normally through mitosis (Miserey-Lenkei, Couedel-Courteille et al. 2006). Since many diseases have been shown to be caused by kinesin deficits as well as by a disturbed microtubule-dependent recycling system, this aspect should not be disregarded due to the fact that a destabilization of microtubles plays a critical role of learning in memory (Yuen, Jiang et al. 2005).

Another candidate gene from this deleted interval is the mitrochondrial uncoupling protein *UCP2*. It has a neuroprotective effect in both the developing brain (Sullivan, Dube et al. 2003) and following traumatic brain injury in adults (Mattiasson, Shamloo et al. 2003). This is compatible with the hypothesis that ADHD is a multifactorial disorder caused by genetic and environmental factors which, in combination, have direct effects on aspects of the cognitive development and function. Whether the last two genes contribute to the general risk towards ADHD in the population remains to be established.

The gene encoding the supershort brain-specific isoform 6 of the phoshodiesterase 4D (*PDE4D6*) (Wang, Deng et al. 2003) including its presumed transcriptional control region is exclusively duplicated in patient 201. The duplication of *PDE4D6* is inherited from the affected mother and located on chromosome 5q11.2, a region adjacent to the 5q13.1 locus of genome-wide significance in a high-resolution linkage study to ADHD (~2.5 Mb 5' of rs895381, family P1) (Romanos, Freitag et al. 2008). It is noteworthy that the 5q12.1 deletion in patient 241 (also see preceding section) is only ~250 kb upstream of the transcription start site for the longest PDE4D isoform. Moreover, the *PDE4D* region is contained in a linkage interval flanked by markers D5S1968-D5S629 in an extended pedigree (Lin et al., manuscript submitted) and nominally significant association of several SNPs (highest ranking SNP rs17780175, $p = 3.41 \times 10^{-9}$) in *PDE4D* was also revealed by a pooling-based genome-wide association (GWA) study in adult ADHD (Lesch, Timmesfeld et al. 2008). Of related interest, *PDE4D* variants that distinguish dependent *versus* non-dependent individuals abusing methamphetamine, alcohol, nicotine and other substances has been previously identified in several GWA studies of addiction vulnerability (Uhl, Drgon et al. 2008). Given the high co-morbidity of ADHD with substance use disorders, the convergence with genes identified in GWA studies of addiction vulnerability and related phenotypes provides further confidence in this data. While previous association to ADHD has not been reported for these genes, those identified by both the present study and findings from other related reports, appear especially relevant of further detailed evaluation. Furthermore, the *PDE4*-specific inhibitor rolipram shows antidepressant effects on animals and humans (Fleischhacker, Hinterhuber et al. 1992; Zhang, Huang et al. 2002). *PDE4D* ko-mice also show antidepressant-like behavior which is further increased by rolipram. Recently, variants in two genes encoding PDEs were found to be associated with major depression (Wong, Whelan et al. 2006). Together, these observations indicate that PDE4D may be involved in the susceptibility to diverse neural diseases.

In summary, often genes which are rarely in population show a larger effect than those found more frequently. From this follows that also a private mutation only in one special family could be a susceptibility factor for the multifactorial disease ADHD. The hypothesis, how the appropriated gene is segregating in the affected family, is further analyzed.

1.6. SYNAPTIC VESICLE PROTEIN 2C

At 5q13.3 *SV2C* encoding the synaptic vesicle protein 2C is partially duplicated in patient 21 with preservation of the 3' segment. SV2C belongs to the sugar transporter family and is only present in a small subset of neurons in phylogenetically old brain regions like pallidum, substantia nigra, midbrain, brainstem and olfactory bulb (Janz and Sudhof 1999). Notably, *SV2C* mediates the uptake of botulinum neurotoxin A into peripheric nerves (Mahrhold, Rummel et al. 2006). In addition, the synaptic vesicle protein shows 20 - 22% sequence identity to the relatively novel vesicle protein called SVOP (SV two-related protein). Immunocytochemical straining of adjacent rat brain section for both genes demonstrated that SV2C and SVOP are co-expressed in most neurons (Janz, Hofmann et al. 1998). Synaptic vesicle-associated proteins are known to be important regulators of neurotransmitter releases at synaptic terminals. They are also often associated with ADHD as in case of NET or DAT, both affect presynaptic nerve terminals. Although our analysis indicates that the three chosen promotor polymorphisms are no susceptibility factors for ADHD, this does not argue against a role of *SV2C* in this psychiatric disorder. However, the physiological role of *SV2C* is suggestive of being associated with ADHD. Because *SV2C* is studied for an association to ADHD only for a short time, there exist no preliminary expression data as for other genes. Its expression remains unaffected by MPH treatment as far as possible. An enhancement of the expression is determined by trend only in the hippocampus of DAT-deficient mice; a significant duplication of expression was detected in the cerebellum of these animals in comparison to wildtype mice (Kreutzfeldt, 2008, diploma thesis). Due to the essential relevance of *SV2C* in Ca^{2+}- dependent secretion (Schivell, Mochida et al. 2005), these chances could be involved in the increased neurotransmitter release in the cerebellum of these mice and contribute to their hyperactive phenotype. Furthermore, genetically modified mice have become important tools to investigate functions of previously unexplored proteins and to define mechanism of action.

Another common copy number polymorphism, found in patient 211, results in a duplication of the gene for the neuronal glucose transporter 3 (*SLC2A3*). Both gene products, SLC2A3 and SV2C, are associated with synaptic vesicles and participate in the regulation of neurotransmitter release. Interestingly, reduced *SLC2A3* expression resulting from a trans-regulation effect of a locus on 4q32.1 was recently implicated in dyslexia 62. Given the remarkable comorbidity of dyslexia and ADHD, and the anecdotal reports of sugar

intolerance in ADHD associated with an exacerbation of the symptomatology, systematic investigation of the role of common CNVs in the *SLC2A3* region in neuronal glucose utilization is warranted.

1.7. FURTHER CANDIDATE GENES

Several aberrations inherited from healthy parents, as well as those were observed at low frequencies in the reference datasets may also represent candidate risk factors for ADHD. Of particular relevance, the *CAMK2D* gene is disrupted by the duplication in patient 461 (as well as in his healthy mother). *CAMK2D* belongs to the family of calcium- and calmodulin-dependent protein kinases. Several isoforms have been described, one of these is expressed exclusively in rodent and human cerebral cortex (OMIM) making it a candidate for brain disorders.

The 5q12.1 deletion in patient 241 (inherited from his affected mother) affects four genes, of which the partially deleted *NDUFAF2* is a plausible candidate. *NDUFAF2* is a Myc-induced mitochondrial NADH dehydrogenase and complex I assembly factor. Complex I catalyses the first step in the mitochondrial respiratory chain, and a homozygous mutation in this gene was found in a child with severe progressive leucencephalopathy (Ogilvie, Kennaway et al. 2005). Disruption and functional loss or a dominant interfering effect of one copy of *NDUFAF2* may have caused neurometabolic deficiency resulting in an allelic disorder with phenotype resembling ADHD.

While several ADHD and other neuropsychiatric disorder-relevant and inherited CNVs involving neurodevelopmental genes, such as A2BP1, *CNTNAP2*, *CNTN6*, and *DPP6*, have recently been reported by Elia and co-worker (Elia, Gai et al. 2009), only a ~635 kb duplication displayed overlap with the *de novo* deletion on chromosomes 3q26 reported here. However, the latter two of these candidates, *CNTN6* and *DPP6*, also gave nominally significant and high-ranking signals in our GWA study of adult ADHD (Lesch, Timmesfeld et al. 2008).

Although our findings implicate rare variants in the pathogenesis of ADHD, GWA studies are by and large considered to support the common disease/common variants (CDCV)

hypothesis, whose validity for psychiatric disorders is currently controversial (Lesch, Timmesfeld et al. 2008; Franke, Neale et al. 2009; Mitchell and Porteous 2009). While several genes affected by CNVs identified in the present study contain SNPs that yield significant signals in GWA studies, there is presently no obvious relationship between the heritability of ADHD and the number or strength of the observed effects. Unlike rare CNVs, common variants for ADHD may be of very small effect and thus require very large samples to be reliably detected. This argues for the requirement of meta-analysis of various whole-genome (including classical or high resolution) linkage, GWA, and CNV scans as well as larger sample collections. In conclusion, our findings from this first array CGH CNV screen in ADHD are consistent with the notion that multiple rare and common CNVs involving genes functioning in shared dosage-sensitive neurobiological pathways contribute to ADHD pathology.

2. DISTRIBUTION OF LPHN3 mRNA IN CNS

Recent genetic studies have shown a susceptibility haplotype for ADHD in the *LPHN3* gene, which could be found in about 22% of the examined patients (Arcos-Burgos, Jain et al.). *LPHN3* is one of at least three closely related forms of latrophilin expressed in vertebrates. Latrophilins are G-protein coupled receptors with unusually large extra- and intracellular sequences. So far, not much is known about the function of *LPHN3*.

The comparison between the *LPHN3* distribution in humans and mice showed an identical staining of the homologous anatomical structures (Kreutzfeldt, 2008, diploma thesis).
To find out more about the distribution of Lphn3 mRNA in CNS we used ISH and IHC for the detection of the protein. Unfortunately, besides these evidences of the ISH experiments not much is known about the *LPHN3* expressing cell type, so the specificity of the LPHN3 IHC staining could not be verified by co-localization with other proteins. However, Arcos-Burgos detected LPHN3 protein in pyramidal and purkinje cells of human brain sections (Arcos-Burgos, Jain et al.). Unspecific bindings between human and murine LPHN3 are not assumed due to the strong homology.

The susceptibility haplotype and a protective one, encompassing the coding sequence of *LPHN3* exons 4 till 19, contain important functional domains. This suggests that the

regulation of LPHN3 expression could be involved in the etiology of ADHD. So *LPHN3* is one of the first genes recognized for association with a substantially increased risk for manifesting ADHD (Bobb, Castellanos et al. 2005). This is in line with the *LPHN3* function as a G-protein coupled receptor and argues for a putative role in neuronal transmission and maintenance of neuron viability. Further the spatial and temporal expression of the protein supports this thesis. IHC straining indicates that *LPHN3*, the most brain-specific of the latrophilin family, is distributed independently from the neurotransmitter systems and expressed in brain regions most affected by ADHD, i.e. in the amygdala, the caudate and serotonergic raphe, the glutamatergic hippocampal granule cell layer as well as in the GABAergic purkinje cells (Krain and Castellanos 2006).

Different data indicates that *LPHN3* is mainly implicated in brain development, during which ADHD is considered to arise (Ichtchenko, Bittner et al. 1999; Krain and Castellanos 2006). In fact, a tendency to ADHD may represent a selected trait from which humans have further evolved. Cladistic analysis has suggested the *LPHN3* susceptibility haplotype for ADHD identified by Arcos-Burgos is phylogenetically older than the complementary protective haplotype (Arcos-Burgos, Jain et al.). Additionally, one of 49 regions in the human genome, identified as “human accelerated regions” reflecting a rapid evolution of human systems, is HAR28 on chromosome 4: 62,506,874-62,506,977, the exact locus of the ADHD-susceptibility haplotype within the *LPHN3* gene (Williams and Taylor 2006).

3. NEW FINDINGS OF MLC

3.1. *MLC1* POLYMORPHISMS ARE ASSOCIATED WITH PERIODIC CATATONIA

the aforementioned results replicate the findings of Verma in 2005 (Verma, Mukerji et al. 2005) that both intronic SNPs, rs2235349 and rs2076137, are associated with schizophrenic psychoses. But as predicted, they were specifically associated only with PC. Also both TCR SNPs were suggestive of an association with PC. These results underscore the notion that *MLC1* variants influence the susceptibility towards PC (Meyer, Huberth et al. 2001).

This data argues for a restriction of the *MLC1* Leu309Met mutation found in two affected families. The presence of a Met-encoding variant in an affected member in each family, both stemming from the same catchment area, supports the thesis of a founder effect, as this variant could not be found in more than 1800 patients (Meyer, Huberth et al. 2001; Devaney, Donarum et al. 2002; Ewald and Lundorf 2002; Rubie, Lichtner et al. 2003; Kaganovich, Peretz et al. 2004; Verma, Mukerji et al. 2005). So the *MLC1* Leu309Met mutation seems to cause PC only in the spoken families. But despite this, other polymorphic genetic variations, preferential in regulatory regions, could also be associated with PC in other cases. This provides an example of how mutations with severe functional consequences in a gene aid in the identification of risk variants which act probabilisticly yet not deterministicly. Despite the tested mutation not beingt found in replication studies, this does not argue against a role of *MLC1* in SCZ, as Ewald and Lundorf demonstrated in 2002 (Ewald and Lundorf 2002). In this context it is noteworthy that the two TCR SNPs are also associated with PC. The region that harbors both SNPs is shown to contain potential binding sites for transcription factors (http://www.genomatrix.de/cgi-bin/matinspector_prof/mat_fam.pl), which could be altered by the polymorphisms.

The Leonard system (Leonard 1999), which was applied in the present study, is mirrored by ICD-10 diagnostic criteria and thus is not equal to “catatonia schizophrenia”. So discrepancies between different studies might be further explained. On the other side, *MLC1* may also be a modifier gene causing psychomotor symptoms specifically in PC rather than being a susceptibility gene in SCZ. This is in line with concepts on the genetic of SCZ suggesting susceptibility, modifier, and mixed SCZ genes (Fanous and Kendler 2005). Finally, another possible explanation could be that both associated SNPs are in linkage disequilibrium with a potential “true” disease causing variants in a gene nearby. Because *TCR1* lies within an intronic region of the adjacent MOV10-like gene, the marker could be useful to determine the borders of LD. The marker is not counted as a candidate for PC as it shows only testis-specific expression. However, together with previous data (Meyer, Huberth et al. 2001; Verma, Mukerji et al. 2005), these results add further evidence to the view that *MLC1* is implicated in the pathogenesis of at least some forms of SCZ.

3.2. GENERATION OF A KNOCKOUT MOUSE BY GENE TARGETING

Gene targeting technology in mice by homologous recombination like knockout or knockdown techniques has become an important method to generate loss-of-function of genes in a predetermined locus.

Several lines of evidence suggest that white matter tract abnormalities observed in this disorder may result from a primary astrocytic defect because of the temporal expression profile of *Mlc1* (Schmitt, Gofferje et al. 2003). Also diverse pathogenetic mutations in the *Mlc1* gene (missense, splice site, insertion, and deletions) are responsible for at least one form of the neurological disorder. The relation of these findings to its pathogenesis is still uncertain. In the context of suggestive human genetic data and because of the high conservation throughout evolution in a variety of different vertebrate species, the generation of a genetic mouse model, whose *Mlc1* gene is inoperable, is essential for the diagnosis of this disease, for genetic counseling and for prenatal diagnosis. The development and characterization of animal models that express molecular defects found in MLC are one of the major achievements in the applied research.

The mouse *Mlc1* gene at chromosome 15 is expressed throughout the brain, the highest expression is in the pituitary gland, spinal cords and pineal gland (smd-www.standford.edu). However, the lack of *Mlc1* allows the examination of the role of this gene. By observation of any differences from normal behavior or condition the still unknown function in the neurodegenerative disorder MLC can be inferred. For this ko mouse motor and cognition tests would be fruitful. Motor deficits were defined best by the Rotarod Test (Wang, Xu et al. 2008) as well as the Inverted Screen Test (Guenther, Deacon et al. 2001). Also the *Weight Lift Test* is often applied to analyze changed motor skills. Here mice were brought to lift a heft to measure the resistance till loosing to a force transducer. Cognitive testing is performed by Morris Water Maze (Morris 1984), the *Two-Object Recognition Test* (Kowal, Degiorgio et al. 2006) or the Cogitat (Heim, Pardowitz et al. 2000). So, a ko mouse may provide a most powerful and necessary tool to dissect this psychiatric disorder in much more detail to understand the complex nervous system and to correct the inherited disorder.

V. APPENDIX

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2. LIST OF FIGURES AND TABLES

FIGURES

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3. LIST OF ABBREVIATIONS

µ	Micro (10^{-6})
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HTP	5-hydroxytryptophan
5-HTT	Serotonin transporter

A

aa	Amino acids
ABC-method	Avidin-biotin-complex method
a. d.	Aqua destillatra (distilled water)
ADD	Attention Deficit Disorder
ADHD	Attention-Deficit/Hyperactivity Disorder
ADR	Adrenergic receptor
aP	Alkaline phosphatase
approx.	Approximately
array CGH	Array comparative genomic hybridization

B

BAC	Bacterial artificial chromosome

BCHE	Butyrycholinesterase
bp	Base pair(s)
BPD	Bipolar affective disorder
BMI	Body mass index
BSA	Bovine serum albumin

C

Ca^{2+}	Calcium
CAM	Cell adhesion molecules
CAMK2D	Calcium- and calmodulin-dependent protein kinase 2D
CC	Corpus callosum
CDCV	Common disease/common variants
cDNA	Copy DNA
CGH	Comparative genomic hybridization
chap.	Chapter
cko	Conditional knockout
CNS	Central nervous system
CNV	Copy number variation
COMT	Catechol-O-methyl transferase
Cot1 DNA	Competitor DNA
cRNA	Copy RNA
CSMD1	CUB and Sushi multiple domains 1
Cy3/5	Cyanine 3/5

D

DA	Dopamine
DAB	3,3´-Diaminobenzidine

dACC	Dorsal anterior cingulated cortex
DAT (SLC6A3)	Dopamine transporter
DBH	Dopamine-beta hydroxylase
DDC	5-HTP decarboxylase
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DNA	Dexoxyribonucleic acid
dNTP	Desoxynucleotide triphosphate
DRD	Dopamine receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
DoGV	Database of Genomic Variants

E

ES	Embryonal stem cells
EtBr	Ethidium bromide

F

FBAT	Family-based association test
Fig.	Figure

G

GABA	γ-aminobutyric acid
GLUT	Glucose transporter
GPATCH1	G patch domain containing 1
GPCR	G-protein coupled, Ca^{2+}-independent receptors
GWAS	Genome-wide association studies

H

HC	Hippocampus
HKS	Hyperkinetic syndrome
HTR	5-HT receptor
HVA	Homovanillic acid
HWE	Hardy-Weinberg equilibrium

I

i. e.	Id est
IHC	Immunohistochemistry
IPTG/X-gal	Isopropyl-ß-D-thiogalactopyranosid/bromo-chloro-indolyl-galactopyranoside (BCIG)
IQ	Intelligence quotient
ISH	*In situ* hybridization

K

kb	Kilobases
ko	Knockout

L

LA	Long arm
LB	Lysogeny broth
LD	Linkage disequilibrium
L-DOPA	L-dihydroxyphenylalanine
LOD	Logarithm of the odds (to the base 10)
LPHN	Latrophilin
LTX	α-latroxin

M

m	Mol
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MAO	Monoamine oxidase
MAO-I	Monoamine oxidase inhibitors
Mb	Megabases
MID	Moneary Incentive Delay
min	Minute(s)
MLC	Megaloencephalic leukoencephalopathy with Subcortical cysts
mM	Millimolar
MOPEG	Methoxy-4-hydroxyphenyethyenglycol
MPH	Methylphenidate, “Ritalin”
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

N

NDUFAF2	NADH dehydrogenase 1 alpha subcomplex, assembly factor 2
NE	Norepinephrine
NET (SLC6A2)	Norepinephrine transporter
ng	Nanogram
NGS	Normal goat serum
NPY	Neuropeptide thyrosine

O

OCD	Obsessive-compulsive disorder

ODD	Oppositional defiant disorder

P

PC	Periodic Catatonia
PCR	Polymerase-chain reaction
PDCD10	Programmed cell death protein 10
PDE4D	Phosphodieserase 4D
PFA	Paraformaldehyde
PFC	Prefrontal cortex
Pgk-1	Phosphoglycerate kinase 1
pH	Pondus Hydrogenii
PLEKHB1	Pleckstrin homology domain-containing protein
pMol	Picomol
PNMT	Phenylamine N-methyltransferase

R

RAB6A	Ras-associated protein 6A
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR

S

SA	Short arm
SAP	Shrimp alkaline phosphatase
SCZ	Schizophrenia
sec	Second(s)
SERT (SLC6A4, 5-HTT)	Serotonin transporter

SERPINI2	Serpin peptidase inhibitor 2
SHR rat	Spontaneous hyperactive rat
SLC	Solute carrier
SNAP-25	Synaptosomal associated protein 25
SNP	Single nucleotide polymorphism
SSC	Sodium saline citrate
SSRI	Serotonin reuptake inhibitor
SV2C	Synaptic vesicle protein 2C
SVOP	SV two-related protein

T

Tab.	Table
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TCR	Transcriptional control region
Tm	Medial temperature
TPH	Tryptophan hydroxylase
tRNA	Transfer RNA

U

UTP	Uridine 5’-triphosphate
UTR	Untranslated region
UCP2	Uncoupling protein 2
UV	Ultraviolet

V

VMAT	Vesicular monoamine transporter
VNTR	Variable number tandem repeat
vs	Versus
VTA	Ventral tegmental area

W

WDR49	WD repeat domain 49
WKL1	MLC1 (Megaloencephalic leukoencephalopathy with subcortical cysts)

Z

ZBBX	B-box domain containing zinc finger protein

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5. DECLARATION / ERKLAERUNG

Declaration

I hereby declare that the submitted dissertation „***The contribution of common and rare variants to the complex genetics of psychiatric disorders***“ was completed by myself and no other at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg. I have not used any sources or materials other than those enclosed.

Moreover, I declare that the following dissertation has not been submitted further in this form or any other form and has not been used for obtaining any other equivalent qualification in any other organization.

Würzburg, April 2010

Sandra Schulz

Eidesstattliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die eingereichte Arbeit „***The contribution of common and rare variants to the complex genetics of psychiatric disorders***“ selbstständig am Lehrstuhl für Psychiatrie, Psychosomatik und Psychotherapie der Universität Würzburg angefertigt und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Weiterhin versichere ich, dass ich die vorliegende Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt habe und ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, April 2010

Sandra Schulz