Molecular Genetics of Emotional Dysregulation in Attention-Deficit/Hyperactivity Disorder

Molekulargenetik emotionaler Dysregulation bei Aufmerksamkeitsdefizit-/Hyperaktivitätssyndrom

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Summary

Attention-deficit/hyperactivity disorder (ADHD) is a genetically complex childhood onset neurodevelopmental disorder which is highly persistent into adulthood. Several chromosomal regions associated with this disorder were identified previously in genome-wide linkage scans, association (GWA) and copy number variation (CNV) studies.

In this work the results of case-control and family-based association studies using a candidate gene approach are presented. For this purpose, possible candidate genes for ADHD have been finemapped using mass array-based SNP genotyping. The genes *KCNIP4*, *CDH13* and *DIRAS2* have been found to be associated with ADHD and, in addition, with cluster B and cluster C personality disorders (PD) which are known to be related to ADHD. Most of the associations found in this work would not withstand correction for multiple testing. However, a replication in several independent populations has been achieved and in conjunction with previous evidence from linkage, GWA and CNV studies, it is assumed that there are true associations between those genes and ADHD.

Further investigation of *DIRAS2* by quantitative real-time PCR (qPCR) revealed expression in the hippocampus, cerebral cortex and cerebellum of the human brain and a significant increase in *Diras2* expression in the mouse brain during early development. *In situ* hybridizations on murine brain slices confirmed the results gained by qPCR in the human brain. Moreover, *Diras2* is expressed in the basolateral amygdala, structures of the olfactory system and several other brain regions which have been implicated in the psychopathology of ADHD.

In conclusion, the results of this work provide further support to the existence of a strong genetic component in the pathophysiology of ADHD and related disorders. *KCNIP4*, *CDH13* and *DIRAS2* are promising candidates and need to be further examined to get more knowledge about the neurobiological basis of this common disease. This knowledge is essential for understanding the molecular mechanisms underlying the emergence of this disorder and for the development of new treatment strategies.

Zusammenfassung

Bei Aufmerksamkeitsdefizit-/Hyperaktivitätssyndrom (ADHS) handelt es sich um eine genetisch komplexe neuronale Entwicklungsstörung, die im Kindesalter einsetzt und eine hohe Persistenz ins Erwachsenenalter aufweist. Mehrere chromosomale Regionen zeigten eine Assoziation mit dieser Erkrankung in genomweiten Kopplungsanalysen, Assoziations- (GWA) und Copie Number Variation (CNV) Studien.

In dieser Arbeit werden die Ergebnisse von Fall-Kontroll- und Familien-basierten Assoziationsstudien, basierend auf der Annahme bestimmter Kandidatengene, vorgestellt. Die möglichen Kandidatengene wurden mit Hilfe eines massenspektrometrischen Verfahrens für SNP Genotypisierungen untersucht. Für die Gene *KCNIP4*, *CDH13* und *DIRAS2* konnte eine Assoziation mit ADHS und zudem mit Persönlichkeitsstörungen gefunden werden. Die meisten der in dieser Arbeit berichteten Assoziationen würden einer Korrektur für multiples Testen nicht standhalten. Dennoch kann von einer tatsächlichen Assoziation dieser Gene mit ADHS ausgegangen werden da eine Replikation in verschiedenen unabhängigen Stichproben stattgefunden hat und zudem vorangegangene Kopplungsanalysen, GWA und CNV Studien auf eine Assoziation hindeuten.

Die weitere Untersuchung des *DIRAS2* Gens mit Hilfe von quantitativer real-time PCR (qPCR) ergab eine Expression des Gens im Hippocampus, dem zerebralen Kortex und dem Kleinhirn des Menschen. Zudem wurde ein signifikanter Anstieg der *Diras2* Expression im murinen Gehirn während der frühen Entwicklungsstadien beobachtet. *In situ* Hybridisierungen auf Maushirnschnitten bestätigten die Ergebnisse der qPCR im menschlichen Gehirn. Außerdem wird *Diras2* in der basolateralen Amygdala, in Komponenten des olfaktorischen Systems und in mehreren anderen Hirnarealen, die vermutlich an der Pathologie von ADHS beteiligt sind, exprimiert.

Zusammenfassend untermauern die Ergebnisse dieser Arbeit die Tatsache dass eine starke genetische Komponente an der Entstehung von ADHS beteiligt ist. *KCNIP4*, *CDH13* und *DIRAS2* sind vielversprechende Kandidatengene und sollten weiter untersucht werden um nähere Einblicke in die Neurobiologie dieser häufigen Erkrankung zu erhalten. Das dadurch erlangte Wissen ist notwendig um die molekularen Mechanismen die ADHS zugrunde liegen zu verstehen und um neue Behandlungsstrategien entwickeln zu können.

1. Introduction

1.1. Attention-deficit/hyperactivity disorder

Phenotype and diagnosis

Attention-deficit/hyperactivity disorder (ADHD) is an early onset childhood neurodevelopmental disorder. With a prevalence of 3% - 5% it is one of the most common psychiatric disorders in children (Renner 2008a).

According to the *Diagnostic and Statistical Manual of Mental Disorders,* fourth edition (DSM-IV), ADHD diagnosis is based on two symptomatic dimensions: inattention and hyperactivity/impulsivity. For the diagnosis of the inattentive or the hyperactive/impulsive subtype of ADHD, at least six of nine criteria for one category have to be met. If six or more symptoms of both dimensions are present, the combined subtype of ADHD is diagnosed. ADHD is observed in males more often than in females. Phenotypic gender differences are also observed. Girls suffering from ADHD are more likely affected by the inattentive subtype which is less salient. Therefore, it is possible that girls with ADHD might stay undiagnosed as compared to boys (Biederman 2002). Although ADHD was first perceived as a childhood disease, it is now well accepted to be highly persistent into adulthood.

A common clinical feature of ADHD is its comorbidity with other psychiatric diseases. In children, the occurrence of oppositional defiant disorder (ODD), conduct disorders (CD), unipolar and bipolar mood disorders, anxiety disorders, learning disorders, autism and other pervasive developmental disorders together with ADHD is often observed (Biederman, 2005). In adults with ADHD, comorbidities with anxiety disorders, mood disorders, personality disorders (PD) and alcohol/substance abuse disorders were reported (Jacob 2007). In addition, it has been reported that patients with ADHD in childhood are more likely to be diagnosed with PD in late adolescence (Miller 2008). According to the DSM – IV, PDs are subdivided in three clusters. Cluster A (odd or eccentric cluster) comprises paranoid and schizoid disorders, cluster B (dramatic, emotional or erratic cluster)

includes antisocial, borderline and narcissistic personality disorders, and cluster C (anxious or fearful cluster) consisting of avoidant, dependent and obsessive-compulsive personality disorders.

A cumulative appearance of ADHD within families can be observed and several studies verified the familiarity of ADHD. In adoption studies a higher likelihood for hyperactivity in biological relatives of children with ADHD as compared to adopted relatives was found. Twin studies revealed a heritability of ADHD of almost 80% (Faraone 2005). Those findings indicate that there exists a strong genetic component in the origin of ADHD.

Molecular genetics of ADHD

To identify chromosomal regions which harbor genes for ADHD, whole genome linkage studies can be conducted. Those studies are based on families with multiple affected siblings and examine if any chromosomal regions are shared among family members more often than expected by chance. For this purpose, genetic markers like single nucleotide polymorphisms (SNPs) which are spread throughout the human genome are investigated. Several of such studies identifying possible susceptibility loci for ADHD have been published in the last years. For example Romanos et al. reported linkage at the chromosomal regions 2q35, 5q13.1, 6q22-23, 7q21.11, 9q22, 14q12 and 16q24.1 across eight investigated families (Romanos 2008a). Zhou et al. performed a meta-analysis of seven independent genome-wide linkage scans including the study described above. A genome-wide significant linkage on chromosome 16 and additional suggestive or nominal evidence of linkage for nine other loci was found (Figure 1.1) (Zhou K. , 2008).

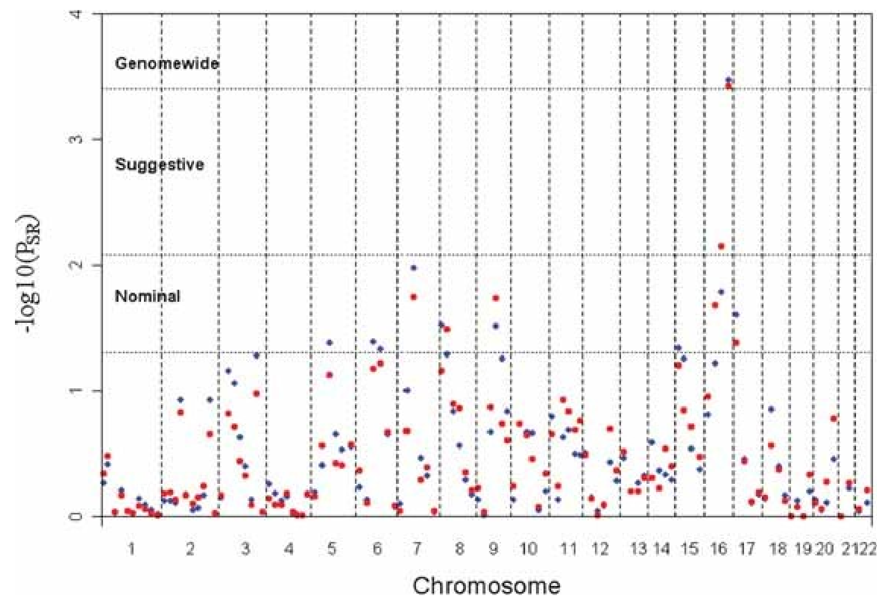


Figure 1.1: Meta-analysis of seven independent genome-wide linkage scans by Zhou et al. Weighted (red) and unweighted (blue) −log10(PSR). The thresholds of nominal (P = 0.05) suggestive (P = 0.0083) and genome-wide significant linkage (P = 0.000417 after Bonferroni correction) are shown (Zhou K. , 2008)

To search for susceptibility genes for ADHD in a hypothesis-free approach, genome-wide association studies (GWAS) can be used. For those studies hundreds of thousands of markers across the human genome are tested for an association with the disease by comparing patients versus control individuals. To overcome the issue of multiple testing, very large sample sizes are needed for GWAS. One of the first GWAS on ADHD was conducted by the International Multicenter ADHD Genetics (IMAGE) project. In their first study (IMAGE I) in 909 affected family trios consisting of the parents and one affected child, none of the 438 784 tested SNPs achieved genome-wide significance (Neale 2008). The data gained in this study were also tested for an association with quantitative traits generated from ADHD symptoms. Two SNPs showed significance within a specific phenotype. Those markers are found in the intronic regions of the *CDH13* and the *GFOD1* gene. Additional 17 genes had association p values < 0.01 (Lasky-Su 2008). A second GWAS of the IMAGE consortium (IMAGE II) testing more than 1 million SNPs for an association with ADHD in 896 cases and 2455 controls failed to identify genome-wide significant associations as well. However, the most significant results implicated some interesting candidate genes. Notably, the *CDH13* gene was amongst those (Neale 2010a). Meta-analysis of the IMAGE I and IMAGE II data and datasets of two additional GWAS on ADHD also revealed

no genome-wide significant findings, suggesting that the effect sizes of common variants must be very small (Neale 2010b). A pooling-based GWAS of adult ADHD (aADHD) found a large number of top-ranked SNPs in the regions of genes which are expressed in the brain including *CTNNA2, ASTN2* and *CDH13 (Lesch 2008)*.

In candidate gene studies selected genes are tested for an association with a disease. Selection of potential candidate genes is usually based on findings of linkage studies or GWAS, animal studies or theoretical considerations implicating a role of the gene in the pathomechanism of ADHD. A case-control design comparing the allele frequencies of patients and control subjects is possible for these studies as well as a family-based design in which the transmission rates of alleles from parents to ADHD children are determined. The genes investigated with this approach so far are for the most part components of the dopaminergic system. But also genes involved in the serotonergic and other neurotransmitter systems as well as genes playing a role in transmitter release have been considered as candidate genes for ADHD. The most frequently studied genes of the dopaminergic system are the dopamine D4 receptor gene (*DRD4*), the dopamine D5 receptor gene (*DRD5*) and the gene coding for the dopamine transporter (*DAT,* SLC6A3). The 7-repeat allele of a tandem repeat polymorphism in exon 3 of *DRD4* was found in several studies to be associated with ADHD. In the *DRD5* gene the 148bp allele of a dinucleotide polymorphism was associated with ADHD in several family-based studies and meta-analyses (Faraone 2005). Significant association between a tandem repeat polymorphism in the 3´ untranslated region (UTR) of the *DAT* gene and ADHD was found in several meta-analyses and pooled analyses (Stergiakouli and Thapar, 2010). A study of 776 ADHD cases found several SNPs located in the 3´ UTR and in the 5´flanking region of *DAT1* to be associated with the disease (Brookes 2006). Also genes involved in other neurotransmitter systems than the dopaminergic one have been reported to be associated with ADHD. To these belong the serotonin 1B receptor gene (*HTR1B*), the serotonin transporter gene (*5-HTT*) and the gene coding for the α-4 subunit of the nicotinic acetylcholine receptor (*CHRNA4*) (Faraone 2005).

The contribution of environmental factors in ADHD

As ADHD is, like other psychiatric disorders a genetically complex disease, it is also influenced by environmental factors. It is documented that maternal smoking and alcohol exposure during pregnancy and low birth weight are risk factors for ADHD. Pregnancy and delivery complications are thought to predispose for ADHD as well. Psychosocial adversities like chronic family conflict, decreased family cohesion and exposure to parental psychopathology are found in ADHD families more often than in control families (Biederman 1995). Such psychosocial factors are considered as nonspecific triggers of an underlying predisposition for ADHD (Biederman, 2005).

Neurobiology of ADHD

A dysbalance in the dopaminergic and noradrenergic systems is often assumed to be a cause for the main symptoms of ADHD. Results of neurobiological, neuroimaging and neuropsychological studies suggest that deficits in frontal lobe function and the connections between the frontal lobe and subcortical regions are of vital importance for this disorder. In structural imaging studies changes in brain anatomy like smaller frontal cortex volumes as well as smaller volumes in subcortical structures and the cerebellum have been found in patients suffering from ADHD (Castellanos 2002) (Sowell 2003). The subcortical structures caudate nucleus, putamen and globus pallidus were reported as altered in ADHD patients by imaging studies. In addition, larger hippocampal volumes have been reported in children and adults suffering from ADHD compared to controls (Plessen 2006). The same study found the basolateral complex of the amygdala to be smaller in children with ADHD than in controls. Most of the fronto-subcortical systems implicated in ADHD are catecholaminergic. Catecholamines are involved in the mechanisms of action of medications for ADHD treatment. Substances like methylphenidate inhibit the dopamine transporter and therefore stop the reuptake of dopamine and norepinephrine from the synaptic cleft. Many models for the effects of ADHD medication are discussed. One is suggesting for example that, via the effect on the dopaminergic and noradrenergic pathways, the inhibition of subcortical structures by frontal cortical activity is increased (Biederman, 2005).

1.2. Kv channel-interacting protein 4 (*KCNIP4)*

A single marker situated in the intron of the *KCNIP4* gene which is located on Chr. 4p15.31 in humans was among the top 25 results of a transmission disequilibrium testing (TDT)-based GWAS on childhood ADHD (cADHD) conducted by the IMAGE project (Neale 2008). The same marker also showed nominal association with ADHD in a GWAS of quantitative traits for ADHD (Lasky-Su 2008). In addition, several SNPs within the *KCNIP4* locus emerged in a GWAS on aADHD (Lesch 2008) and in a GWAS on PD conducted in our group. The location of the markers found to be associated with ADHD and PD is shown in Figure 1.2. Remarkably, the SNPs that were found to be associated with PD are clustered at the 5´region of the gene while those that emerged in the two GWAS on ADHD are mainly located in the 3´region and central regions of *KCNIP4*. Additionally, the KCNIP4 locus was among the top 25 results of GWAS on schizophrenia (Sullivan 2008), emphasizing its possible role in psychiatric disorders.

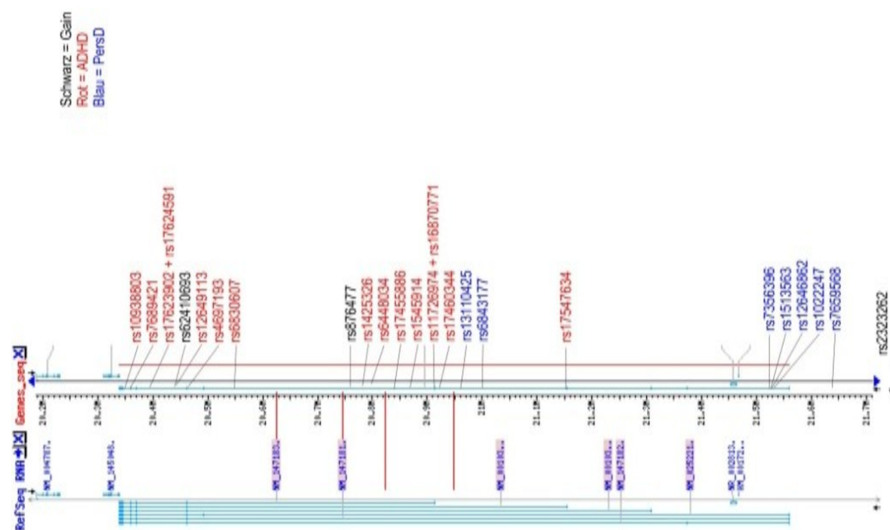


Figure 1.2: Location of SNPs at the *KCNIP4* locus associated with ADHD or PD
SNP IDs are shown in different colors according to the GWAS in which they emerged. Most SNPs associated with PD are clustered at the 3´region while the ADHD associated ones are located more upstream. Black = GWAS on cADHD (Neale 2008); Red = GWAS on aADHD (Lesch 2008); Blue = GWAS on PD

The Kv channel-interacting protein 4 (KCNIP4, KChIP4) which is encoded by the KCNIP4 gene was first described as a binding protein of presenilin (PS) and as a novel KCNIP pro-

tein by Morohashi et al. in 2002 (Morohashi 2002). KCNIP proteins are known as calcium binding proteins that are interaction partners of the voltage-gated potassium channel subunit Kv4 family. This family of subunits is believed to be responsible for the A type potassium current in neurons which is defined as a low-threshold, rapidly activating current that inactivates very fast. This current regulates the firing rate of neurons and the sensitivity to synaptic inputs at the soma and the dendrites. KCNIP4 has been reported to be co-localized with the potassium channel subunit Kv4.2 in the apical and basal dendrites of hippocampal and neocortical pyramidal cells in the rat brain (Rhodes 2004). In the mouse brain it was present in the mitral cell layer of the olfactory bulb, the piriform cortex, in layers II – IV of the cerebral cortex, in the hippocampus, the thalamus and the Purkinje cells of the cerebellum (Xiong 2004) (Pruunsild and Timmusk, 2005).

While the C terminal domains of the four KCNIP proteins (KCNIP1 – 4) are conserved core regions containing EF-hand Ca^{2+} binding motifs, the N termini of those proteins are highly variable and share no homology with other calcium binding protein domains. There are different isoforms of KCNIP4 which differ in their N termini due to alternative splicing or alternative transcription start sites. So far, six predominant transcripts of the human *KCNIP4* gene have been described (Pruunsild and Timmusk, 2005). One isoform of KCNIP4 (KCNIP4a) which contains a K-channel inactivation suppressor (KIS) domain has been found to slow down the closing of Kv4.3 channels and therefore to retard the inactivation of the A type potassium current (Holmqvist 2002). The same splice variant *KCNIP4a* was found to be expressed in a cell type specific manner in the rat brain, leading to a slower potassium current inactivation in cells in the globus pallidus and the basal forebrain compared to striatal and hippocampal neurons which do not express this isoform (Baranauskas, 2004).

In addition to its function in the regulation of potassium currents through its interaction with Kv4 subunits, KCNIP4 has been initially reported to interact with presenilin which is known to be involved in early onset Alzheimer´s disease. A possible implication of this interaction has been found by a group that examined the orphan nuclear receptor Nurr1. This receptor plays an essential role in the development of dopaminergic neurons in the

midbrain that regulate motor control. According to their results, Kitagawa et al. postulate a model (see Figure 1.3) in which Nurr1 is bound to co-repressor molecules as long as there is no signal of the Wnt family of secreted glycoproteins. After Wnt mediated β-catenin transport to the nucleus, Nurr1 associated proteins are switched from co-repressors to co-activators. Nurr1 and β-catenin induce KCNIP4 which associates with a PS complex and promotes β-catenin degradation in a negative feedback loop (Kitagawa 2007). As the Wnt/β-catenin pathway plays an important role in the development of the nervous system, it can be assumed that KCNIP4 is also involved in developmental processes in the brain.

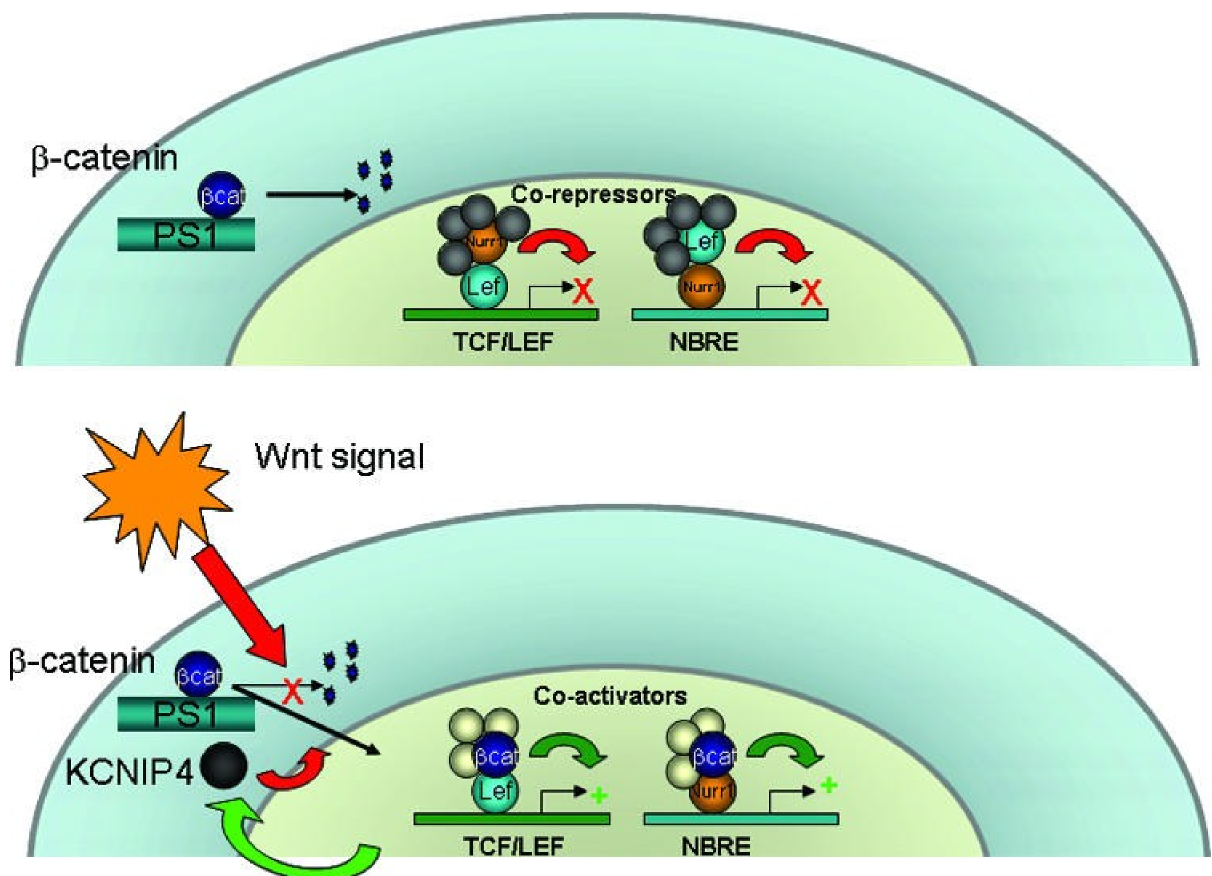


Figure 1.3: Model of the role of KNIP4 interaction with Presenilin (Kitagawa 2007)
Molecules acting as co-repressors are bound to Nurr1 as long as there is no Wnt signal. After Wnt mediated β-catenin accumulation in the nucleus, co-repressors are switched to co-activators. Nurr1 and β-catenin induce KCNIP4 which associates with a PS complex and promotes β-catenin degradation in a negative feedback loop

1.3. Cadherin 13 (CDH13)

The gene coding for Cadherin 13 (*CDH13*; also called T-Cad for Truncated Cadherin or H-Cad for Heart Cadherin) emerged in several GWAS on ADHD and other psychiatric disorders. The *CDH13* gene is located on chromosome 16, in a chromosomal region (16q24) that was described as a linkage locus in a genome-wide linkage analysis of ADHD (Romanos 2008a). Moreover, the gene lies within the only significant linkage region found in a meta-analysis of genome-wide linkage scans of ADHD (Zhou K. , 2008). An intronic SNP in the *CDH13* gene was one of the top 30 single marker association findings in a GWAS on adult ADHD (Lesch 2008). In addition, a marker situated upstream of the *CDH13* gene was found to be over-transmitted in a family-based GWAS of ADHD (Neale 2008). Another intronic SNP within this gene was the most significant association finding in a GWAS of quantitative traits in ADHD conducted with the data gained in the family-based study from Neale et al. 2008 (Lasky-Su 2008). In a case control-based GWAS of childhood ADHD, the *CDH13* gene again was one of the top 100 candidates (Neale 2010a). Finally, a candidate gene approach in a meta-analysis of GWAS of ADHD revealed no genome-wide significant results, but 26 SNPs within the *CDH13* gene were among the top 50 results (Neale 2010b). Beyond its association with ADHD, the *CDH13* gene has been found to be associated with a wide range of other psychiatric disorders such as alcoholism (Treutlein 2009), methamphetamine dependence (Uhl 2008) and schizophrenia (Sullivan 2008).

Besides its strong expression in the cardiovascular system, *CDH13 is* highly expressed in the brain. In humans, lower expression levels have been found in the developing brain than in the adult brain. In human adult brains, *CDH13* expression is reported in the cerebral cortex, the medulla oblongata, the thalamus and the midbrain (Takeuchi 2000).
Cadherins are known to mediate calcium-dependent intercellular adhesion. Cadherin13 is an atypical cadherin since it shares the 5 extracellular cadherin repeat domain (EC1-5) structure but lacks a transmembrane domain and has no cytoplasmic domain. Instead it contains a hydrophobic sequence that serves as a signal for glycosylphosphatidylinositol (GPI) anchor attachment. Via this GPI anchor, Cadherin13 is attached to the plasma membrane of the cell. Cadherin13 has been found to act as a negative guidance signal to

motor axons (Fredette 1996). In endothelial cells, Cadherin13 promotes deadhesion and polarization of cells via the RhoA/ROCK and the Rac pathway (Philippova 2005). Furthermore, signaling of Cadherin13 through PI3K/Akt/GSK3β pathways, in which integrin linked kinase (ILK) is an essential mediator, has been shown in endothelial cells (Joshi 2007). The membrane proteins Grp78 and Intergrin β3 are thought to play an important role in Cadherin13 dependent endothelial cell survival mediated via the Akt pathway (Philippova 2008). It has also been shown that Cadherin13 can mediate cell proliferation and migration initiated by low density lipoprotein (LDL) via the phospholipase C (PLC) which is activating the ERK1/2 pathway (Kipmen-Korgun 2005). The different signaling mechanisms via which Cadherin13 acts are illustrated in Figure 1.4 (borrowed from Philippova et al.).

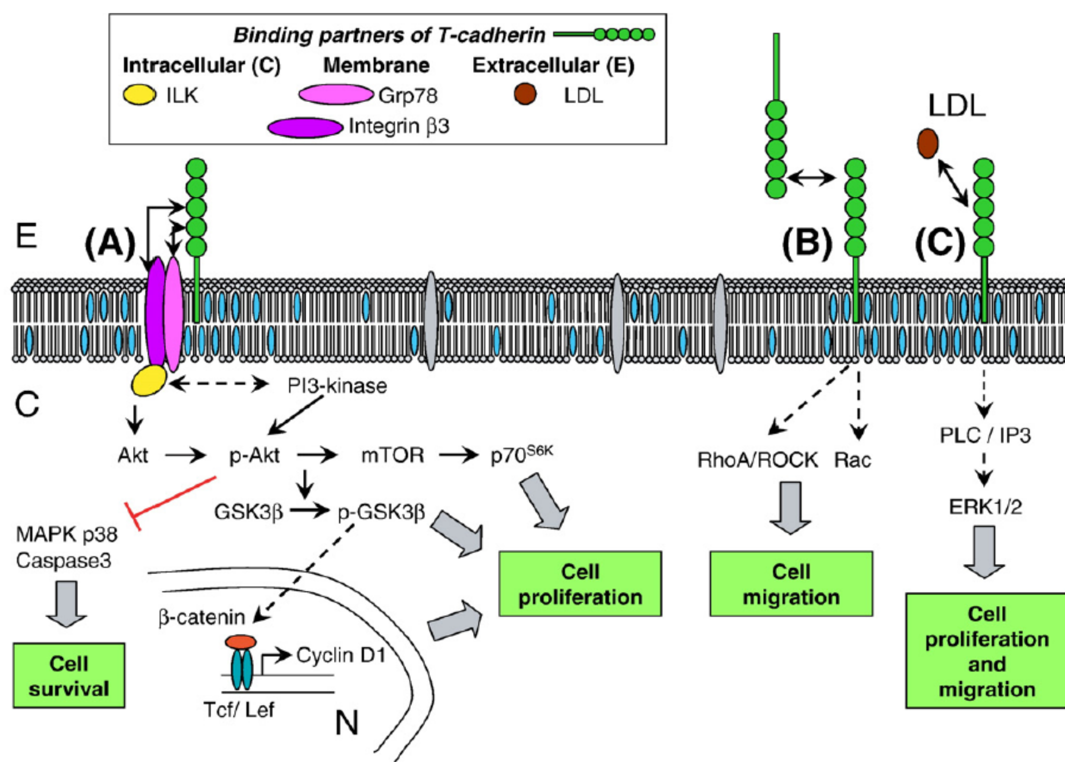


Figure 1.4: Signaling mechanisms activated by Cadherin13 (Philippova 2009)
(A) Cadherin13 promotes proliferation and survival of endothelial cells via activation of the PI3K/Akt/mTOR survival pathway. To exert these functions GPI-anchored Cadherin13 must engage molecular transmembrane adaptors like Grp78 (pink), integrin-β3 (purple) and/or intracellular adaptors e.g. integrin linked kinase (ILK) (yellow) which facilitate inward signal transduction pathway activation. (B) Homophilic Cadherin13 ligation in endothelial cells induces cell deadhesion and polarization via RhoA/ROCK- and Rac-dependent signaling mechanisms. (C) Cadherin13 is able to bind the low-density lipoprotein (LDL). This interaction leads to activation of phospholipase C (PLC), inositol trisphosphate (IP3) formation, intracellular Ca^{2+} mobilization, activation of the tyrosine kinases ERK1/2 followed by mitogenic responses.

1.4. Distinct subgroup of the Ras family member 2 *(DIRAS2)*

Two SNPs on chromosome 9q2.22 were among the 500 top ranking markers in a GWAS on aADHD (Lesch 2008). Additionally, this locus emerged in two linkage studies. One was conducted in individuals recruited in the same environs as those in the aADHD GWAS (Romanos 2008a), the other one was performed in the UK (Asherson 2008). The chromosomal region comprises the *Distinct subgroup of the Ras family member 2* (*DIRAS2*) gene which is known to be uniquely expressed in the brain with high expression levels in the cerebral cortex, occipital pole, frontal and temporal lobe and cerebellum (Kontani 2002).

The human Ras superfamily of small guanosine triphosphatases (GTPases) consists of more than 150 members and is divided in five subfamilies: Ras, Rho, Rab, Ran and Arf. Small GTPases share a common biochemical mechanism and act as molecular switches that regulate many cell functions like proliferation and differentiation. A common feature of small GTPases is their high affinity binding of guanosine diphosphate (GDP) and guanosine triphosphate (GTP), with the GTP-bound molecule representing the active state. GDP/GTP cycling is controlled by two classes of regulatory proteins. Activation of small GTPases is promoted by guanine nucleotide exchange factors, whereas GTPase-activating proteins accelerate the intrinsic GTPase activity to promote formation of the inactive GDP-bound form (Wennerberg 2005).

Although related in sequence, Di-ras proteins (Di-ras1 and Di-ras2) differ in their biochemical and functional properties from other Ras family members. Like most Ras kinases they have a highly conserved GTP-binding domain, an effector domain and a membrane localizing motif at the carboxyl terminus. However, there are amino acid substitutions present at positions that are critical for GTP hydrolysis. This biochemical property fits to the finding that Di-ras proteins are found mainly in GTP-bound form in living cells. Kontani et al. found that Di-ras2 neither activates the mitogen activated protein kinase (MAPK) pathway, nor induces Akt activation which is a downstream target of phosphoinositide 3 kinase (PI3K).

As Di-ras2 has significant homology to Aplasia Ras homolog member I (ARHI, NOEY2, Di-ras3) which has been identified as a tumor suppressor, both proteins may have similar roles in cell function (Kontani 2002). In breast and ovarian cancer a loss of ARHI expression due to promoter methylation is observed. There is evidence that ARHI inhibits cell growth through several pathways including the MAPK, the PI3K and the STAT3 pathways. Reexpression of ARHI in human ovarian cancer cell lines and ARHI overexpression in xenograft tumors leads to an inactivation of the mammalian target of rapamycin (mTOR) (Lu 2008) (Zhao 2010). mTOR regulates protein synthesis through the inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein (4E-BP1), and through the activation of S6 kinase (S6K1). One of the upstream effectors of mTOR is Akt which, as mentioned above, is part of the PI3K signaling cascade (Hay and Sonenberg, 2004). The mTOR pathway is rapidly activated by ketamine and results in rapid and sustained elevation of synapse associated proteins and spine number in the prefrontal cortex (PFC) of rats. This might represent a mechanism for the rapid antidepressant actions of ketamine (Li 2010). Therefore, the mTOR pathway plays an important role not only in the origin of psychiatric diseases but also in their treatment. Taken together, these facts indicate a possible role of Di-ras2 in the control of cell growth.

A study investigating the distinct transcriptomes of serotonergic neurons found *Diras2* expression increased 13 fold in serotonergic neurons isolated from the caudal hindbrain of mouse embryos, pointing to a role in the regulation of the caudal serotonergic system (Wylie 2010). A GWAS on cognitive performance of the aging brain revealed DIRAS2 to be associated with performance in the Boston naming test indicating a role of DIRAS2 in cognitive processes (Seshadri 2007). However, it is still not known via which pathways Di-ras2 acts and which function it accomplishes.

2. Material and Methods

2.1. Material

2.1.1. Human samples

A total of 1659 patients suffering from aADHD were available for genotyping. All patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders) criteria along a semi-structured interview. Consensus eligibility criteria for this study across all study sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years by retrospective diagnosis (which was confirmed by a family member, wherever possible), life-long persistence and current diagnosis. Additionally, scales on disease severity as well as co-morbid conditions have been included. 624 patients were recruited in Germany, 285 in The Netherlands, 497 in Norway and 253 in Spain. Individuals were aged 18 – 65 years. Patients suffering from addictive disorders, bipolar disorders, schizophrenia or mental retardation were excluded. The healthy control group consisted of 2478 individuals altogether, 630 of which were collected in the Lower Franconia area of Germany, 467 in Munich in Germany, 508 in The Netherlands, 562 in Norway and 311 in Spain (Jacob 2007) (Franke 2010).

The family-based childhood ADHD sample from Würzburg comprises of 171 families, of which 113 are trios, 43 are quartets and 15 are multi sibling families. 142 family trios were recruited in Homburg, Germany. All of the children in both samples were affected by the combined subtype of ADHD according to DSM-IV criteria. Exclusion criteria were an intelligence quotient < 80, autism, Tourette syndrome and severe primary psychiatric disorders (Renner 2008b) (Neale 2010a).

In addition to the adult and childhood ADHD samples, genotyping of a PD sample and of a bipolar disorder sample was conducted for some candidate genes. The PD sample consisted of 708 patients from Germany diagnosed with personality disorder according to criteria of DSM-IV and assigned to cluster B and cluster C using the SCID-II. The bipolar

sample consisted of 214 unrelated bipolar patients from the German Lower Franconia area for whom an ICD-10 diagnosis was established by means of an extensive, semi-structured interview carried out by two experienced psychiatrists at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg. A further 166 unrelated bipolar patients were ascertained according to ICD-10 diagnostic criteria for research (DCR) by means of a semi-structured interview at the Center for Psychiatric Research, Århus University Hospital, Risskov, Denmark, giving a total number of 380 patients suffering from bipolar disorder (Jacob 2005) (Scholz 2010).

2.1.2. Animals

All animals were housed in a colony room under controlled temperature and humidity conditions, with lights on between 6:00 a.m. and 6:00 p.m., with food and water freely available. All animal protocols have been reviewed and approved of the review boards of the University of Würzburg and of the Government of Unterfranken (Bavaria, Germany), and were in accordance with international guiding principles of the care and use of animals.

For in situ hybridization, qPCR and western blotting experiments adult C57BL/6J wild-type mice were decapitated in isoflurane anesthesia. Brains were dissected and immediately frozen in dry ice-cooled isopentane (AppliChem, Darmstadt, Germany) and tissue was stored at -80°C.

For hippocampal primary cell culture the embryos (C57BL/6J background, wild-type) of a 2 – 3 month old pregnant mouse were obtained at embryonic day 18 (E18).The mother animals were sacrificed by cervical dislocation or exposure to isoflurane (Forene®, Abbott, Illinois, USA).

2.1.3. Cell lines

The human cell lines A172 (glioblastoma), U373 (astrocytoma), SK-N-SH (neuroblastoma) and HEK293 (immortalized human embryonic kidney cells) have been used for western blotting analyses.

2.2. Association studies

2.2.1. SNP selection

The SNP IDs (rs numbers) of SNPs located within the sequence of a potential candidate gene plus the 5´ and 3´ flanking sequences were downloaded from the HapMap genome browser. In the case of genes with very long sequences, the selection was narrowed down to the SNPs located in the exons and their flanking intronic sequences. Tagging SNP selection was performed using the tagger algorithm of the Haploview software for pair-wise tagging with a r^2 threshold > 0.8 and a minor allele frequency > 5% (Barrett 2005).

2.2.2. Mass array-based SNP genotyping

SNP genotyping was conducted using the MassArray® system (Sequenom, San Diego, CA). For the so called iPLEX™ assay, genomic DNA isolated from blood conduced as template for a multiplex PCR. Up to 30 different DNA fragments, each harboring one SNP were amplified in this reaction using 100 nM primers (Metabion, Martinsried, Germany), 0.5 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 500 µM dNTPs (Promega, Madison, WI, USA), 1.25x Hotstar PCR buffer (Qiagen) and 1.625 mM MgCl2 (Qiagen) in a total volume of 5 µl. PCRs were performed using a Biometra thermocycler (Biometra, Göttingen, Germany) The reaction conditions were as follows:

Step	Purpose	Temperature	Time	Repeats
1	Denaturation	94°C	15 min	1
2	Denaturation	94°C	20 sec	
3	Annealing	56°C	30 sec	44
4	Amplification	72°C	1 min	
5	Amplification	72°C	3 min	1
6	Cooling	10°C	until end	

PCR products underwent treatment with shrimp alkaline phosphatase (SAP) at 37°C for 20 min to dephosphorylate unincorporated dNTPs. The next step was a single base extension of primers that bind to the amplified DNA immediately adjacent to the SNPs of interest. This reaction was done using mass modified ddNTPs and 0.625 µM of low mass primers and 1.25 µM of high mass primers under the following conditions:

Step	Purpose	Temperature	Time	Repeats
1	Denaturation	94°C	30 sec	1
2	Denaturation	94°C	5 sec	40
3	Annealing	52°C	5 sec	
4	Extension	80°C	5 sec	
5	Extension	72°C	3 min	1

After desalting the samples with 6 mg SpectroCLEAN™ resin (Sequenom), 40 nl of each reaction was spotted onto a SpectroCHIP™ (Sequenom) using the Sequenom MassARRAY™ Nanodispenser. Mass spectrometric analysis was carried out on a Bruker Autoflex time-of-flight mass spectrometer (MALDI-TOF; Bruker Daltonics, Billerica, MA, USA).

2.2.3. Statistical analyses

Mass spectra were all checked by eye for quality control before the statistical evaluation. Statistical analyses of genotyping data was done by Thuy Trang Nguyen at the institute for medical biometry and epidemiology of the Philipps University in Marburg, Germany. Only SNPs passing quality check, which was defined by MAF >0.01, call rate >80% and p value of χ^2 test for Hardy-Weinberg equilibrium >0.01 in controls or parents, were analyzed. Inconsistent genotypes in the family sample were revealed and removed by means of PedCheck (O'Connell and Weeks, 1998). Generalized linear models (GLM) implemented in the R function glm() were carried out to test for an association between SNP genotype as independent variable and a trait of interest as dependent variable (binary: ADHD, bipolar, cluster B and C PD; quantitative: NEO-PI R and TPQ). Binomial logit link or Gaussian identi-

ty link was used for binary or quantitative traits, respectively. In addition, Fisher's exact test was performed in order to assure the asymptotic test results of glm(). Family-based association analysis was carried out using pedigree disequilibrium test (Martin 2000) to assess whether an allele is more frequently transmitted to affected offspring.

The omnibus test implemented in famhap (10,000 simulations; (Becker and Knapp, 2004)) was used to test the global hypothesis that association with ADHD may be contributed to by at least one of the estimated haplotypes. The LD blocks were defined by pairwise D'>0.65 assessed using Haploview. For haplotype-specific association testing Unphased (Dudbridge, 2008) was used.

For the meta analysis, Woolf's test for heterogeneity, obtained with the R function meta.MH(), was performed. A joint analysis assessing the common odds ratio (OR) was done by means of GLM described above, adjusted for fixed country effect.
Unless otherwise specified, p values are two-sided p values unadjusted for multiple testing. The significance level was set to 0.05. The Bonferroni method was used to calculate p values adjusted for testing multiple SNPs and haplotypes for association with ADHD. Power calculations were performed under a two-sided nominal significance level of 0.05 using QUANTO Version 1.0 (http://hydra.usc.edu/gxe).

2.3. Expression studies

2.3.1. Quantitative real-time analyses

RNA isolation and cDNA synthesis

RNA isolation from mouse brain tissue was conducted by Dr. Claudia Kriegebaum using the trizol procedure (Invitrogen™, LIFE TECHNOLOGIES, USA) followed by purification and removal of potential remaining genomic DNA with the RNeasy kit (QIAGEN, Hilden, Germany) in combination with DNase I treatment. RNA concentration and quality were determined using the automated electrophoresis system Experion™ (Biorad, Munich, Germany) as described in the appertaining manual. In addition, human total RNA of different

brain regions (cerebral cortex, hippocampus, putamen, caudate nucleus, cerebellum, pons and medulla oblongata) was obtained from BD Biosciences (Erembodegem, Belgium). This RNA was pooled from 10-35 male and female Caucasians (16-70 years).
500 ng of total RNA were reversely transcribed into complementary DNA in a 20 µl reaction volume using the iScript™ cDNA synthesis kit (Biorad, Munich, Germany). After the reverse transcription reaction the cDNA was diluted 1:5 with DEPC treated water.

Quantitative real-time polymerase chain reaction (qPCR)

For quantitative real-time PCR the IQ™ SYBR® Green supermix (Biorad, Munich, Germany) and Quantitec primer assays (QIAGEN, Hilden, Germany) or self-designed primers were used. The oligonucleotides used in this work are listed in Table 2.1 and Table 2.2. The volume of each reaction was 10 µl containing 1x IQ™ SYBR® Green supermix, 1x Quantitec primers or 500 nM of each oligonucleotide primer and 1µl of the diluted cDNA. PCR and fluorescence measurements were run in the CFX384™ Real-Time PCR detection system (Biorad, Munich, Germany). The reaction conditions were as followed:

Step	Purpose	Temperature	Time	Repeats
1	Denaturation	95°C	15 min	1
2	Denaturation	95°C	30 sec	40
3	Annealing Amplification Fluorescence measurement	60°C	45 sec	
4	Denaturation	95°C	1 min	1
5	Melting curve	55°C – 95°C (0.5°C steps)	10 sec per step	
6	Cooling	15°C	until end	

Species	Gene	GenBank accession No	Primer	Amplicon length
Mus musculus	Beta-actin	NM_007393	Mm_Actb_2_SG	149 bp
	Glyceraldehyde-3-phosphate dehydro-genase	NM_01001303	Mm_Gapdh_3_SG	100 bp
	Ubiquitin C	NM_019639	Mm_Ubc_1_SG	75 bp
	18s ribosomal RNA	X00686	Mm_Rn18s_2_SG	149 bp
	Beta-2 microglobulin	NM_009735	Mm_B2m_2_SG	143 bp
	Ribosomal protein, large, P0	NM_007475	Mm_Rplp0_1_SG	125 bp
	Latrophilin 3	NM_198702	Mm_Lphn3_2_SG	104 bp
Homo sapiens	Beta-actin	NM_001101	Hs_ACTB_2_SG	104 bp
	Glyceraldehyde-3-phosphate dehydro-genase	NM_002046	Hs_GAPDH_2_SG	119 bp
	18s ribosomal RNA	X03205	Hs_RRN18S_1_SG	149 bp
	Aminolevulinate delta synthase 1	NM_000688	Hs_ALAS1_1_SG	113 bp
	Peptidylprolyl isomer-ase A	NM_001008741	Hs_PPIA_4_SG	121 bp

Table 2.1: Quantitec primer assays used for quantitative real-time PCR

Species	Gene	GenBank ac-cession No	Primer	Sequence 5´- 3´
Mus musculus	Diras2	NM_001024474	Mm_Diras2_Ex1_for	GAGCTGCGCCTGGAGACCTG
	Diras2	NM_001024474	Mm_Diras2_Ex2_rev	CCGCCACCCGGTAGTCGTTG
Homo sapiens	Diras2	NM_017594	Diras2_Ex1_for	CGAGTGGAGCTCTGAAGAAG
	Diras2	NM_017594	Diras2_Ex2_rev	GGCATGTTGCTGGAGAGC

Table 2.2: Self-designed primer pairs used for quantitative real-time PCR

Determination of expression levels and statistical analyses

PCR efficiencies were determined based on raw data using the software tool LinReg. This tool calculates the PCR efficiency for each individual sample from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase (Ramakers 2003). Baseline correction of threshold cycle (Ct) values was performed by the CFX Manager™ software (Biorad, Munich, Germany). Relative quantities (Q values) were obtained by applying the following formula:

$$\text{Q value} = \text{PCR efficiency}^{[\text{Minimum (Ct values)} - \text{Ct value}]}$$

To normalize the Q values of the investigated target genes, normalization factors were calculated based on the relative quantities of several reference genes. Firstly, the gene expression stability values (M) for those reference genes were calculated. Then the Q values of the two to three genes showing the most stable expression were used for normalization factor determination using the GeNorm algorithm. (Vandesompele 2002) To get the normalized expression levels, the Q values of the target genes were divided by the normalization factor for each sample. Statistical evaluation of expression data was done running a Kruskal-Wallis test over all groups testing for a group effect. If this test showed significant group differences (p value< 0.05), a Mann-Whitney test for detecting differences between two groups was performed. The significance threshold was Bonferroni corrected for multiple testing.

2.3.2. RNA *in situ* hybridization

cRNA probe synthesis

A *Diras2* specific cDNA fragment was synthesized by reverse transcription of total RNA isolated from mouse brains followed by a PCR using the following primers:

Sonde_Diras2_Mm F (forward):	5´- GAGAAGCTCAAGGGCAAGTG -3´
Sonde_Diras2_Mm R (reverse):	5´- CATCTGCCTGCCTACTCCTC -3´

The 445bp PCR product was cloned into the pCRII® dual promotor vector using the TOPO® TA cloning® kit dual promotor according to the manufacturer´s instructions (Invitrogen™, LIFE TECHNOLOGIES, USA).

The vector construct served as template for the generation of digoxigenin (DIG) labeled cRNA probes by in vitro transcription. Therefore, the vector was linearized by a restriction digest using either XhoI (sense) or BamHI (antisense) and cleaned up using the QIAquick PCR purification kit (Qiagen, Inc. Valencia, CA, USA). cRNA probe synthesis and DIG labeling was performed using SP6 RNA polymerase for the sense and T7 RNA polymerase for the antisense probe generation and the DIG RNA labeling mix (Roche, Mannheim, Germany). For riboprobe clean-up RNeasy Mini columns (Qiagen, Inc. Valencia, CA, USA) were used according to the manufacturer´s directions. To the eluted probes the same volume of 100% formamide was added.

In situ hybridization (ISH)

In situ hybridization was performed as described by Palop et. al. (Palop 2011). 10 µm cryostat sections of native mouse brains were fixed for 10 min with 4% PFA in 1x PBS. After washing with PBS sections were digested with 1 µg/ml proteinase K in 50 mM Tris-HCl (pH 8.0)/5 mM EDTA buffer for 30 min and fixed again in 4% PFA for 5 min. Once washed with PBS, sections were incubated in acetylation solution containing 1.355% triethanolamine, 0.175% HCl and 0.25% acetic anhydride for 10 min followed by three washes with PBS. For pre hybridization slides were covered with hybridization buffer (50% formamide, 5× salt sodium citrate (SSC) buffer, pH 7.0, 5× Denhardt's solution, 0.25 mg/ml salmon sperm DNA) and incubated for 4 h at room temperature in a humidified chamber. DIG labeled riboprobes and hybridization buffer were heated at 70°C for 3 min. The riboprobes were added to the hybridization buffer in a final concentration of 500 – 1000 ng/ml. 100 µl of this hybridization solution was added on each slide, covered with parafilm and incubated at 72°C overnight. To remove unbound probes the sections were washed with 5x SSC for 10 min and three times with 0.2x SSC for 30 min at 72°C followed by a 5 min wash with 0.2x SSC and a 5 min wash with 1x TBS at room temperature. To block nonspecific binding, the slides were transferred to a humidified chamber and 500 µl of 1x TBS with 10%

heat inactivated sheep serum (HISS) were added on each slide for 1 h at room temperature. Blocking solution was replaced with 500 µl of a 1:5,000 dilution of anti-digoxigenin antibody-AP (Roche, Mannheim, Germany) in TBS with 3% HISS and incubated at 4°C overnight. After several washes with TBS and equilibration with NTMT buffer (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl2) color development was conducted using a 1:50 dilution of NBT/BCIP stock solution (Roche, Mannheim, Germany) in NTMT buffer overnight in the dark. Color reaction was stopped by washing the slides in PBS/EDTA (0.1 M PBS, 1 µM EDTA pH 8.0), fixed for 10 min with 4%PFA in PBS and washed in PBS. Finally, sections were rinsed with distilled water to remove salts and coverslipped using Aquatex® (Merck, Darmstadt, Germany).

2.3.3. Western blot analyses

Protein lysates

Cells and tissues were lysed using Nonidet-P40 (NP40) buffer consisting of 150 mM sodium chloride, 1 % NP40 and 50 mM Tris (pH 8.0). For the preparation of cell lysates 166 µl ice cold NP40 buffer containing proteinase inhibitor cocktail (Roche, Mannheim, Germany) was added per 25 cm^2 cell culture flask. Adherent cells were removed from the flask using a plastic cell scraper and the cell suspension was transferred to a microfuge tube and sonicated for 15 sec.

For tissue lysates 100 µl NP40 buffer containing proteinase inhibitor cocktail (Roche, Mannheim, Germany) was added per 20 mg of brain tissue. The tissue was separated using the Tissue LyserII (Qiagen, Inc. Valencia, CA, USA) for 30 sec followed by sonication for 15 sec. Protein concentrations were determined by a Bradford assay for which 1 µl protein lysate was added to 1 ml Bradford reagent (Sigma-Aldrich, Munich, Germany) and the optical density was measured at 595 nm. The total protein concentration was calculated based on a BSA standard curve.

Denaturing reduced protein electrophoresis

The NuPAGE® system (Invitrogen™, LIFE TECHNOLOGIES, USA) was used for electrophoretic separation of proteins. 50 µg total protein were mixed with appropriate volumes of NuPAGE® LDS sample buffer and NuPAGE® reducing agent according to the NuPAGE® user manual for denaturing gel electrophoresis with reduced protein samples. Samples were loaded on a NuPAGE® Novex 4-12% Bis-Tris-Gel and separated at 200V for 50 min in an electrophoresis apparatus filled with NuPAGE® MOPS SDS running buffer.

Western blotting

After electrophoretic separation proteins were transferred to PVDF or nitrocellulose membranes using the XCell II™ blot module (Invitrogen™, LIFE TECHNOLOGIES, USA). Protein gel, membrane, filter papers and blotting pads were assembled as shown in Figure 2.1. All components were soaked in NuPAGE® transfer buffer with 10% methanol and the closed blot module was filled with transfer buffer for wet blotting. The protein transfer took place at 30V for 1h. Afterwards, successful transfer was verified by reversible Ponceau S staining.

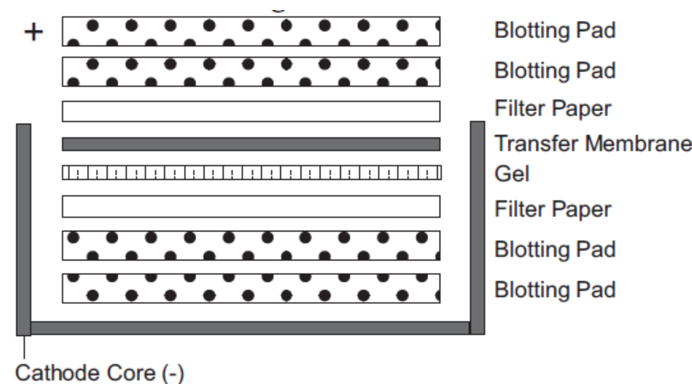


Figure 2.1: Assembly of blotting pads, filter papers, protein gel and transfer membrane for western blotting (Invitrogen)

Immunological protein detection

To prevent unspecific background binding, the membrane was incubated with 5% milk powder in TBS with 0.1% Tween-20 (TBS-T) for 1h at room temperature. For specific detection of proteins, primary antibodies were diluted in 5% milk in TBS-T as listed in Table 2.3. Primary antibody incubation was conducted at 4°C overnight. After three washes with TBS-T, the primary antibody was detected using a suitable horseradish peroxidase (HRP) coupled secondary antibody (dilutions are given in Table 2.3). Secondary antibody incubation took place for 1h at room temperature. The specific protein bands were visualized by enhanced chemiluminescence (ECL) using the Amersham™ ECL plus reagent (GE Healthcare, Buckinghamshire, UK) or the SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) depending on signal intensity. The emerged light signal was detected using the ChemiDoc™ system (Biorad, Munich, Germany).

Antigen	**Antibody**	**Company**	**Dilution**
Di-ras2	15557-1-AP	Proteintech	1:1000
β-Actin	ab8224	Abcam	1:1000
Mouse Ig	Goat anti-Mouse HPR conjugated	BD Pharmingen	1:5000
Rabbit Ig	Goat anti Rabbit HRP conjugated	Sigma	1:20 000

Table 2.3: Antibodies and dilutions used for western blotting analyses

Blocking with immunizing peptide

To determine if an antibody staining is specific or which band is specific if several bands occur, blocking of the antibody with the immunizing peptide was performed. For this, the blocking peptide was added to the diluted primary antibody to a final concentration of 1 µg/ml and the solution was incubated with agitation for 30 min at room temperature. Then, the staining procedure was carried out as described above.

Stripping blots for reprobing

In order to detect different targets in the same samples the primary and secondary antibodies bound to the membrane were removed by a method called stripping. Whenever the primary antibody used for the second staining originated from a different species than the one used for the previous staining, a mild stripping method was used. The membrane was incubated in stripping buffer containing 1.5% glycine, 0.1% SDS and 1% Tween 20 (pH2.2) for 10 min, twice. After two 10 min washes with PBS and two 5 min washes with TBS-T the membrane was ready for blocking. When both antibodies originated from the same species, a harsher stripping protocol was used. This was done by incubating the membrane in a buffer containing 10% SDS, 0.5M Tris HCl (pH 6.8) and 0.8% β-mercaptoethanol at 50°C for 30 min. The membrane was blocked after several washing steps with TBS-T.

2.3.4. Fluorescence immunocytochemical double stainings

Mouse primary hippocampal cell culture

A 2 – 3 month old pregnant mouse (embryonic day 18 (E18), wild type, C57BL/6 background) was sacrificed by cervical dislocation or exposure to isoflurane (Forene®, Abbott, Illinois, USA). After opening the abdominal cavity the uterus with the embryos was transferred to a petri dish. The embryos were decapitated using a forceps and the heads were put in a new dish filled with pre-warmed HBSS buffer. The skin and the skull were cut along the middle vein and bent away to remove the brain. For dissection of the hippocampi the hemispheres were removed from diencephalon and brainstem. The meninges were removed carefully and hippocampi were dissected by cutting along the convex outer side using a forceps.

All dissected hippocampi were transferred to pre warmed 0.05% Trypsin/EDTA and digested at 37°C for 5 min. Hippocampi were washed with warm HBSS buffer twice. After removal of the HBSS, 2 ml of neurobasal medium were added. To separate the cells, hippocampi were dissolved by pipetting up and down using a fire polished glass Pasteur pi-

pette. Cells were counted using a Neubauer chamber. 800 000 cells were plated per 25 cm^2 culture flask and 25 000 cells per 2 cm^2 cover slip. Culture flasks and cover slips were both poly-D-lysine coated. Cells were cultured for 5-30 days in an incubator at 37°C. For immunological staining cells were usually cultured for 12 days.

Fixation and staining of cells

For fixation, cells were washed with PBS and ice cold methanol was added. Cells were fixed for 20 min at -20°C. This was followed by three washes with PBS. Fixed cells can be stored in PBS at 4°C for several weeks. To prevent unspecific staining, cells were blocked for 2h at room temperature with PBS containing 2% normal goat serum (NGS). Primary antibody incubation was performed at 4°C overnight with antibodies diluted in 2% NGS in PBS as listed in Table 2.4. After washing with PBS, the cells were incubated with a 1:500 dilution of fluorescence dye coupled secondary antibodies for 1-2h at room temperature. The cells were washed with PBS, rinsed with distilled water and afterwards coverslipped with Fluoro-Gel (EMS, Hatfield, PA, USA) containing a 1:1000 dilution of DAPI for nuclei staining.

Antigen	Antibody	Company	Dilution
Di-ras2	15557-1-AP	Proteintech	1:500
GFAP	Monoclonal Anti-GFAP clone G-A-5	Sigma	1:2000
Synyaptophysin	Monoclonal Anti-Synaptophysin	Sigma	1:200
MAP2	Mouse Anti-MAP-2 monoclonal	Chemicon	1:500
TH	Mouse Anti-Tyrosine Hydrox-ylase	Chemicon	1:500
Calbindin	Monoclonal Anti-Calbindin D-28K	Swant	1:500
Parvalbumin	Monoclonal Anti-Parvalbumin	Swant	1:500
Rabbit Ig	Alexa Fluor 488 goat Anti-Rabbit	Sigma	1:400
Mouse Ig	Alexa Fluor 555 goat Anti-Mouse	Sigma	1:400

Table 2.4: Antibodies and dilutions used for immunocytological staining

3. Results

3.1. Association studies

3.1.1. KCNIP4

Adult ADHD

49 of 56 SNPs within or near the *KCNIP4* gene passed quality control (Minor allele frequency > 0.01, p value of χ^2 test for Hardy-Weinberg equilibrium (HWE) > 0.01, Call rate > 80%) for association testing in 594 patients suffering from adult ADHD (aADHD) and 974 healthy controls from Germany (Würzburg and Munich). Six SNPs showed a nominally significant association with aADHD with p values < 0.05 (see Table 3.1). In addition, eight markers were found to be associated with co-morbid disorders within the aADHD sample compared to controls (p values 0.0471 – 0.0067; Table 3.2). Haplotype testing revealed one (block 4) of the nine haploblocks (Figure 3.1) to be associated with the disease (p = 0.0079). This block consists of the SNPs rs1388321, rs6447994, rs10516367, rs6850182, rs17557419 and rs16870168. The risk haplotype CACTCG of block 4 showed an odds ratio of 1.99. None of the SNPs within this haploblock showed an association with aADHD on the single marker level. The chromosomal position of all genotyped SNPs is depicted in Figure 3.2.

SNP ID	P value	OR (allele)	95%-CI
rs4697192	**0.0166**	1.23 (A)	1.0389 - 1.4653
rs16870771	**0.0349**	1.51 (C)	1.0298 - 2.2211
rs13110425	**0.0092**	1.24 (C)	1.0540 - 1.4525
rs1349384	**0.0490**	1.19 (G)	1.0007 - 1.4184
rs7356396	**0.0138**	1.28 (G)	1.0508 - 1.5472
rs12646862	**0.0147**	1.27 (C)	1.0481 - 1.5371

Table 3.1: Significant markers of the *KCNIP4* gene of case control-based association testing for adult ADHD
Six of the 49 tested SNPs showed an association with aADHD (p value < 0.05). OR = odds ratio; CI = confidence interval.

Phenotype	SNP ID	P value	OR (allele)	95%-CI
Anxiety disorder	rs4697192	**0.0201**	1.40 (A)	1.0539 - 1.8525
	rs1388321	**0.0169**	1.39 (C)	1.0615 - 1.8326
	rs6850182	**0.0383**	1.28 (T)	1.0134 - 1.6137
	rs983071	**0.0358**	1.87 (A)	1.0426 - 3.3577
	rs16870771	**0.0305**	1.91 (C)	1.0625 - 3.4204
Mood disorder	rs4697192	**0.0244**	1.28 (A)	1.0328 - 1.5939
	rs6448034	**0.0067**	1.31 (C)	1.0782 - 1.5950
	rs17455886	**0.0172**	1.28 (C)	1.0448 - 1.5711
	rs13110425	**0.0471**	1.22 (C)	1.0025 - 1.4835
Substance abuse disorder	rs4697192	**0.0169**	1.35 (A)	1.0552 - 1.7261

Table 3.2: SNPs that showed a nominally significant association with co-morbidities within the aADHD sample
Eight markers were found to be associated with co-morbid disorders. rs4697192 was associated with anxiety disorder, mood disorder and substance abuse disorder. The other seven markers were associated with anxiety disorder or mood disorder.

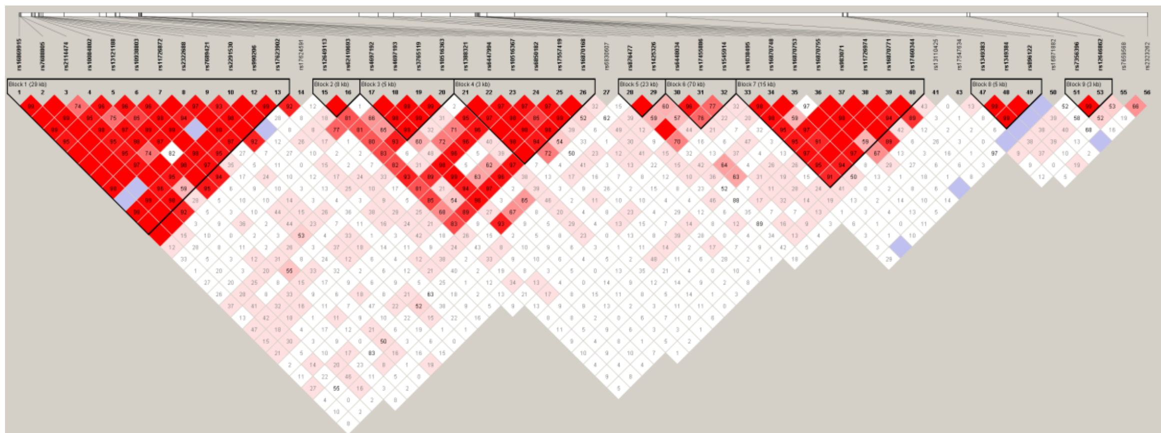


Figure 3.1: Haploblock structure of the *KCNIP4* gene
Due to the linkage disequilibrium between SNPs, the *KCNIP4* gene showed a haploblock structure consisting of nine blocks. The CACTCG haplotype of block 4 was nominal significant associated with aADHD (p = 0.0079; OR = 1.9870).

Childhood ADHD

Pedigree disequilibrium testing for 51 SNPs which passed quality check was performed with genotyping data gained from 171 families (113 trios, 43 quartets and 15 multi sibling) consisting of 592 individuals in total. Two SNPs indicated a nominally significant association with ADHD (rs16869915 p = 0.0477 and rs358834 p = 0.0411). Nine markers showed an over-transmission of one allele for co-morbid disorders, but only two of them acquired a sufficient significance level (rs11726872: learning disability p = 0.0038; rs6447994: oppositional defiant disorder p = 0.0023).

Personality disorders

To investigate the possible role of *KCNIP4* in PD, 48 SNPs in 630 cases and 974 controls were genotyped. After eight markers did not fulfill quality criteria, 40 SNPs were tested for an association with cluster B and cluster C PD. Six SNPs were found to be associated with cluster B and four SNPs with cluster C PD. For the markers rs1425326, rs7356396 and rs12646862 risk alleles for both cluster B and C PD were identified (Table 3.3).

Phenotype	SNP ID	P value	OR (allele)	95% Cl
Cluster B	rs11726872	**0.02639**	1.33 (C)	1.0344 - 1.7202
	rs1425326	**0.00910**	1.35 (T)	1.0769 - 1.6849
	rs6448034	**0.03561**	1.20 (C)	1.0123 - 1.4220
	rs983071	**0.04367**	1.43 (A)	1.0102 - 2.0173
	rs7356396	**0.03393**	1.22 (G)	1.0151 - 1.4616
	rs12646862	**0.01907**	1.24 (C)	1.0361 - 1.4875
Cluster C	rs2291530	**0.03311**	1.87 (T)	1.0515 - 3.3296
	rs1425326	**0.03552**	1.40 (T)	1.0229 - 1.9061
	rs7356396	**0.00428**	1.40 (G)	1.1123 - 1.7716
	rs12646862	**0.00849**	1.36 (C)	1.0823 - 1.7171

Table 3.3: Markers of the *KCNIP4* gene associated with cluster B and cluster C PD
The markers rs1425326, rs7356396 and rs12646862 were found to be associated with cluster B as well as with cluster C PD.

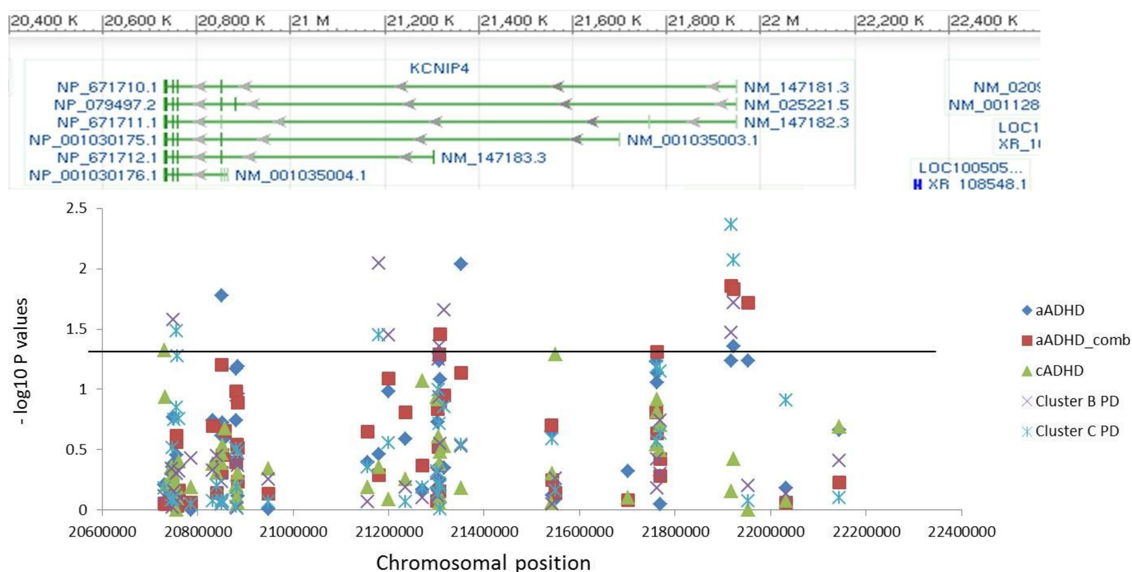


Figure 3.2: Chromosomal position of the genotyped *KCNIP4* SNPs
The chromosomal position of the SNPs is plotted against the negative logarithmic p values for an association with aADHD (blue diamonds), aADHD combined subtype only (red squares), cADHD (green triangles), cluster B PD (grey cross) and cluster C PD (light blue crosses). The threshold for nominal significance at 1.3 is indicated by a black line. Most SNPs are situated in the 5´region or the first intron of the gene depending on the variant.

3.1.2. CDH13

Adult ADHD

In the initial study 86 SNPs were genotyped in 602 adult ADHD patients and 422 healthy controls from Germany. 73 SNPs survived quality control and passed through case control-based association testing. Ten markers showed p values indicating an association of the CDH13 gene with aADHD (Table 3.4). Bonferroni correction for 73 tests leads to a significance threshold of p = 0.0007. rs11642219 is the only SNP showing a significant association after correction. Twelve haploblocks could be defined of which block 4 consisting of the markers rs4591132, rs9888896, rs7189859, rs7197530, rs2228685 revealed a protective haplotype (TTGTT) after statistical evaluation (p = 0.0394, OR = 0.40).

To replicate this, genotyping of 58 *CDH13* SNPs and association testing in three samples from the IMpACT consortium consisting of 1018 aADHD cases and 1384 controls from The Netherlands, Norway and Spain was performed. Two markers (different from the ten

which showed an association with the disease in the initial study) revealed a nominal significance in the IMpACT sample (rs 16958788 p = 0.0070 and rs16961122 p = 0.0116).

SNP ID	**German discovery sample**			**IMpACT replication sample**			**Pooled data**		
	P value	**OR (allele)**	**95%-CI**	**P value**	**OR (allele)**	**95%-CI**	**P value**	**OR (allele)**	**95%-CI**
rs4445897	0.0119	1.41 (T)	1.07 – 1.84	0.2694	1.10 (C)	0.93 – 1.31	0.8069	1.02 (T)	0.88 – 1.17
rs12925602	0.0133	1.45 (G)	1.08 – 1.96	0.1158	1.17 (A)	0.96 – 1.43	0.9430	1.01 (G)	0.85 – 1.19
rs16958148	0.0286	1.32 (G)	1.03 – 1.68	0.4631	1.06 (T)	0.90 – 1.25	0.5438	1.04 (G)	0.91 – 1.19
rs4591132	0.0032	1.33 (C)	1.10 – 1.61	0.3866	1.05 (C)	0.93 – 1.19	**0.0204**	**1.13 (C)**	**1.02 – 1.25**
rs2228685	0.0399	1.23 (T)	1.01 – 1.51	0.0749	1.11 (T)	0.99 – 1.25	**0.0099**	**1.14 (T)**	**1.03 – 1.25**
rs4077621	0.0171	1.41 (A)	1.06 – 1.88	*0.0131**	1.29 (C)	1.05 - 1.57	0.5254	1.05 (C)	0.90 – 1.23
rs17211336	0.0156	1.39 (A)	1.06 - 1.81	0.1610	1.12 (G)	0.95 - 1.32	0.9434	1.01 (A)	0.87 – 1.16
rs11642219	0.0003	1.62 (G)	1.25 - 2.11	0.7311	1.03 (G)	0.85 - 1.26	**0.0150**	**1.22 (G)**	**1.04 – 1.42**
rs153653	0.0055	1.43 (C)	1.11 - 1.85	0.5339	1.06 (C)	0.89 - 1.26	**0.0355**	**1.17 (C)**	**1.01 - 1.35**
rs692612	0.0010	1.45 (G)	1.16 - 1.80	0.8950	1.01 (G)	0.87 - 1.17	0.0524	1.13 (G)	1.00 – 1.28

Table 3.4: Significant markers of the *CDH13* gene in the case control-based association study on aADHD Ten markers showed an association with aADHD in the initial sample from Germany. The association of rs11642219 is significant even after correcting for multiple testing (significance threshold = 0.0007). The only SNP which had a p value < 0.05 in the IMpACT replication sample had to be excluded due to HWE < 0.01 (indicated by a star). Bold face, significant findings in the pooled samples. The pooled analysis of all 1620 cases and 1806 controls revealed four SNPs with nominally significant p values

The sole SNP among the initially associated ones which had a p value < 0.05 in the replication study, had to be excluded due to HWE < 0.01. However, in a pooled analysis of all 1620 cases and 1806 controls four of the SNPs had nominally significant p values (Table 3.4). A two stage design meta-analysis revealed marker rs11642219 to be associated with ADHD robust to correction (empirical p value = 0.00014; corrected p value = 0.0102).

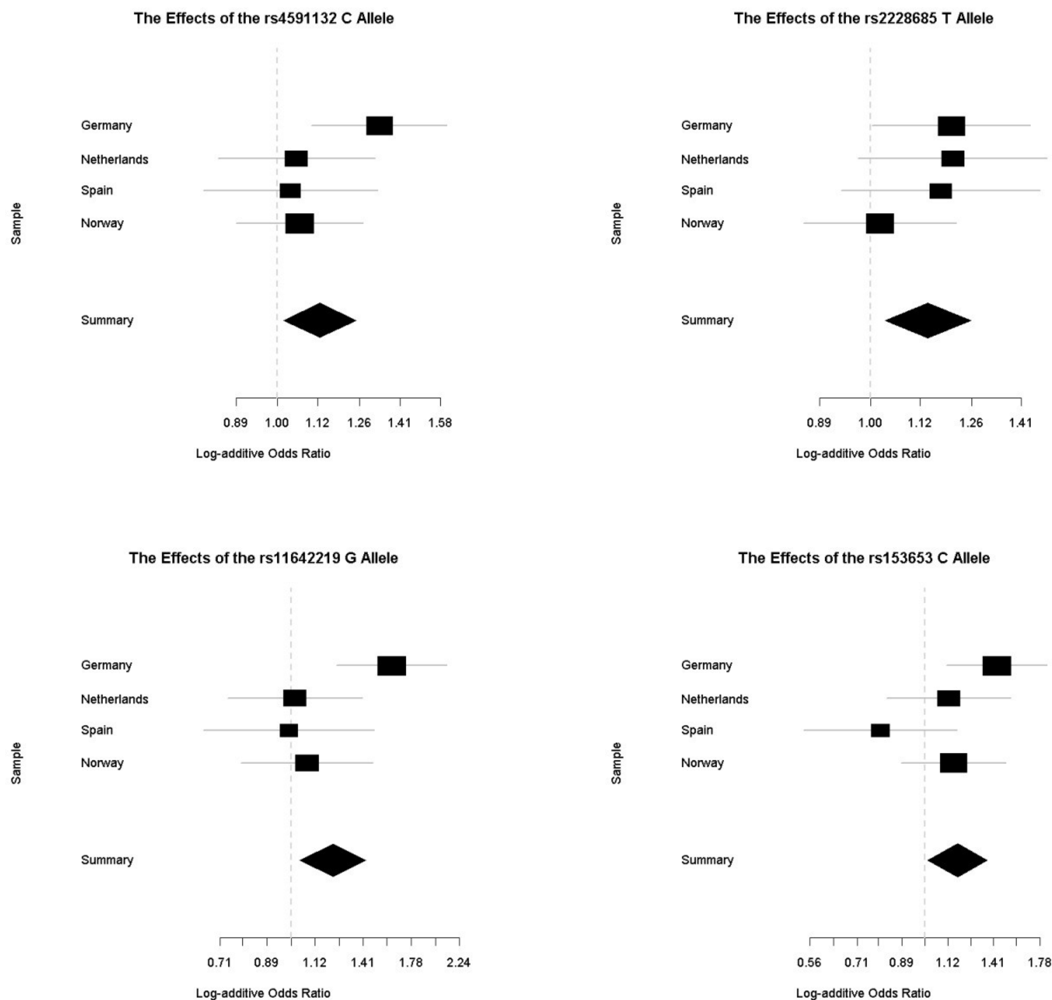


Figure 3.3: Meta-analyses on the effects of four risk alleles found in the *CDH13* gene
Odds ratios of the risk alleles of rs4591132, rs2228685, rs11642219 and rs153653 in the four independent aADHD samples and in summary

Childhood ADHD

To investigate the role of *CDH13* in childhood ADHD, 170 families from Franconia (112 trios, 43 quartets and 15 multi sibling) were genotyped. This family-based association

study revealed six markers which showed an allele over-transmission to affected children (see Table 3.5). Only one of those SNPs (rs4077621) was found to be associated with ADHD in the German adult sample, as well. No haplotype was found to be associated with cADHD in this sample.

For a replication of the single marker associations found in the families from Würzburg, a second independent family-based sample from Homburg (Germany) was examined. Pedigree disequilibrium testing in 142 trios resulted in three SNPs with an over-transmitted allele (rs7198517, rs13338647, rs16961122) with p values ranging from 0.0357 to 0.0184 different from those identified in the initial family sample. There was no overlap of the SNPs identified in this replication sample with the associated SNPs in the aADHD sample.

SNP ID	**Over transmission**		**PDT**
	Allele	**Rate (%)**	**P value**
rs1019959	C	52.7	**0.0168**
rs4077621	C	51.4	**0.0326**
rs12716966	C	52.4	**0.0413**
rs8182163	T	52.0	**0.0413**
rs10514575	A	51.1	**0.0389**
rs2326025	G	51.8	**0.0367**

Table 3.5: Over transmitted alleles of *CDH13* in the family-based cADHD sample from Würzburg
Six SNPs showed over transmission of one allele to affected children. None of these markers showed an over transmission in the replication sample from Homburg.

Comorbid conditions in the aADHD and the cADHD samples

As the cADHD samples from Germany and the aADHD samples from Germany and The Netherlands were also assessed for comorbid conditions, the genotyping data have been tested for associations with those co-morbidities, as well.

In the German aADHD sample, 20 SNPs associated with different comorbid conditions could be identified (p values 0.0432 – 0.0011). Twelve SNPs of which five were identical to those encountered in the sample from Germany were found in the Dutch sample to be associated with comorbidities in ADHD with p values of 0.0492 to 0.0006. Except of one

marker (rs16958148), all SNPs that have been identified as associated with aADHD in the initial sample showed an association with one or even more comorbid disorders, too.

For cADHD, 15 markers in the sample from Würzburg and eight in the Homburg sample were associated with comorbid disorders (mainly conduct disorder, oppositional defiant disorder and depression). None of those markers was found to be associated with ADHD within the same sample. Though, rs13338647 was associated with depression in the Würzburg sample and with ADHD in the replication sample, rs1019959 and rs8182163 were associated with ADHD in the initial study and with conduct disorder in the replication.

Association of *CDH13* with personality disorders

To test whether *CDH13* may be involved in PD which are known to be related to ADHD, case control-based association testing of a PD sample consisting of 629 patients suffering from cluster B or cluster C PD was conducted. The single marker association findings are listed in Table 3.6.

Three out of 40 analyzed SNPs showed nominally significant association with both cluster B and cluster C PD. One SNP (rs16958148) was found to be associated with cluster C disorders and showed tendencies of an association with cluster B disorders with a p value slightly exceeding the significance threshold ($p = 0.0552$). Bonferroni correction for 40 tests results in a significance threshold of $p = 0.00125$ which is slightly exceeded by rs692612 with a p value for association with cluster B PD of 0.0013. The association found for rs11642219 with cluster B PD withstands correction.

	PD Cluster B			PD Cluster C		
SNP ID	P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI
rs16958148	0.0552	n.a.	n.a.	**0.0335**	1.40 (G)	1.0266 - 1.9134
rs11642219	**0.00001**	1.90 (G)	1.4269 - 2.5197	**0.0071**	1.60 (G)	1.1360 - 2.2489
rs16961669	**0.0261**	1.56 (C)	1.0546 - 2.3172	**0.0041**	2.34 (C)	1.3074 - 4.1852
rs692612	**0.0013**	1.49 (G)	1.1688 - 1.9057	**0.0173**	1.45 (G)	1.0672 - 1.9585

Table 3.6: Results of case control association analyses of *CDH13* with PD
Four SNPs showed a nominally significant association with cluster C PD. Three out of those four markers also showed an association with cluster B PD. rs11642219 is significant even after correction for multiple testing (significance threshold after correction = 0.00125).

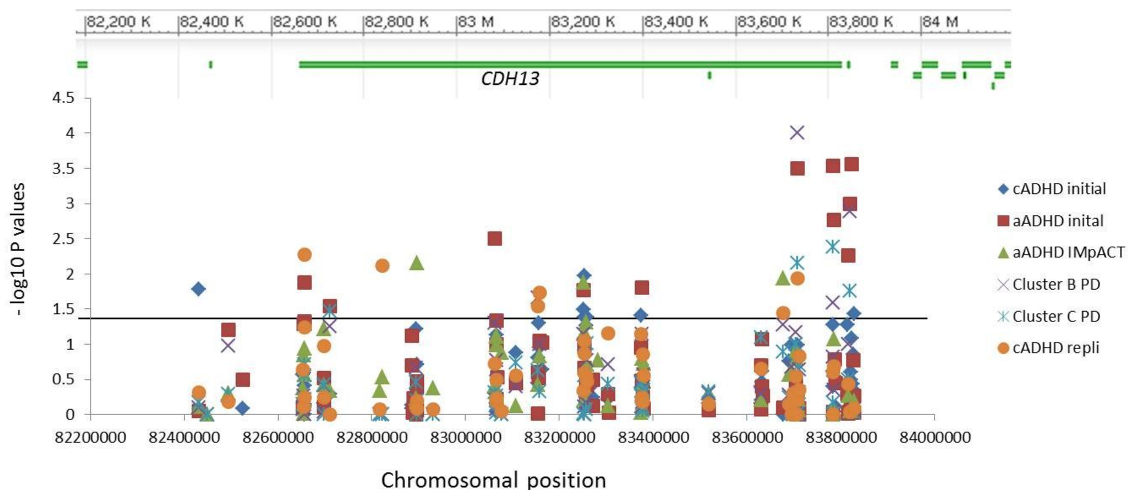


Figure 3.4: Chromosomal distribution of the genotyped *CDH13* SNPs
The chromosomal position of the SNPs is plotted against the negative logarithmic p values for an association with cADHD in the initial sample from Würzburg (blue diamonds), cADHD in the replication sample from Homburg (orange dots), aADHD in the initial sample from Germany (red squares), aADHD in the IMpACT sample from Norway, The Netherlands and Spain (green triangles), cluster B PD (grey cross) and cluster C PD (light blue crosses). The threshold for nominal significance at 1.3 is indicated by a black line. Most of the initial found SNPs for aADHD and PD are clustered in the 3´region of the gene or the 3´UTR.

3.1.3. DIRAS2

Adult ADHD

In the German aADHD sample 600 patients were compared to 420 healthy controls. Four out of 12 SNPs tested for an association with the disease showed a nominal significance (Table 3.7). The replication of this single marker association finding in the IMpACT sample consisting of 1035 aADHD patients and 1381 controls from The Netherlands, Norway and Spain failed. Nevertheless, meta-analyses for the best four SNPs of the discovery sample in all 1635 patients and 1801 controls revealed the marker rs1412005 to be associated with adult ADHD with a nominal p value of 0.0437 (adjusted for five tests p = 0.175). The effects of the T risk allele of this marker in the four independent aADHD samples and in summary are shown in Figure 3.5.

SNP ID	**German discovery sample**			**IMpACT replication sample**			**Pooled data**		
	P value	**OR (allele)**	**95%-CI**	**P value**	**OR (allele)**	**95%-CI**	**P value**	**OR (allele)**	**95%-CI**
rs7854469	**0.0394**	1.40 (T)	1.02 - 1.93	0.0720	1.19 (A)	0.98 - 1.43	0.6023	1.04 (A)	0.89 - 1.23
rs16906711	**0.0453**	1.97 (G)	1.01 - 3.81	0.7288	1.07 (C)	0.74 - 1.55	0.4271	1.14 (G)	0.83 - 1.55
rs1331503	**0.0125**	1.27 (A)	1.05 - 1.54	0.5812	1.03 (A)	0.92 - 1.17	0.0678	1.10 (A)	0.99 - 1.22
rs1412005	**0.0182**	1.29 (T)	1.04 - 1.58	0.3785	1.06 (T)	0.93 - 1.21	**0.0437**	1.12 (T)	1.00 - 1.25

Table 3.7: Significant SNPs of the *DIRAS2* gene of case control-based association analysis for aADHD
Four SNPs were nominally significant associated with aADHD in the discovery sample. None of those single marker association findings could be replicated in the IMpACT sample. Meta-analysis for the four SNPs revealed rs1412005 to be associated with aADHD.

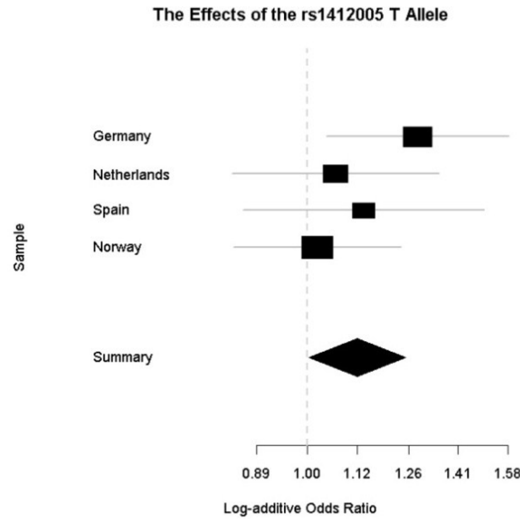


Figure 3.5: Effects of the rs1412005 T risk allele
Odds ratios of the T risk allele of rs1412005 in the four independent aADHD samples and in summary

For each of the two haplotype blocks of the *DIRAS2* gene (see Figure 3.6) one risk haplotype could be identified in the aADHD sample from Germany (block 1: GTGGGT p = 0.006; block 2: ACGCTT p = 0.033). Association testing for the haplotype ACGCTT from block 2 in the IMpACT sample replicated the significant finding from the discovery sample (p = 0.026). Meta-analyses of this risk haplotype revealed an association with an increased risk for aADHD and this effect was statistically significant even after adjusting for multiple testing (p=0.00028, p value adjusted for five tests: 0.001, OR=1.45, 95% CI: 1.19-1.77; Figure 3.7). This haplotype contains the T allele of SNP rs1412005.

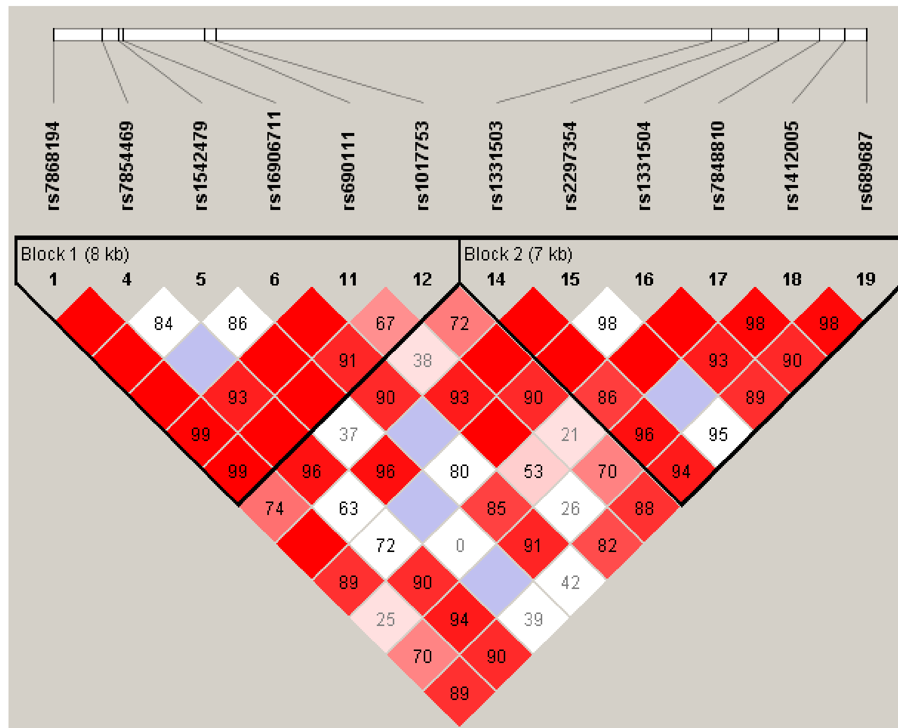


Figure 3.6: Haploblock structure of the *DIRAS2* gene
Linkage disequilibrium (LD) plot of DIRAS2. rs1017753 is the last SNP of block 1, while rs1331503 is the first SNP of block 2. rs7854469 and rs16906711 are associated with aADHD; rs1331503 with aADHD and personality domains; rs7848810 with cADHD and personality domains; and rs1412005 with aADHD, bipolar disorders, Cluster C PD and personality domains; a risk haplotype for aADHD was identified for block 2.

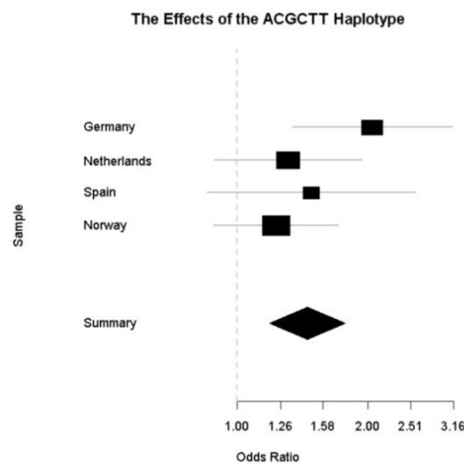


Figure 3.7: Effects of the risk haplotype of block 2 in the *DIRAS2* gene
Odds ratios of the ACGCTT haplotype in the four independent aADHD samples and in summary

Childhood ADHD

To extend the association findings for *DIRAS2* to cADHD, 166 independent nuclear families with offspring affected by cADHD were analyzed. The rs1412005 T allele was not significantly over-transmitted (p=0.65), but the C allele of the neighboring SNP rs7848810, which is also located in the promoter region, was (p=0.046, transmission rate: 53%). Haplotype analysis provided evidence of significant association of the haplotype block 2 (p=0.0231) with a p value for the specific haplotype ACGCTT slightly exceeding 0.05 (p=0.0692, frequency among transmitted versus non-transmitted haplotypes: 11% versus 6.5%, OR=2.01, 95% CI: 0.80-5.00). rs7848810 was also associated with co-morbid dyslexia (p=0.015).

Related disorders and personality traits

To check for possible associations of *DIRAS2* with other psychiatric disorders known to be related to ADHD, SNP genotyping of a bipolar disorder sample and a PD samples (cluster B and cluster C) was conducted.
Marker rs1412005 was found to be nominally significantly associated with bipolar disorders and cluster B PD. Also, a tendency (p = 0.0855) for an association with cluster C PD could be observed for this SNP (Table 3.8). The T allele of this SNP represents the risk allele for all three disorders (OR = 1.26 – 1.32), which is consistent with the findings in ADHD.

SNP ID	Biplolar			Cluster B			Cluster C		
	P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI
rs16906711	0.4935	1.32 (G)	0.59 - 2.94	***0.0454****	*1.95 (G)*	*1.01 - 3.75*	***0.0220****	*2.33 (G)*	*1.13 - 4.80*
rs1412005	**0.0256**	1.32 (T)	1.03 - 1.67	**0.0305**	1.27 (T)	1.02 - 1.59	0.0855	1.26 (T)	0.97 - 1.64

Table 3.8: *DIRAS2* SNPs associated with disorders related to ADHD
Nominally significant p values are shown in bold letters
*pHWE=0.001

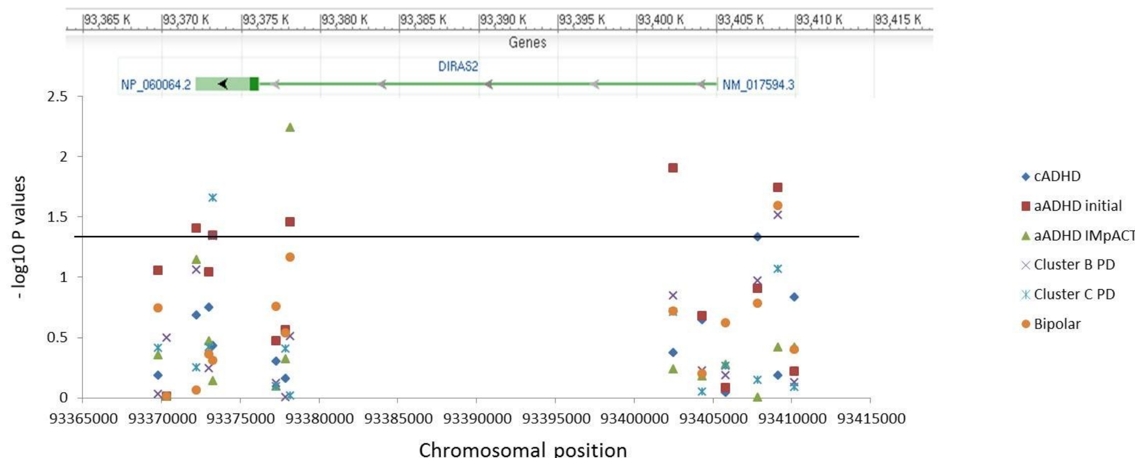


Figure 3.8: Chromosomal position of the genotyped *DIRAS2* SNPs
The chromosomal distribution of the SNPs is plotted against the negative logarithmic p values for an association with cADHD (blue diamonds), aADHD in the initial sample from Germany (red squares), aADHD in the IMpACT sample from Norway, The Netherlands and Spain (green triangles), cluster B PD (grey cross), cluster C PD (light blue crosses) and bipolar disorders (orange dots). The threshold for nominal significance at 1.3 is indicated by a black line. The SNPs associated with aADHD are clustered at the protein coding exon 2 of the DIRAS2 gene and at the promoter region.

The aADHD and PD samples recruited in Würzburg had also been assessed for personality dimensions using the NEO-PI R (testing for Neuroticism, Extraversion, Openness to Experience, Agreeableness, Conscientiousness) and the TPQ (Novelty Seeking, Harm Avoidance, Reward Dependence). This enabled an additional investigation to clarify whether personality scores may also be affected by *DIRAS2* variants. The nominally significant association results are depicted in Table 3.9. In the aADHD sample, p values < 0.05 were found for Neuroticism, Extraversion, Agreeableness, Conscientiousness and Harm Avoidance. The three SNPs rs1331503, rs1412005 and rs689687, which were associated with these personality traits, are located in haplotype block 2 and the transcriptional control region of the gene. In the PD sample, nominal evidence of association was found for Extraversion, Agreeableness, Conscientiousness, Novelty Seeking and Harm Avoidance (p values between 0.008 and 0.040). In comparison to the aADHD sample, different SNPs showed association in this sample and most of them are located in the haplotype block 1. However, rs7854469 was associated with both aADHD and the ADHD-relevant personality trait Extraversion in PD (p=0.0075, Table 3.9). Also, the aADHD associated SNP rs7848810

was associated with Novelty Seeking (p=0.0396) and Conscientiousness (p=0.0123) in the PD sample (Table 3.9).

Personality trait	SNP ID	aADHD cases			PD patients		
		P value	**Beta (allele)**	**Se**	**P value**	**Beta (allele)**	**Se**
NEO-N	rs1331503	**0.0241**	3.51 (A)	1.55	0.3113	1.72 (A)	1.70
	rs689687	**0.0327**	4.14 (C)	1.93	0.5747	1.15 (C)	2.05
NEO-E	rs7854469	0.4172	2.07 (A)	2.55	**0.0075**	6.32 (T)	2.36
	rs1331503	**0.0065**	3.68 (T)	1.35	0.6695	0.59 (T)	1.38
	rs1412005	**0.0088**	3.83 (G)	1.46	0.7600	0.44 (T)	1.42
NEO-A	rs7854469	0.8963	0.26 (T)	1.96	**0.0116**	4.24 (A)	1.67
	rs2297354	0.2065	4.78 (C)	3.78	**0.0288**	10.03 (C)	4.57
	rs689687	**0.0284**	2.80 (C)	1.28	0.2645	1.28 (T)	1.15
NEO-C	rs690111	0.9152	0.17 (T)	1.60	**0.0448**	2.87 (T)	1.43
	rs1331503	**0.0173**	3.24 (T)	1.36	0.1876	1.77 (T)	1.34
	rs7848810	0.9051	0.18 (C)	1.52	**0.0123**	3.49 (A)	1.39
	rs1412005	**0.0135**	3.61 (G)	1.46	0.1013	2.26 (G)	1.38
	rs689687	**0.0043**	4.84 (T)	1.69	0.1166	2.50 (T)	1.59
TPQ-NS	rs7854469	0.8075	0.16 (A)	0.65	**0.0224**	1.35 (T)	0.59
	rs7848810	0.3585	0.35 (A)	0.38	**0.0396**	0.73 (C)	0.36
TPQ-HA	rs7868194	0.1634	0.62 (A)	0.45	**0.0126**	1.08 (G)	0.43
	rs1017753	0.0897	0.74 (C)	0.43	**0.0257**	0.95 (T)	0.43
	rs1331503	**0.0023**	1.30 (A)	0.42	0.7309	0.16 (A)	0.46
	rs1412005	**0.0018**	1.43 (T)	0.46	0.7284	0.17 (T)	0.48
	rs689687	**0.0033**	1.54 (C)	0.52	0.6540	0.25 (T)	0.55

Table 3.9: Results of association analysis for quantitative personality traits in the aADHD and the PD sample.
Nominal significant p values are printed in bold. NEO-E = Extraversion; NEO-A = Agreeableness; NEO-C = Conscientiousness; TPQ-NS = Novelty Seeking; TPQ-HA = Harm Avoidance.

3.2. *Diras2* expression studies

3.2.1. Expression of *Diras2* during development

Diras2 expression levels were determined by qPCR using total RNA isolated from mouse brains at different developmental stages as described in 2.3.1. Samples were grouped as followed: Prenatal (n = 6; embryonic day 11, 13 and 15), early postnatal (n = 12; postnatal days 0, 1, 2, 4, 5 and 7), late postnatal (n = 12; postnatal days 9, 10, 11, 13 and 15), adolescence (n = 6; postnatal days 16, 17, 18, 19, 22 and 23) and adult (n = 5; aged two to four months). Normalized expression levels showed a significant increase in *Diras2* expression in the mouse brain during early developmental stages. After significant group differences ($p < 0.05$) were detected using the Kruskal-Wallis test, significant increases in expression levels were found between the prenatal and the early postnatal (P 0 – 7) group and between the early postnatal and the late postnatal (P 9 – 15) group using the Mann-Whitney test ($p < 0.0083$; significance threshold adjusted for six tests). From the late postnatal development to adulthood, no differences in *Diras2* expression in the mouse brain could be observed (see diagram in Figure 3.9).

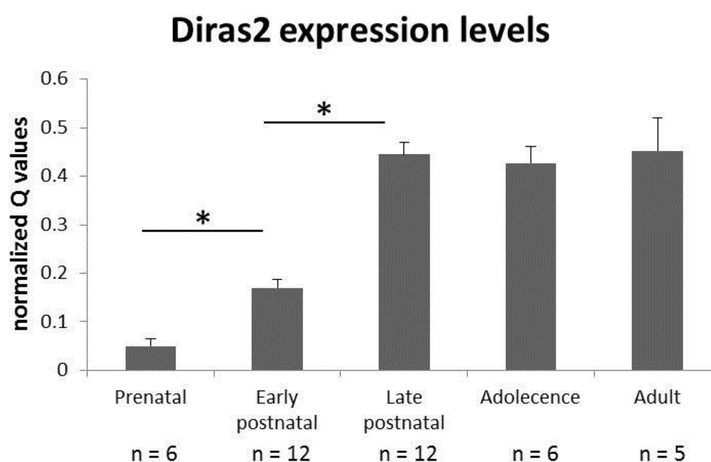


Figure 3.9: *Diras2* expression levels during brain development
Normalized expression levels of *Diras2* in the mouse brain at different developmental stages determined by qPCR. A significant increase in expression levels could be observed between the prenatal and the early postnatal group and between the early and late postnatal group.

3.2.2. Expression of *DIRAS2* in the human brain

The expression of *DIRAS2* in the human brain was investigated by conducting qPCR analyses using purchased pooled total RNA isolated from different human brain regions. The highest DIRAS2 expression was found in the hippocampus followed by the cerebral cortex and the cerebellum. The expression levels in the caudate nucleus, putamen and the raphe nuclei containing regions medulla oblongata and pons were strikingly lower.

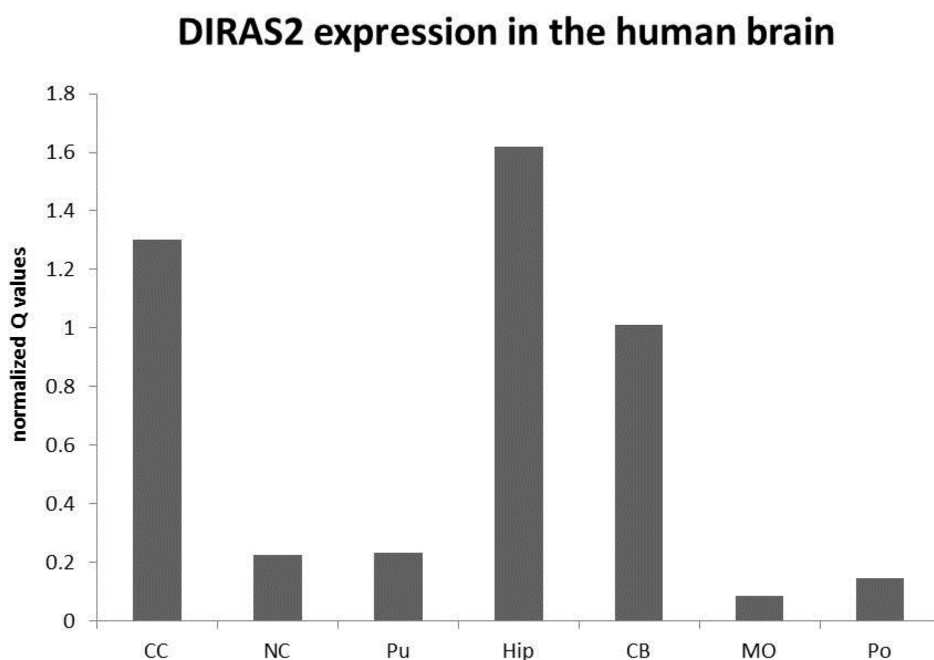
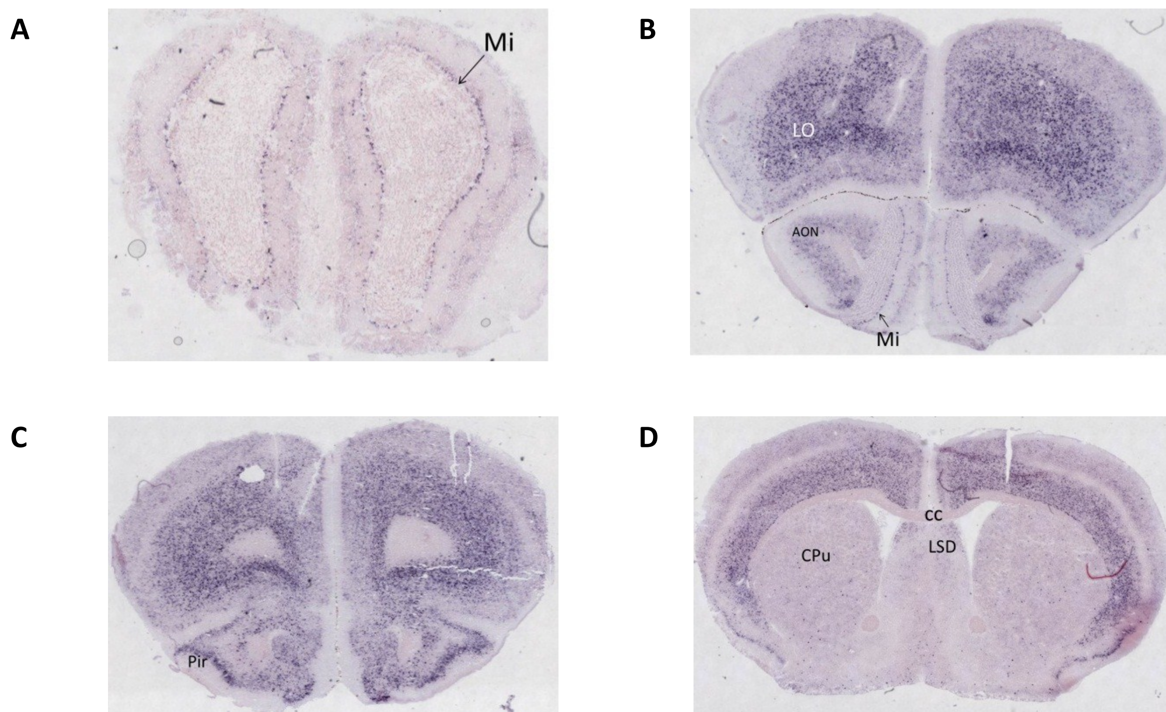


Figure 3.10: Expression of *DIRAS2* in the human brain
Normalized expression levels of *DIRAS2* in different human brain regions. Highest *DIRAS2* expression was observed in the hippocampus, the cerebral cortex and the cerebellum. CC = cerebral cortex; NC = caudate nucleus; Pu = putamen; Hip = hippocampus; CB = cerebellum; MO = medulla oblongata; Po = pons.

3.2.3. Expression of *Diras2* in the mouse brain

To investigate the expression pattern of *Diras2* in the mouse brain, *in situ* hybridization was performed as described in 2.3.2. The results of the detection of the cRNA probe specific for the *Diras2* mRNA in the mouse brain are shown in Figure 3.11.

In the olfactory bulb, *Diras2* expression is mainly localized in the mitral cell layer (Figure 3.11 A). The deep cellular zone of the anterior olfactory nucleus as well as the orbital cortex showed a notable expression of the gene (Figure 3.11 B). In the posterior regions of the cerebral cortex a strong staining of distinct cortical layers, probably layers V and VI and layers II and/or III, could be observed (Figure 3.11 D,E and H). In addition, *Diras2* is strongly expressed in the piriform cortex, the amygdala, the hippocampus and the granular cell layer of the cerebellar cortex. Expression in the amygdala seems to be localized in the basolateral nucleus exclusively (Figure 3.11 E and G). In the hippocampus, a very intense staining is visible in the dentate gyrus, CA3 and CA1 region (Figure 3.11 E and F). Isolated *Diras2* positive spots in the hilus may correspond to interneurons located in this area. Besides those areas showing a very strong *Diras2* signal, a weaker or more punctual expression could also be observed in the striatum (caudate putamen), the lateral septal nucleus (Figure 3.11 D), the substantia nigra, the parafascicular thalamic nucleus (Figure 3.11 I) and the locus coeruleus (Figure 3.11 J).



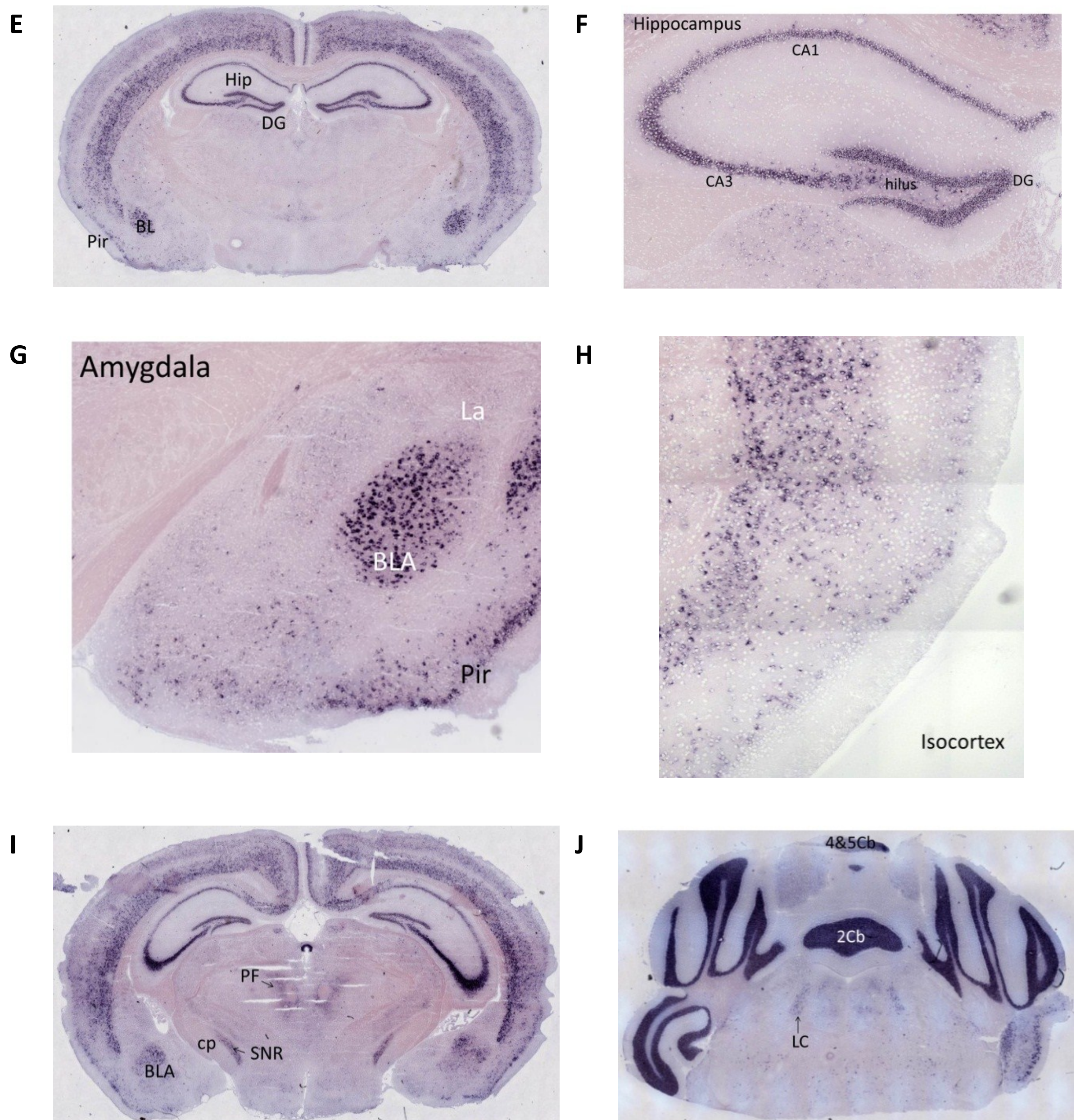


Figure 3.11: ***In situ*** **hybridization specific to** ***Diras2*** **on native mouse brain slices**

Visualization of Diras2 mRNA on mouse brain cryosections of 10 µm thickness using *in situ* hybridization. Highest expression levels can be observed in the cerebral cortex, the amygdala, the hippocampus and the cerebellar cortex. 2Cb = 2nd Cerebellar lobule; 4&5Cb = 4&5th Cerebellar lobule; AON = anterior olfactory nucleus; BLA = basolateral amygdaloid nucleus; CA1 = field CA1 of hippocampus; CA3 = field CA3 of hippocampus; cc = corpus callosum; cp = cerebral peduncle; CPu = caudate putamen (striatum); DG = dentate gyrus; Hip = hippocampus; La = lateral amygdaloid nucleus; LC = locus coeruleus; LO = lateral orbital cortex; LSD = lateral septal nucleus, dorsal part; Mi = mitral cell layer of the olfactory bulb; PF = parafascicular thalamic nucleus; Pir = piriform cortex; SNR = substantia nigra

3.2.4. Expression of *Di-ras2* in different cell types

To detect the Di-ras2 protein, at first a purchased antibody was tested for specific binding in western blotting analyses using human and mouse brain lysates. After ensured antibody specificity, the human cell lines A172, SK-N-SH, U373 and HEK293 were tested for Di-ras2 occurrence. In all of the four investigated human cell lines there was no or just a weak Di-ras2 signal (Figure 3.12 A). In addition, mouse hippocampal primary cells were examined at different times after seeding. As primary cells showed the highest protein expression after 12 days in culture (Figure 3.12 B), cells were cultured for 12 days for fluorescence double staining.

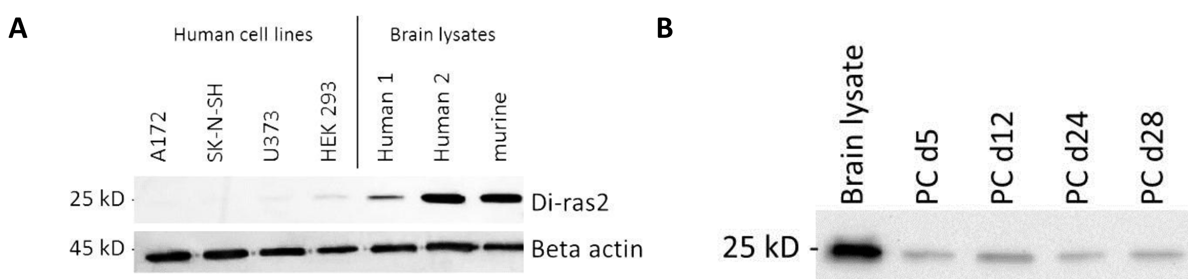


Figure 3.12: Western blot analyses of Di-ras2
Detection of the Di-ras2 protein after electrophoretic separation of proteins A) in lysates of the four human cell lines A172, SK-N-SH, U373; HEK293, two different human brain lysates and a murine brain lysate; B) in murine brain lysate and four lysates of murine hippocampal primary cells harvested at different times after seeding. A172 = glioblastoma cell line; SK-N-SH = neuroblastoma cell line; U373 = astrocytoma cell line; HEK293 = immortalized human embryonic kidney cells; kD = kilo Dalton; PC = murine hippocampal primary cells; d = days after seeding.

To investigate in which cell types Di-ras2 is present, mouse hippocampal primary cells were fixed and stained after 12 days in culture. The antibody against Di-ras2 was detected with an alexa488 labeled secondary antibody. Antibodies against several cell markers were detected using an alexa555 coupled secondary antibody. Double staining against Di-ras2 and the glial fibrillary acidic protein (GFAP), which is a glia cell marker, showed no overlap in expression, indicating that Di-ras2 is not present in glia cells (Figure 3.13 A). Di-ras2 is found in microtubule associated protein 2 (MAP2) positive cells. Since MAP2 is a neuronal marker, this result leads to the assumption that *Diras2* is expressed in neurons. Other than the MAP2 staining, the Di-ras2 staining is as well present in the axonal and dendritic branches far off the cell body (Figure 3.13 B). Simultaneous detection of Di-ras2

and Synaptophysin, a synaptic vesicle glycoprotein, is shown in Figure 3.13 C. This staining also indicates expression in the same cell type, but not with exactly the same subcellular localization, as Di-ras2 is located rather at the cell soma then at the synapses.

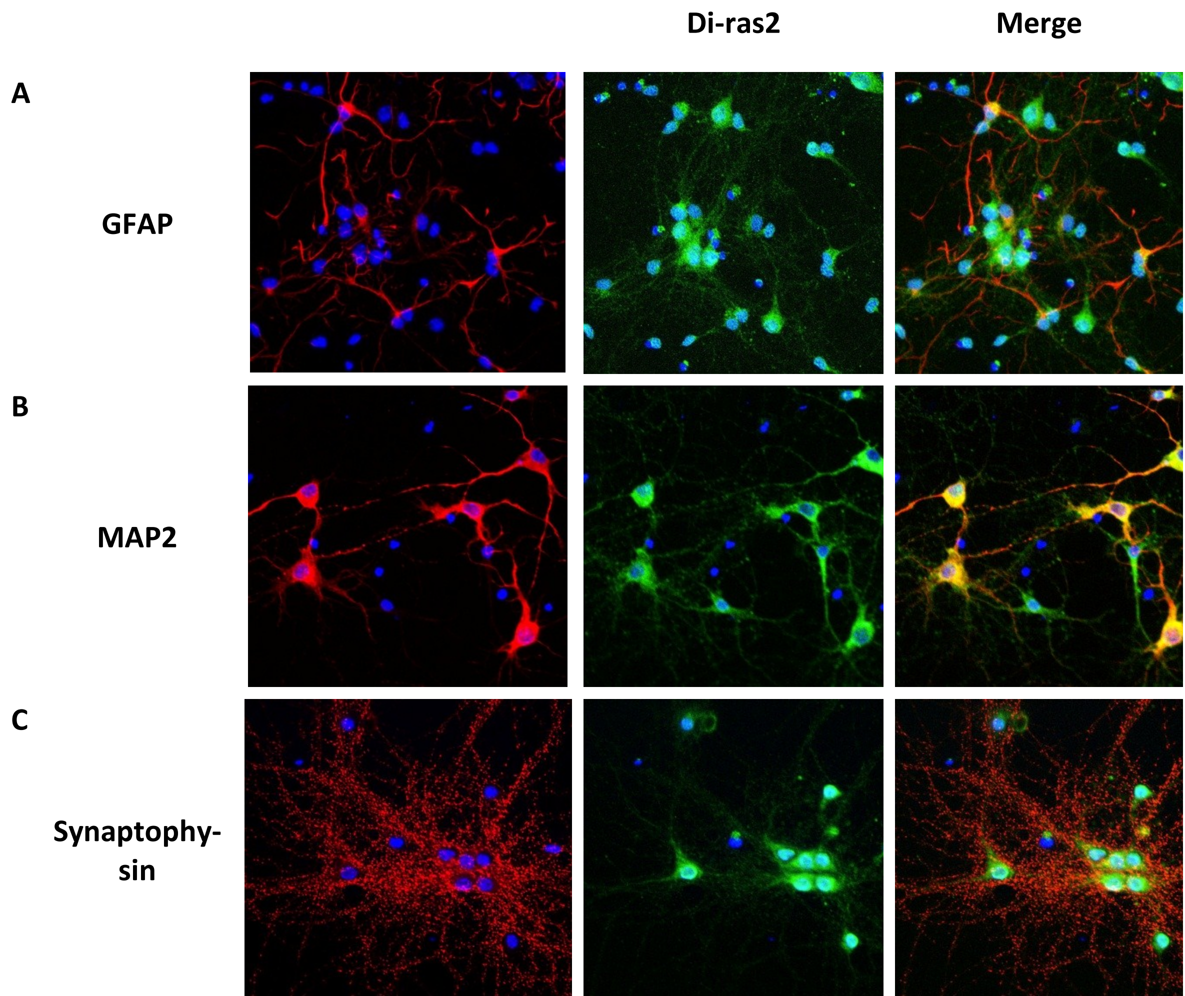


Figure 3.13: Immunocytological double staining of primary cells
Murine hippocampal primary cells were stained with the Di-ras2 antibody (green) and antibodies against cell markers (red). Yellow color in the merged pictures indicates a co-expression. A) No co-expression of Di-ras2 with GFAP could be found; B) MAP2 stained cells are Di-ras2 positive; C) Detection of Di-ras2 and Synaptophysin in the same cells. GFAP = glial fibrillary acidic protein; MAP2 = microtubule associated protein 2

To check whether Di-ras2 is present in all neuronal cells or only in specific neuronal cell types, additional double staining against Di-ras2 and Parvalbumin, Calbindin and Tyrosine Hydroxylase were conducted (Figure 3.14). Parvalbumin and Calbindin are both markers for GABAergic interneurons. While Parvalbumin occurs in cells with thick myelinated axons, Calbindin antibodies mainly label cells with thin, unmyelinated axons (Celio, 1990).

The enzyme Tyrosine Hydroxylase (TH) is responsible for the synthesis of dihydroxy-phenylalanine (DOPA) which is the precursor molecule for dopamine. As dopamine is a precursor for norepinephrine and epinephrine, TH is a marker for dopaminergic, nora-drenergic and adrenergic neurons. All three of the marker proteins were found in Di-ras2 positive cells (see Figure 3.14 B). Hence, Di-ras2 protein could be detected in all neuronal subtypes investigated so far.

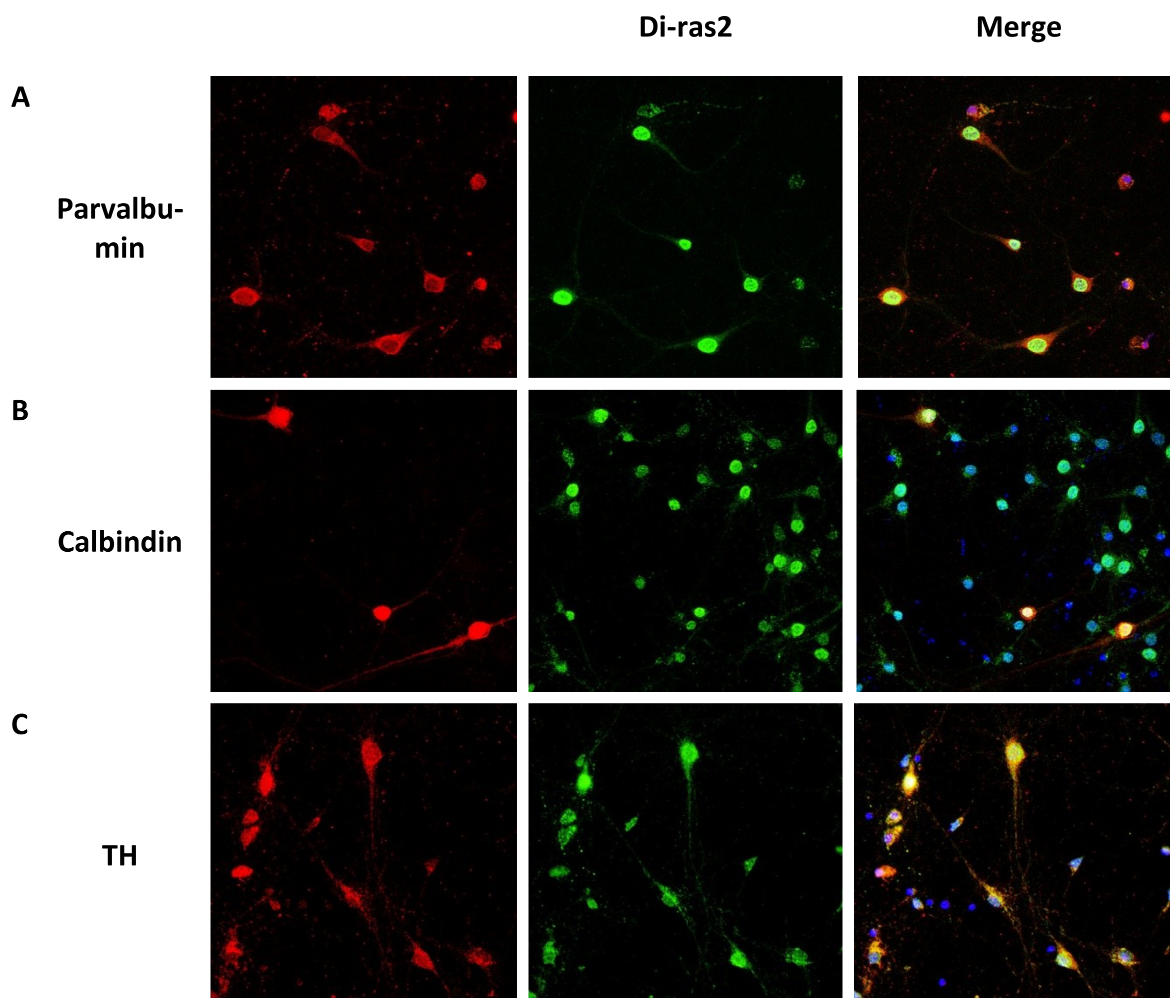


Figure 3.14: Immunocytochemical double staining of mouse hippocampal primary cells
Staining against Di-ras2 is shown in green, detection of Parvalbumin (A), Calbindin (B) and TH (C) is represented in red. All cells positive for one of the three markers (A-C) also showed Diras2 expression. TH = tyrosine hydroxylase.

4. Discussion

The present work aims to further examine three candidate genes which are potentially involved in the genetic predisposition towards ADHD.

4.1. KCNIP4

The *KCNIP4* gene was considered as a potential candidate gene for ADHD because its chromosomal locus on chromosome 4 emerged from a GWAS of cADHD, a GWAS of aADHD and a GWAS of PD. For the candidate gene-based association studies, tagging SNPs of the exons and their flanking regions as well as tagging SNPs situated in the promoter region and the 3´UTR of the *KCNIP4* gene have been genotyped in an adult ADHD sample, a family-based childhood ADHD sample and a PD sample. On the single marker level, six out of 49 SNPs were found to be associated with aADHD. The two markers which showed an association with childhood ADHD were different from those found in the adult sample with one marker (rs16869915) being the only associated one located in the 3´UTR of the gene. However, due to the p values calculated for those two markers barely exceeding the nominal significance threshold and the given likelihood for false positive results, the association finding of KCNIP4 with childhood ADHD seems not to be very robust. Two SNPs (rs7356396 and rs1264862) were found to be nominally significant associated with aADHD and cluster B and cluster C PD. Additionally, two markers which showed an association with cluster B PD were also found to be associated with co-morbid disorders in aADHD (rs6448034, mood disorders; rs983071, anxiety disorders) indicating a role of *KCNIP4* not only in ADHD but also in personality and other co-morbid disorders. Noticeably, the major part of the SNPs associated with one of the investigated diseases is located in intron1 or the 5´upstream sequence of the *KCNIP4* gene depending on the isoform (Figure 3.2). This is true for nine out of 13 associated SNPs, whereas the genotyped SNPs are allocated across all exons and flanking sequences of the gene. The first intron of the *KCNIP4* gene comprises up to 1,065,861 base pairs, depending on the isoform. Many predicted transcription factor binding sites can be found in this region. For example the rs1264862 risk allele identified for aADHD and cluster B and C PD leads to a loss of a Homeobox B9, a TATA box and a Pax 2/5/8 binding site but gives rise to a new

Hmx2/Nkx5-2 homeodomain transcription factor binding site. Pax and homeodomain transcription factors are known to be essential for the control of the genesis of the mid-brain and cerebellum in the vertebrate embryo (Pfeffer 2000). Therefore, it is conceivable that *KCNIP4* expression or rather the expression of certain *KCNIP4* isoforms through al-ternate promoters, transcription start sites or splicing is altered in risk allele carriers and may have an impact on neurodevelopment. There is not much known about the expres-sion and different functions of the six described KCNIP4 isoforms. Cell type specific differ-ential expression of two of the isoforms has been reported. Additionally, these isoforms seem to differ in their rate of potassium current inactivation (Baranauskas, 2004) and thus may cause differences in the neuronal excitability of the cells in which they are ex-pressed. The functions and expression patterns of the other four *KCNIP4* isoforms remain unknown. Hence, further investigation of expression patterns, transcriptional and transla-tional regulation as well as functional analyses of the different isoforms of this candidate gene are clearly needed.

It has to be noted that the association findings presented in this work are nominally sig-nificant and do not withstand correction for multiple testing, rendering the positive asso-ciation data preliminary at best. Moreover, as only one sample each was investigated for aADHD, cADHD and PD, a replication of the association findings in additional independent samples is necessary.
However, the described association study results together with the existing GWAS results provide evidence that *KCNIP4* is a promising candidate gene not only for aADHD but also for PD. This assumption is further supported by the possible function of KCNIP4 via Nurr1 in the development of dopaminergic neurons in the midbrain that regulate motor control as hyperactivity is a core symptom of the hyperactive/impulsive and of the combined sub-type of ADHD.

4.2. CDH13

The CDH13 gene represents one of the most promising candidate genes in the genetics of ADHD. The chromosome 16q24 locus which harbors this gene was found to be associated with ADHD in several linkage scans and GWAS. Moreover, CDH13 has been found to be

associated with alcoholism, substance abuse and schizophrenia. The functions of Cadherin13 in cell proliferation and survival via the PI3K/Akt/mTOR pathway and in cell adhesion and polarization as well as in cell migration underline the likelihood of its role in neurodevelopment. To confirm an association of CDH13 with ADHD and related psychiatric disorders, a SNP fine-mapping of the gene in seven independent samples was conducted. For the initial German aADHD sample, the most significant associated SNPs are clustered at the 3´region of the gene (see Figure 3.4). A total of 10 SNPs was found to be nominally significant associated with aADHD. The replication of those single marker findings in three additional independent aADHD samples was not successful. However, a meta-analytic approach for the ten top SNPs of the first association study revealed four SNPs to be associated with aADHD on a nominal significance level. A two-stage analysis resulted in one SNP being associated with aADHD even after correction for multiple testing. Therefore, the replication of the association findings for aADHD can be deemed as successful. The protective haplotype found in the aADHD sample from Germany did neither emerge in one of the other aADHD samples nor in one of the family samples for ADHD. Thus, this finding with a p value of 0.0394 has maybe to be considered as a false positive one. The single marker association findings in the cADHD sample from Würzburg mainly show no overlap with those in the aADHD samples. The only marker which showed an association with both adult and childhood ADHD is rs4077621, located in intron 5 of the CDH13 gene. The single marker findings in the replication sample for cADHD from Homburg were also different from those in the aADHD sample as well as from those in the initial cADHD sample. However, genetic variations which are in high linkage disequilibrium to the SNPs that showed an association with ADHD in this study may represent the risk alleles for the disease. As with every association study, the possibility of false positive findings has to be considered. With 86 SNPs genotyped in the initial samples and 58 in the respective replication samples and a significance threshold of 0.05, 3 – 4 false positive findings (5% of the tested SNPs) per association analysis are to be expected. Nevertheless, the fact that ten SNPs were found to be associated with aADHD and six to be associated with cADHD in the first studies, taken together with the association of up to 20 SNPs (20 SNPs in the German aADHD sample, 12 in the Dutch aADHD sample, 15 SNPs in the cADHD sample from Würzburg and 8 in the cADHD sample from Homburg) with co-morbidities within the

ADHD samples, strongly indicates an involvement of CDH13 in ADHD genetics. The results gained in the association studies of CDH13 with PD further substantiate the assumption that this gene also plays a role in psychiatric disorders related to ADHD. The result of four SNPs associated with PD with one of them being significantly associated with cluster B PD after Bonferroni correction can be considered as a robust association finding. Three of those SNPs associated with PD were also found to be associated with aADHD. However, a replication in an independent sample is desirable. Notably, the significant SNPs found in the PD sample are also clustered at the 3´region of the CDH13 gene the same way as most significant SNPs associated with aADHD are (Figure 3.4). Two of those clustering SNPs showed exceptionally high associations with both aADHD and PD (rs11642219: aADHD $p = 0.0003$, PD $p = 0.00001$ significant after correction; rs692612: aADHD $p = 0.001$, PD $p = 0.0013$). The risk allele of rs692612 produces a predicted homeodomain transcription factor binding site while the risk allele for the other SNP leads to the generation of an E2F binding site and the loss of a binding site for the mammalian transcriptional repressor RBP-Jkappa/CBF1. The nuclear factor E2F is known to be involved in cell cycle regulation and the human MYC promoter is a target of this transcription factor (Thalmeier 1989). Although these polymorphisms are located at the 3´part of the *CDH13* gene they might alter the expression of the gene. According to the Ensembl genome browser one isoform of *CDH13* has its transcriptional start site near the 3´end of the gene and may therefore be influenced by variations in this region. Another possibility is that those regulatory elements have an effect on the expression of other genes located downstream the *CDH13* gene. In this case CDH13 itself would not be the candidate gene for ADHD but would contain critical DNA features for the regulation of the de facto candidate gene situated nearby. Possible alternative candidates are the *NECAB2* gene which is also highly expressed in the brain and which is coding for the Neuronal calcium-binding protein 2 (also called Synaptotagmin-interacting protein 2) or the *HSFBP1* gene that is coding for the heat shock factor binding protein 1. As a consequence, despite all the evidences that point to *CDH13* as a potent candidate gene for ADHD there is still clear need to further investigate the Chromosome 16q24 locus and the genes it harbors as well as the translational regulation of the *CDH13* gene and its adjacent genes.

4.3. *DIRAS2*

Based on the findings of four independent hypothesis free approaches- two linkage scans on childhood ADHD, a GWAS of adult ADHD and a GWAS of PD- *DIRAS2* was considered as a candidate gene for ADHD and a fine-mapping of this gene located on chromosome 9q22 was performed. Tagging SNPs situated in the promoter region, the coding sequence and the 3´UTR of the gene were analyzed in seven independent patient samples. Two of the four SNPs which were found to be associated with aADHD in the initial sample from Germany are located in the 3´UTR (rs7854469 and rs16906711) and two are located in the upstream regulatory region of the *DIRAS2* gene (rs1331503 and rs1412005). The initial association findings in aADHD were successfully replicated in the independent sample of the IMpACT collaboration using a meta-analytic approach. The risk haplotype identified for aADHD spans the promoter region of the *DIRAS2* gene and the associated SNP which was found in the meta-analysis (rs1412005) is contributing to this haplotype. The only marker (rs7848810) that showed an association with childhood ADHD is also located within this regulatory region and component of this haplotype as well. Searching for transcription factor binding motifs revealed that the sequence carrying the major allele of rs7848810 contains predicted response elements for PBX1 and for the PBX-HOXA9 dimer. It is known that PBX1 plays a role in the developmental regulation of neuropeptide Y expression (Mayer 2003), whereas PBX-HOX dimers are involved in vertebrate development in general (Moens and Selleri, 2006). Risk allele carriers may therefore have an impaired developmental expression of DIRAS2. Due to the fact that all markers and the haplotype block that showed an association with ADHD are located in potential control regions of gene expression, it can be hypothesized that changes in the expressional control of DIRAS2 underlie the ADHD association. The role of the risk alleles identified in this work in respect to the regulation of gene expression needs to be further investigated using tools like luciferase promoter assays.

The association study results gained from the genotyping data of the bipolar and PD samples show that association of *DIRAS2* with ADHD is not specific for this disease, but also occurs for other disorders. Considering the co-morbidity of these disorders with aADHD,

those findings may provide a genetic basis for these clinical observations. It has to be reflected which neurobiological phenotype is modulated by *DIRAS2*. Indications might be obtained from personality genetics, as nine *DIRAS2* SNPs were associated with personality domains in aADHD or PD patients. For example, aADHD patients carrying the rs1412005 T allele show on average 3.6 point lower Conscientiousness and 1.4 point higher Harm Avoidance scores than non-carriers (Table 3.9). Therefore, *DIRAS2* seems to predispose to a characteristic personality profile observed in ADHD, which might best be characterized by reduced Conscientiousness and increased Harm Avoidance. However, it has to be noted that associations varied between samples so that these data can only be considered preliminary and there is clear need of further replication.

To get more knowledge about candidate genes which have been identified in association studies, expression studies as well as functional studies are needed. As almost nothing is known about the *DIRAS2* gene, its expression in the brain was further investigated in this work. The expression pattern of *Diras2* in the mouse brain provides further support to the assumption that this gene is involved in the pathophysiology of ADHD.
Volume anomalies of subcortical structures, specifically of the basal ganglia, hippocampus and amygdala in patients suffering from ADHD have been reported in structural neuroimaging studies (Shaw and Rabin, 2009). Therefore, the expression of a candidate gene for this disorder in regions like the amygdala and hippocampus like it was observed for *Diras2* expression is not surprising.

The hippocampus is mainly known for its function in memory processing. It belongs to the limbic system of which the amygdala is also a part. The entorhinal cortex (EC) is considered a part of the hippocampal region because of its anatomical connections. Within the hippocampus, the flow of information from the EC is largely unidirectional. First , the perforant fiber pathway projects to the granule cells of the dentate gyrus, then the mossy fibers of those cells project to the pyramidal cells in the CA3 layer which are connected to the pyramidal cells of the CA1 layer through the Schaffer collateral pathway. The information is then transmitted to the subiculum and there it is forwarded out of the hippocampus to the EC. Beyond the output to the EC, additional output pathways go to other

cortical areas including the prefrontal cortex. There is also output from the hippocampus to the lateral septal area. It is hypothized that that hippocampal dysfunction could produce an alteration of dopamine release in the basal ganglia, especially in the nucleus accumbens (NAcc), thereby indirectly affecting the integration of information in the prefrontal cortex. Adaptive modifications in dopaminergic system balance are important for enabling the NAcc to shift between limbic and cortical drives like the amygdala and the hippocampus and the PFC (Goto and Grace, 2008).

Diras2 expression in the amygdala was limited to the basolateral nucleus. The basolateral amygdala (BLA) projects heavily to the nucleus accumbens and also reciprocally to the hippocampus. Bidirectional projections connect the medial prefrontal cortex and basolateral amygdala. This circuit has been suggested to play a role in fear extinction. Abnormalities in the projections from the medial prefrontal cortex to the basolateral amygdala have been observed in schizophrenics. A recent study in rats revealed that the perinatal exposure to bisphenol-A (BPA), which causes GABAergic disinhibition and dopaminergic enhancement, leads to an abnormal cortical-BLA synaptic transmission and plasticity. Those changes might be responsible for the observed hyperactivity and attention-deficit in BPA-rats (Zhou R. , 2011).

In a MRI study which compared children with ADHD to healthy controls an enlargement of the hippocampus and reduced size of the basolateral complex of the amygdala was found. Correlations with prefrontal measures suggested abnormal connectivity between the amygdala and prefrontal cortex in the ADHD group. Those findings again suggest an involvement of the limbic system in the pathophysiology of ADHD (Plessen 2006).

The mitral cells of the olfactory bulb receive input from the olfactory receptor neurons. They convey information through the anterior olfactory nucleus to several brain regions including the piriform cortex, the amygdala and the entorhinal cortex. Romanos et al. reported an increase in odor sensitivity in children suffering from ADHD (Romanos 2008b). The expression of Diras2 in the mitral cells, the anterior olfactory nucleus and the

piriform cortex might indicate an involvement of this gene in the observed changes in olfactory perception in ADHD.

Since the cerebral cortex is much smaller and less complex in rodents than it is in humans, it is difficult to compare particular cortical areas in these two species. Especially the prefrontal cortex - one of the brain regions strongly linked to ADHD - was considered to be unique to primates. Nonetheless, today there are several studies on rats indicating the existence of cortical structures with homologies to the prefrontal cortex in primates (Uylings 2003) (Seamans 2008). Less is known about the prefrontal cortical areas of mice which have been characterized cytoarchitectonically and chemoarchitectonically quite recently (Van De Werd 2010). In one study it was found that mice exhibit affective and attentional sets like rats and primates and that those functions are disrupted by medial and orbital prefrontal cortical lesions (Bissonette 2008) indicating a possible role of the mouse prefrontal cortical areas similar to that of the prefrontal cortex in primates. Hence, the expression of a candidate gene for ADHD in the orbital cortex of mouse brains is in line with the involvement of the prefrontal cortex in the pathophysiology of ADHD in humans.

The lateral septum (LS) is thought to be of central importance for the control of mood and motivation. There is evidence that this brain region is involved in several psychiatric disorders like schizophrenia, depression or anxiety disorders due to its role in affective regulation. The LS receives glutamatergic input from the hippocampus and is composed of GABAergic neurons projecting to various hypothalamic cell groups and midbrain regions. Moreover, the LS receives input from the amygdala, the frontal cortex, the bed nucleus of the stria terminalis and the entorhinal cortex, which are structures known for their regulatory impact on emotional states (Sheehan 2004). Therefore, the lateral septum may also play a part in the emotional dysregulation in ADHD and co-morbid disorders.

As the dopaminergic system is assumed to be highly involved in the pathophysiology of ADHD, the substantia nigra as a core structure of this system may be of interest for this disease as well. The dopaminergic terminations in the striatum originate mainly from the

substantia nigra. Thus, the spot-wise *in situ* hybridization staining in the striatum could constitute neuronal endings deriving from the substantia nigra which was found to contain *Diras2* mRNA. An enlargement of the echogenic substantia nigra area could be observed in children with ADHD compared to controls in two independent studies (Romanos 2010) (Krauel 2010). In one study a correlation with symptoms of inattention, hyperactivity, and impulsivity was found. The results of those studies support the idea that the nigrostriatal system is altered in ADHD.

In addition to the dopaminergic system, the noradrenergic system is considered to be involved in ADHD. A majority of the noradrenergic neurons of the brain are concentrated in the locus coeruleus, a brain stem nucleus. This nucleus is the primary source of noradrenergic innervation of the forebrain and the sole source of norepinephrine to the hippocampus and the neocortex. Norepinephrin modulates the collection and processing of noticeable sensory information through its action on sensory, memory, attentional and motor processes. It is assumed that dysfunction of the locus coeruleus noradrenergic system may lead to deficits in cognitive and affective processes that are associated with disorders like ADHD. There is evidence that the action of psychostimulant drugs used for the treatment of ADHD involves the locus coeruleus norepinephrine system (Berridge and Waterhouse, 2003). A study on the effect of methylphenidate (MPH) on the locus coeruleus suggested that the behavioral-calming and cognition-enhancing actions of low-dose MPH involve moderate actions on locus coeruleus discharge combined with alterations in catecholamine neurotransmission within the prefrontal cortex (Devilbiss and Berridge, 2006). As the locus coeruleus is innervated by dopamingergic fibers from the ventral tegmental area and therefore is connected to the dopaminergic system, it is not clear if the detection of *Diras2* mRNA in this area indicates the expression of this gene in noradrenergic neurons or in dopaminergic afferences.

The parafascicular thalamic nucleus (PF) in which *Diras2* expression was also observed is a part of the centromedian-PF complex in the caudal region of the intralaminar thalamus. The intralaminar thalamic nuclei seem to be important for attention and action selection

(Brown 2010). Therefore, the expression of *Diras2* within this nucleus supports the assumption that it is a potential candidate gene for ADHD.

All of the brain regions in which *Diras2* expression could be detected have been reported to be involved in the pathomechanisms of ADHD and/or to be altered in the disease. The observed expression pattern matches the idea of *DIRAS2* as a candidate gene for ADHD. The expression levels of *DIRAS2* in the human brain determined by qPCR fit the pattern found in the mouse brain. The highest expression levels were found in the hippocampus, the cerebral cortex and the cerebellum. RNA isolated from the human amygdala was not available. Therefore, no statement about *DIRAS2* expression in this region in the human brain can be made. To review the expectation that the *DIRAS2* expression pattern is the same or similar in the human and in the murine brain, *in situ* hybridization on human brain tissue would be necessary.

To determine if *Diras2* is expressed in components of the dopaminergic system and in GABAergic interneurons exclusively or if it is expressed for example in noradrenergic and serotonergic neurons as well, further studies are needed, especially those working with double labeling on brain tissues and primary cells. The results of the immunocytochemical double staining of mouse hippocampal primary cells revealed a presence of Di-ras2 in GABAergic and dopaminergic neurons but not in glia cells. Expression of *Diras2* in serotonergic cells is to be expected, as a study investigating the distinct transcriptomes of rostral and caudal embryonic serotonergic neurons found a 13-fold increase of *Diras2* in caudal serotonergic cells (Wylie 2010). Also, *in situ* hybridizations and immunohistochemical staining on sagittal and horizontal brain sections will be useful to recognize the brain regions in which Di-ras2 occurs.

The fact that no clear Di-ras2 expression was found in any of the investigated human cell lines, especially in cancer cell lines, may be due to a down regulation of the expression of this Ras kinase in tumor cells like it is the case for the homologue kinase ARHI. Further examination of the function of Di-ras2 in neuroblastoma cells, such as over expression studies, may give insights into its cellular function.

The investigation of *Diras2* expression during mouse brain development by qRT PCR revealed a significant increase of expression levels during the early developmental stages. The expression reaches its summit in the late postnatal phase which was defined as postnatal day 9 – 15. This expression maximum stays stable until adulthood. Those results could be interpreted in a way that *Diras2* being important in the brain at later developmental stages. However, developmental time lines between different species cannot be matched precisely. Clancy et al. developed a model for translating time across different mammalian species during neural development (Clancy 2001). In this model, postnatal days 8.8 to 11.2 in the mouse brain are comparable to 138 to 201 days after conception in humans. Therefore, a similar time course in *Diras2* expression in the human brain could mean that its expression reaches its maximum during prenatal brain development. To elucidate the possible role of *Diras2* during human brain development, further studies to clarify its function on a cellular level are needed and have to be carried out with human tissue.

4.4. Conclusion and outlook

In conclusion the results of this work provide further support to the existence of a strong genetic component in the pathophysiology of ADHD and related disorders.

However, several points have to be considered in the interpretation of the data presented in this work. Given the genetic effect sizes (odds ratio) estimated in the aADHD discovery sample (1020 German individuals), the replication sample (2416 individuals from IMpACT) had sufficient power to detect the effects of the rs1412005 T allele and the ACGCTT haplotype of the *Diras2* gene and the effects of the risk alleles of the markers rs4591132, rs2228685, rs11642219 and rs153653 in the *CDH13* gene. Despite this fact, there still is a chance of false-positive findings. This is relevant as most associations on the single marker level would not withstand correction for multiple testing, which becomes an even more prominent issue when examining multi-axial dimensional data such as personality traits. However, in case of a successful replication in several independent populations as achieved for the association studies concerning the *DIRAS2* and the *CDH13* gene, and in conjunction with previous evidence from linkage studies and GWAS, it might be assumed

that there are true associations between those genes and ADHD. It can be speculated that genetic variations within the investigated candidate genes, in linkage disequilibrium with the genotyped SNPs, are the risk alleles, a hypothesis which will have to be tested in further studies using re-sequencing approaches followed by functional studies. Finally, as strong positive findings were not obtained from all samples, small individual sample size, background, epistatic or founder effects or even gene-environment interaction may also play a role in explaining the fact that genetic variants contribute to ADHD and related phenotypes in some, but not all populations.

The results of the expression studies of *Diras2* promote this gene as an interesting candidate gene for ADHD. However, its function and the pathways via which it acts are still unknown. One can speculate that Di-ras2, like other Ras kinases, may be involved in cell growth and cell morphology and therefore may also be involved in the volume changes of several brain regions observed in ADHD patients. To investigate if the identified risk alleles and the risk haplotype situated in the promoter region of the *Diras2* gene alter the expression levels of this gene, sequencing as well as promoter studies are needed. Also, a closer look at the promoter methylation might give new insights into the expressional regulation of this gene, as the expression of the homologous *ARHI* gene is known to be down regulated via methylation of the promoter region.

Taken together, the investigated genes are promising candidates and need to be further examined to acquire more knowledge about the neurobiological basis of this common disease. This knowledge is essential for understanding the molecular mechanisms underlying the emergence of this disorder and for the development of new treatment strategies.

5. Appendix

5.1. References

Asherson P, et al. (2008) A high-density SNP linkage scan with 142 combined subtype ADHD sib pairs identifies linkage regions on chromosomes 9 and 16. Molecular psychiatry 13: 514-521.

Baranauskas G (2004) Cell-type-specific splicing of KChIP4 mRNA correlates with slower kinetics of A-type current. Eur J Neurosci 20: 385-391.

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

Becker T, Knapp M (2004) Maximum-likelihood estimation of haplotype frequencies in nuclear families. Genet Epidemiol 27: 21-32.

Berridge CW, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain research. Brain research reviews 42: 33-84.

Biederman J, Milberger S, Faraone SV, Kiely K, Guite J, Mick E, Ablon S, Warburton R, Reed E (1995) Family-environment risk factors for attention-deficit hyperactivity disorder. A test of Rutter's indicators of adversity. Archives of general psychiatry 52: 464-470.

Biederman J, Mick E, Faraone SV, Braaten E, Doyle A, Spencer T, Wilens TE, Frazier E, Johnson MA (2002) Influence of gender on attention deficit hyperactivity disorder in children referred to a psychiatric clinic. The American journal of psychiatry 159: 36-42.

Biederman J (2005) Attention-deficit/hyperactivity disorder: a selective overview. Biological psychiatry 57: 1215-1220.

Bissonette GB, Martins GJ, Franz TM, Harper ES, Schoenbaum G, Powell EM (2008) Double dissociation of the effects of medial and orbital prefrontal cortical lesions on attentional and affective shifts in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 28: 11124-11130.

Brookes K, et al. (2006) The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. Molecular psychiatry 11: 934-953.

Brown HD, Baker PM, Ragozzino ME (2010) The parafascicular thalamic nucleus concomitantly influences behavioral flexibility and dorsomedial striatal acetylcholine output in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 30: 14390-14398.

Castellanos FX, et al. (2002) Developmental trajectories of brain volume abnormalities in children and adolescents with attention-deficit/hyperactivity disorder. JAMA : the journal of the American Medical Association 288: 1740-1748.

Celio MR (1990) Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35: 375-475.

Clancy B, Darlington RB, Finlay BL (2001) Translating developmental time across mammalian species. Neuroscience 105: 7-17.

Devilbiss DM, Berridge CW (2006) Low-dose methylphenidate actions on tonic and phasic locus coeruleus discharge. The Journal of pharmacology and experimental therapeutics 319: 1327-1335.

Dudbridge F (2008) Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. Hum Hered 66: 87-98.

Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA, Sklar P (2005) Molecular genetics of attention-deficit/hyperactivity disorder. Biological psychiatry 57: 1313-1323.

Franke B, et al. (2010) Multicenter analysis of the SLC6A3/DAT1 VNTR haplotype in persistent ADHD suggests differential involvement of the gene in childhood and persistent ADHD. Neuropsychopharmacology 35: 656-664.

Fredette BJ, Miller J, Ranscht B (1996) Inhibition of motor axon growth by T-cadherin substrata. Development 122: 3163-3171.

Goto Y, Grace AA (2008) Limbic and cortical information processing in the nucleus accumbens. Trends in neurosciences 31: 552-558.

Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes & development 18: 1926-1945.

Holmqvist MH, et al. (2002) Elimination of fast inactivation in Kv4 A-type potassium channels by an auxiliary subunit domain. Proceedings of the National Academy of Sciences of the United States of America 99: 1035-1040.

Jacob CP, Muller J, Schmidt M, Hohenberger K, Gutknecht L, Reif A, Schmidtke A, Mossner R, Lesch KP (2005) Cluster B personality disorders are associated with allelic variation of monoamine oxidase A activity. Neuropsychopharmacology 30: 1711-1718.

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

Joshi MB, Ivanov D, Philippova M, Erne P, Resink TJ (2007) Integrin-linked kinase is an essential mediator for T-cadherin-dependent signaling via Akt and GSK3beta in endothelial cells. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 21: 3083-3095.

Kipmen-Korgun D, Osibow K, Zoratti C, Schraml E, Greilberger J, Kostner GM, Jurgens G, Graier WF (2005) T-cadherin mediates low-density lipoprotein-initiated cell proliferation via the Ca(2+)-tyrosine kinase-Erk1/2 pathway. Journal of cardiovascular pharmacology 45: 418-430.

Kitagawa H, Ray WJ, Glantschnig H, Nantermet PV, Yu Y, Leu CT, Harada S, Kato S, Freedman LP (2007) A regulatory circuit mediating convergence between Nurr1 transcriptional regulation and Wnt signaling. Molecular and cellular biology 27: 7486-7496.

Kontani K, Tada M, Ogawa T, Okai T, Saito K, Araki Y, Katada T (2002) Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. J Biol Chem 277: 41070-41078.

Krauel K, Feldhaus HC, Simon A, Rehe C, Glaser M, Flechtner HH, Heinze HJ, Niehaus L (2010) Increased echogenicity of the substantia nigra in children and adolescents with attention-deficit/hyperactivity disorder. Biological psychiatry 68: 352-358.

Lasky-Su J, et al. (2008) Genome-wide association scan of quantitative traits for attention deficit hyperactivity disorder identifies novel associations and confirms candidate gene associations. American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 147B: 1345-1354.

Lesch KP, et al. (2008) Molecular genetics of adult ADHD: converging evidence from genome-wide association and extended pedigree linkage studies. J Neural Transm 115: 1573-1585.

Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329: 959-964.

Lu Z, et al. (2008) The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. The Journal of clinical investigation 118: 3917-3929.

Martin ER, Monks SA, Warren LL, Kaplan NL (2000) A test for linkage and association in general pedigrees: the pedigree disequilibrium test. Am J Hum Genet 67: 146-154.

Mayer CM, Cai F, Cui H, Gillespie JM, MacMillan M, Belsham DD (2003) Analysis of a repressor region in the human neuropeptide Y gene that binds Oct-1 and Pbx-1 in GT1-7 neurons. Biochem Biophys Res Commun 307: 847-854.

Miller CJ, Flory JD, Miller SR, Harty SC, Newcorn JH, Halperin JM (2008) Childhood attention-deficit/hyperactivity disorder and the emergence of personality disorders in adolescence: a prospective follow-up study. The Journal of clinical psychiatry 69: 1477-1484.

Moens CB, Selleri L (2006) Hox cofactors in vertebrate development. Dev Biol 291: 193-206.

Morohashi Y, Hatano N, Ohya S, Takikawa R, Watabiki T, Takasugi N, Imaizumi Y, Tomita T, Iwatsubo T (2002) Molecular cloning and characterization of CALP/KChIP4, a novel EF-hand protein interacting with presenilin 2 and voltage-gated potassium channel subunit Kv4. J Biol Chem 277: 14965-14975.

Neale BM, et al. (2008) Genome-wide association scan of attention deficit hyperactivity disorder. American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 147B: 1337-1344.

Neale BM, et al. (2010a) Case-control genome-wide association study of attention-deficit/hyperactivity disorder. Journal of the American Academy of Child and Adolescent Psychiatry 49: 906-920.

Neale BM, et al. (2010b) Meta-analysis of genome-wide association studies of attention-deficit/hyperactivity disorder. J Am Acad Child Adolesc Psychiatry 49: 884-897.

O'Connell JR, Weeks DE (1998) PedCheck: a program for identification of genotype incompatibilities in linkage analysis. Am J Hum Genet 63: 259-266.

Palop JJ, Roberson ED, Cobos I (2011) Step-by-step in situ hybridization method for localizing gene expression changes in the brain. Methods Mol Biol 670: 207-230.

Pfeffer PL, Bouchard M, Busslinger M (2000) Pax2 and homeodomain proteins cooperatively regulate a 435 bp enhancer of the mouse Pax5 gene at the midbrain-hindbrain boundary. Development 127: 1017-1028.

Philippova M, Ivanov D, Allenspach R, Takuwa Y, Erne P, Resink T (2005) RhoA and Rac mediate endothelial cell polarization and detachment induced by T-cadherin. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19: 588-590.

Philippova M, Ivanov D, Joshi MB, Kyriakakis E, Rupp K, Afonyushkin T, Bochkov V, Erne P, Resink TJ (2008) Identification of proteins associating with glycosylphosphatidylinositol-anchored T-cadherin on the surface of vascular endothelial cells: role for Grp78/BiP in T-cadherin-dependent cell survival. Molecular and cellular biology 28: 4004-4017.

Philippova M, Joshi MB, Kyriakakis E, Pfaff D, Erne P, Resink TJ (2009) A guide and guard: the many faces of T-cadherin. Cellular signalling 21: 1035-1044.

Plessen KJ, et al. (2006) Hippocampus and amygdala morphology in attention-deficit/hyperactivity disorder. Archives of general psychiatry 63: 795-807.

Pruunsild P, Timmusk T (2005) Structure, alternative splicing, and expression of the human and mouse KCNIP gene family. Genomics 86: 581-593.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339: 62-66.

Renner TJ, Gerlach M, Romanos M, Herrmann M, Reif A, Fallgatter AJ, Lesch KP (2008a) [Neurobiology of attention-deficit hyperactivity disorder]. Nervenarzt 79: 771-781.

Renner TJ, Walitza S, Dempfle A, Eckert L, Romanos M, Gerlach M, Schafer H, Warnke A, Lesch KP, Jacob C (2008b) Allelic variants of SNAP25 in a family-based sample of ADHD. J Neural Transm 115: 317-321.

Rhodes KJ, et al. (2004) KChIPs and Kv4 alpha subunits as integral components of A-type potassium channels in mammalian brain. J Neurosci 24: 7903-7915.

Romanos M, et al. (2008a) Genome-wide linkage analysis of ADHD using high-density SNP arrays: novel loci at 5q13.1 and 14q12. Mol Psychiatry 13: 522-530.

Romanos M, Renner TJ, Schecklmann M, Hummel B, Roos M, von Mering C, Pauli P, Reichmann H, Warnke A, Gerlach M (2008b) Improved odor sensitivity in attention-deficit/hyperactivity disorder. Biol Psychiatry 64: 938-940.

Romanos M, Weise D, Schliesser M, Schecklmann M, Loffler J, Warnke A, Gerlach M, Classen J, Mehler-Wex C (2010) Structural abnormality of the substantia nigra in children

with attention-deficit hyperactivity disorder. Journal of psychiatry & neuroscience : JPN 35: 55-58.

Scholz CJ, et al. (2010) Functional variants of TSPAN8 are associated with bipolar disorder and schizophrenia. Am J Med Genet B Neuropsychiatr Genet 153B: 967-972.

Seamans JK, Lapish CC, Durstewitz D (2008) Comparing the prefrontal cortex of rats and primates: insights from electrophysiology. Neurotoxicity research 14: 249-262.

Seshadri S, et al. (2007) Genetic correlates of brain aging on MRI and cognitive test measures: a genome-wide association and linkage analysis in the Framingham study. BMC Medical Genetics 8: S15.

Shaw P, Rabin C (2009) New insights into attention-deficit/hyperactivity disorder using structural neuroimaging. Current psychiatry reports 11: 393-398.

Sheehan TP, Chambers RA, Russell DS (2004) Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain research. Brain research reviews 46: 71-117.

Sowell ER, Thompson PM, Welcome SE, Henkenius AL, Toga AW, Peterson BS (2003) Cortical abnormalities in children and adolescents with attention-deficit hyperactivity disorder. Lancet 362: 1699-1707.

Stergiakouli E, Thapar A (2010) Fitting the pieces together: current research on the genetic basis of attention-deficit/hyperactivity disorder (ADHD). Neuropsychiatric disease and treatment 6: 551-560.

Sullivan PF, et al. (2008) Genomewide association for schizophrenia in the CATIE study: results of stage 1. Molecular psychiatry 13: 570-584.

Takeuchi T, Misaki A, Liang SB, Tachibana A, Hayashi N, Sonobe H, Ohtsuki Y (2000) Expression of T-cadherin (CDH13, H-Cadherin) in human brain and its characteristics as a negative growth regulator of epidermal growth factor in neuroblastoma cells. Journal of neurochemistry 74: 1489-1497.

Thalmeier K, Synovzik H, Mertz R, Winnacker EL, Lipp M (1989) Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. Genes & development 3: 527-536.

Treutlein J, et al. (2009) Genome-wide association study of alcohol dependence. Archives of general psychiatry 66: 773-784.

Uhl GR, et al. (2008) Genome-wide association for methamphetamine dependence: convergent results from 2 samples. Archives of general psychiatry 65: 345-355.

Uylings HB, Groenewegen HJ, Kolb B (2003) Do rats have a prefrontal cortex? Behavioural brain research 146: 3-17.

Van De Werd HJ, Rajkowska G, Evers P, Uylings HB (2010) Cytoarchitectonic and chemoarchitectonic characterization of the prefrontal cortical areas in the mouse. Brain structure & function 214: 339-353.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

Wennerberg K, Rossman KL, Der CJ (2005) The Ras superfamily at a glance. Journal of cell science 118: 843-846.

Wylie CJ, Hendricks TJ, Zhang B, Wang L, Lu P, Leahy P, Fox S, Maeno H, Deneris ES (2010) Distinct Transcriptomes Define Rostral and Caudal Serotonin Neurons. Journal of Neuroscience 30: 670-684.

Xiong H, Kovacs I, Zhang Z (2004) Differential distribution of KChIPs mRNAs in adult mouse brain. Brain Res Mol Brain Res 128: 103-111.

Zhao X, Li J, Zhuo J, Cai L (2010) Reexpression of ARHI inhibits tumor growth and angiogenesis and impairs the mTOR/VEGF pathway in hepatocellular carcinoma. Biochemical and biophysical research communications 403: 417-421.

Zhou K, et al. (2008) Meta-analysis of genome-wide linkage scans of attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet 147B: 1392-1398.

Zhou R, Bai Y, Yang R, Zhu Y, Chi X, Li L, Chen L, Sokabe M (2011) Abnormal synaptic plasticity in basolateral amygdala may account for hyperactivity and attention-deficit in male rat exposed perinatally to low-dose bisphenol-A. Neuropharmacology 60: 789-798.

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5.4. List of abbreviations

µ	micro
A172	glioblastoma cell line
ADHD	attention-deficit/hyperactivity disorder
bp	base pair
BSA	bovine serum albumin
CD	conduct disorder
CDH13	Cadherin13
cDNA	complimentary deoxyribonucleic acid
CI	confidence interval
cRNA	complimentary ribonucleic acid
Ct	threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DAT1	dopamine transporter 1
ddNTP	dideoxynucleotide
DEPC	diethylpyrocarbonate
DIG	digoxigenin
Diras2	distinct subgroup of the Ras family member 2
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DRD4	dopamine D4 receptor
DRD5	dopamine D5 receptor
DSM-IV	diagnostic and statistical manual of mental disorders
E18	embryonic day 18
ERK	extracellular-signal-regulated kinase
g	gram
GAP43	growth Associated Protein 43
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GPI	glycosylphosphatidylinositol

GRP78	78 kDa glucose-regulated protein
GSK3β	glycogen synthase kinase 3 beta
GTP	guanosine triphosphate
h	hours
HBSS	Hanks' Balanced Salt Solution
HEK293	immortalized human embryonic kidney cells
HISS	heat inactivated sheep serum
HWE	Hardy-Weinberg equilibrium
ILK	integrin linked kinase
IMAGE	International Multicenter ADHD Genetics
IMpACT	International Multicentre persistent ADHD Collaboration
ISH	*in situ* hybridization
KCNIP4	Kv channel-interacting protein 4
kD	kilo Dalton
KIS	K-channel inactivation suppressor
l	liter
LDL	low density lipoprotein
m	milli
M	molar
MAP2	Microtubule-associated protein 2
MAPK	mitogen activated protein kinase
min	minutes
mTOR	mammalian target of rapamycin
n	nano
NEO-PI R	revised Neuroticism-Extraversion-Openness personality inventory
NGS	normal goat serum
ODD	oppositional defiant disorder
OR	odds ratio
PBS	phosphate buffered saline
PD	personality disorders

PFA	paraformaldehyde
PI3K	phosphoinositide 3 kinase
PS	presenilin
qPCR	quantitative real-time polymerase chain reaction
RhoA	Ras homolog gene family, member A
RNA	ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
SCID-II	structured clinical interview for DSM disorders
SDS	sodium dodecyl sulfate
sec	seconds
SK-N-SH	neuroblastoma cell line
SNP	single nucleotide polymorphism
SSC	salt sodium citrate
TBS	tris buffered saline
TDT	transmission disequilibrium testing
TH	tyrosine hydroxylase
TPQ	tridimensional Personality Questionnaire
U373	astrocytoma cell line
UTR	untranslated region

5.5. Publications

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Affidavit

I hereby declare that my thesis entitled *Molecular Genetics of Emotional Dysregulation in Attention-Deficit/Hyperactivity Disorder* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg…………………………………………………………………………………………

Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *Molekulargenetik der emotionalen Dysregulation bei Aufmerksamkeitsdefizit-/Hyperaktivitätssyndrom* eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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