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Acetylcholine-metabolizing butyrylcholinesterase (*BCHE*) copy number and single nucleotide polymorphisms and their role in attention-deficit/hyperactivity syndrome

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ABSTRACT

A previous genome-wide screen for copy number variations (CNVs) in attention deficit/hyperactivity disorder (ADHD) revealed a *de novo* chromosome 3q26.1 deletion in one of the patients. Candidate genes at this locus include the acetylcholine-metabolizing butyrylcholinesterase (BCHE) expressing gene (OMIM #177400), which is of particular interest. The present study investigates the hypothesis that the heterozygous deletion of the *BCHE* gene is associated with adult ADHD (aADHD). Ina first step, we screened 348 aADHD patients and 352 controls for stretches of loss of heterozygosity (LOH) across the entire *BCHE* gene to screen for the deletion. Our second aim was to clarify whether *BCHE* single nucleotide polymorphisms (SNPs) themselves influence the risk towards ADHD. Putative functional consequences of associated SNPs as well as their un-typed proxies were predicted by several bioinformatic tools. 96 individuals displayed entirely homozygous genotype reads in all 12examined SNPs, making them possible candidates to harbor a heterozygous *BCHE* deletion. DNA from these 96 probands was further analyzed by real-time PCR using a *BCHE*-specific CNV assay. However, no deletion was found. Of the 12 tag SNPs that passed inclusion criteria, rs4680612 and rs829508were significantly associated with aADHD, as their minor alleles occurred more often in cases than in controls ($p = 0.018$ and $p = 0.039$, respectively). The risk variant rs4680612 is located in the transcriptional control region of the gene and predicted to disrupt a binding site for MYT-1, which has previously been associated with mental disorders. However, when examining a second independent adult ADHD sample of 353 cases, the association did not replicate. When looking up the deletion in three genome-wide screens for CNV in ADHD and combining it with the present study, it became apparent that 3 from a total of 1030 ADHD patients, but none of 5787 controls, featured a deletion of the *BCHE* promoter region including rs4680612 ($p = 0.00004$). Taken together, there are several lines of evidence suggesting a potential involvement of *BCHE* in the etiopathology of ADHD, as a rare hemizygous deletion as well as a common SNP in the same region are associated with disease, although with different penetrance. Both variations result in the disruption of the binding site of the transcription factor MYT-1 suggesting epistatic effects of BCHE and MYT-1 in the pathogenesis of ADHD. As we were not able to replicate the SNP association, our findings should be considered preliminary and call for larger studies in extended phenotypes.

1. Introduction

The polygenetic nature of attention-deficit/hyperactivity disorder (ADHD; OMIM #143465) indicates that complex interactions of multiple genes of mild to moderate effect with environmental influence are involved in the etiological basis of ADHD. Candidate gene approaches examine *a priory* hypotheses based on known neurobiological foundations that have an impact on the disease in question. In contrast, genome-wide association studies (GWAS) scan the entire genome for common genetic variation. There is evidence from recent GWAS for the involvement of candidate genes that influence neurodevelopment such as *CDH13* and *ASTN2* (cell

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adhesion molecules) (Lesch et al, 2008), which is in line with clinical manifestations of delayed neurodevelopment (McLaughlin et al., 2010).

There is a considerable debate about the issue whether multiple common variants interact to finally exceed a certain risk threshold and cause disease ("common variant, common disease" model), or whether rare variants with high penetrance individual to each family underlie psychiatric disorders ("clan genomics"; "multiple rare variant, common disease" model). In line with this assumption, alternative genome-wide molecular genetic approaches such as copy number variation (CNV) analyses, have recently been used to identify polymorphic loci underlying several neuropsychiatric disorders. A genome-wide screen for CNVs that was previously performed by our group identified a total of 17 potentially ADHD-associated CNVs by using high-resolution array comparative genomic hybridization (aCGH) in a cohort of 99 children and adolescents with severe ADHD (Lesch et al, 2011). Among the aberrations that comprise 4 deletions and 13 duplications with approximate sizes ranging from 110 kb to 3 Mb was a *de novo* chromosome 3q26.1 deletion (Pat 991 m) overlapping with two recently described potentially disease-associated CNVs in childhood ADHD (Elia et al., 2010; Lionel et al., 2011). Candidate genes located at this locus include the acetylcholine-metabolizing butyrylcholinesterase (*BCHE*) expressing gene (OMIM #177400) which is particularly of interest. This gene spans about 70 kb and is composed of four exons (E1–E4) and three introns (I1–I3) (Darvesh et al., 2003).

There are several lines of evidence that *BCHE* might be involved in the pathogenesis of ADHD. The enzyme BCHE is involved in the regulation of neuronal proliferation and differentiation (Mack and Robitzki, 2000). For example, Kostovic and colleagues describe the influence of *BCHE* in the development of the frontal lobe by influencing the differentiation of thalamo-prefrontal connections both in humans and rhesus monkeys (Kostovic and Goldman-Rakic, 1983). In the brain, *BCHE* is strongly expressed in cholinergic neurons of the pedunculopontine tegmentum that, in interaction with dopaminergic, noradrenergic and serotonergic networks, regulate sleep–wake behavior and vigilance (Darvesh et al., 2003), suggesting that this gene may also directly influence locomotor activity, attention, reward-related behavior and information processing. This might relate to ADHD, as the Attention Network Model of ADHD defines an alerting or vigilance network, an orientation network and an executive or conflict network (Lundervold et al., 2011). Interestingly, a rare functional null mutation of BCHE was shown to influence reaction times in neuropsychological tests (Manoharan et al., 2007). Finally, BCHE may also be involved in the inactivation of exogenous neurotoxic compounds. In mice and rats, a single prophylactic administration of human BCHE acts as an antidote as it inactivates the lethal effects of highly toxic organophosphates (Raveh et al., 1993).

In view of these results, the present study investigates the hypothesis that the heterozygous deletion of the *BCHE* gene influences the categorical phenotype of aADHD, thus extending the results from previous studies (Elia et al., 2010; Lesch et al, 2011; Lionel et al., 2011). In a first step we screened 348 adult AHDH (aADHD) patients and 352 controls for stretches of loss of heterozygosity (LOH) across the entire *BCHE* gene by inspecting 15 single nucleotide polymorphisms (SNPs) representative for SNP variation within the *BCHE* gene and a −100 kb upstream/+ 8 kb downstream region. Of the 700 examined individuals, 96 were homozygous for all 15 polymorphisms and were therefore further analyzed with a *BCHE* specific CNV assay. Our second aim was to clarify whether *BCHE* SNPs themselves influence the risk towards ADHD. Putative functional consequences of associated SNPs as well as their un-typed proxies were predicted by several bioinformatic tools.

2. Method and materials

2.1. Participants and clinical assessment

As part of an ongoing study on aADHD multilevel endophenotyping, 348 in- and outpatients (thereof 43.7% females; mean age 34.3 ± 10.0 years) of the Department of Psychiatry, Psychosomatic and Psychotherapy, University of Wuerzburg, referred for diagnostic assessment and treatment of aADHD, were examined with the Structured Clinical Interview of DSM-IV axis-I disorders between 2003 and 2011 (Jacob et al., 2007). Diagnosis of childhood manifestation of ADHD was retrospectively assessed with the DSM-IV symptom list for ADHD (17 items) that was used as a structured clinical interview (Jacob et al., 2008). Additional information from the Wender-Utah-Rating Scale (WURS-K 21 items) was obtained. School report cards/certificates and medical history taken by the parents were included if available but were not obligatory. Adult manifestations were assessed with the DSM-IV symptom lists for ADHD. The diagnostic checklist of ADHD (ADHD-DC) was used to obtain additional information. To ensure diagnostic validity informative input from partners, relatives, and friends was also collected. For a more detailed description of the sample see also (Franke et al., 2010).

Inclusion criteria were aADHD, i.e. persistence of a clinically relevant ADHD syndrome into adulthood, according to the diagnostic criteria of DSM-IV, onset before the age of 7 years via retrospective diagnosis of childhood manifestation, life-long persistence, and current diagnosis of adult manifestation. Age at recruitment was between 18 and 65 years. Probands affected with substance use disorders underwent detoxification in an in-patient setting. Exclusion criteria were: the symptoms occur exclusively during the course of a pervasive developmental disorder, schizophrenia, or other psychotic disorder or symptoms are better accounted for by another mental disorder (criterion E of DSM-IV). Further exclusion criteria were: IQ level below 80 (MWT-B < 13 points) and life-time diagnosis of bipolar affective disorder (excluded due to the unsolved problems of differential diagnosis).

For replication of our strongest finding, a second independent aADHD sample, comprising 353 German in- and outpatients (thereof 41.8% females; mean age 37.2 ± 10.5 years), was recruited under identical conditions at the Saarland University Hospital, Homburg/Saar, Germany.

The control group consisted of 352 healthy subjects (thereof 49.0% females; mean age 30.5 ± 9.8 years), composed of blood donors, staff members and other controls all coming from the Lower Franconia region. The Ethics Committee of the University of Würzburg approved the study and written informed consent was obtained from all patients and healthy volunteers after procedures and aims of the study had been fully explained.

2.2. Genotyping of SNPs and detection of heterozygous BCHE deletions

In order to capture the common SNP variation in the *BCHE* gene (for an overview on the LD structure of the gene, see Fig. 1), the Haploview version 4.2 (Barrett et al., 2005) *Tagger* function (default settings) was used to choose 15 tag SNPs from HapMap CEU data (Frazer et al., 2007). SNP genotyping was performed using the Sequenom MassArray® system according to the manufacturer's instructions. All PCR reactions were done with the iPlex® chemistry following the MassArray® iPlex® standard operation procedure. Primer sequences can be found in Supplementary Table 1.

Deletions of the *BCHE* gene were detected using a custom TaqMan® Copy Number Assay (FAM labeled, ID: Hs00413437_cn) and a TaqMan® Copy Number Reference RNase P Assay (VIC labeled,