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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,

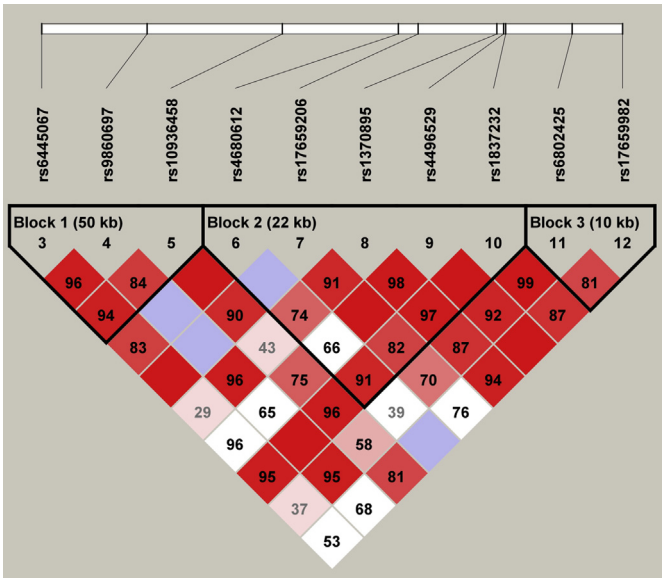

Fig. 1. Linkage disequilibrium (LD) plot of *BCHE*. LD is displayed as D′ haplotype blocks, defined with the four-gamete rule.

ID: 4403326) from Applied Biosystems (Darmstadt, Germany). Reactions were performed in four replicates per sample in a 384-well microplate format with 10 µl PCR reaction volumes as recommended by the manufacturer. For amplification the CFX384 Touch real-time PCR Detection System with CFX™ Manager Software version 1.5 was used (Bio-Rad, Munich, Germany). Real-time raw data were analyzed with the free CopyCaller™ Software version 1.0 from Applied Biosystems on the basis of the comparative CT (ΔΔCT) relative quantitation. The ΔΔCT method first calculates the difference (ΔCT) between the threshold cycles (CT) of the target and reference assay and then compares the ΔCT values of the test samples to a defined calibrator sample with a known copy number of the target sequence. For quality control the confidence value threshold for was set to 95; measurements below were repeated until the threshold was reached.

2.3. Statistical analysis

Prior to statistical analysis, all SNPs were tested for several quality criteria. Only variants with a genotyping call rate of at least 90% and a minor allele frequency (MAF) above 1% were further examined. Also significant ($p < 0.05$) deviations from Hardy–Weinberg equilibrium (HWE) were considered to be indicative for the presence of genotyping errors; SNPs rs1511979, rs9847379 and rs4365667 were thus excluded from further analysis.

Statistical analysis of genotype data was performed with PLINK V1.07 (Purcell et al., 2007) and HaploView V4.1 (Barrett et al., 2005). Single marker associations were calculated by comparison of allele counts in 1 degree-of-freedom χ^2 tests; adjustment for multiple testing was done with the conservative Bonferroni correction, which assumes independence of tests. Due to the presence of extended linkage disequilibrium, this does not hold for the SNPs examined in the present study, thus reducing the likelihood to find significant associations. For multimarker association tests, haplotype blocks were defined according to the four-gamete rule (Wang et al., 2002); inferred haplotype counts were compared with 1-degree-of-freedom χ^2 tests between groups. For each haplotype, multimarker association tests were permuted 10,000 times to generate an empirical probability distribution; this was used to estimate *p*-values that control the family-wise error rate (FWER).

2.4. Assessment of SNP function

The analysis of tag SNPs reduces genotyping effort with minimal loss of information on the one hand, but implies on the other hand that associated SNPs may not be functionally relevant themselves. To estimate which polymorphisms, in LD with the associated tag SNPs rs4680612 and rs829508, may contain putative causal variants conveying the genetic risk, eight perfect proxies ($r^2 = 1$ and $D' = 1$) within a distance of 500 kb were further characterized using bioinformatic tools contained in the GenEpi toolbox (Coassin et al., 2010). Annotation of SNPs in perfect linkage disequilibrium (LD) with associated SNPs ($D' = R^2 = 1$) in a distance of 500 kb was retrieved from the SNAP website version 2.2 (Johnson et al., 2008). Differential transcription factor binding site (TFBS) predictions were made using the web-based tool MatInspector version 2.1 (Cartharius et al., 2005). TFBs with a core similarity below 0.7 were excluded. To indicate an SNP's possible influence on splice junctions such as predictions of splice sites as well as binding sites for splicing regulatory elements (SREs) such as Splicing Enhancer (SE) and Splicing Silencer (SS) were made with the Human Splicing Finder software (HSF) version 2.4.1 (Desmet et al., 2009). For detection of SREs a motif value above 60.0 was required.

3. Results

Previous studies (Elia et al., 2010; Lesch et al, 2011; Lionel et al., 2011) indicate that deletion of a region covering the *BCHE* gene (see Fig. 2) may increase the risk towards ADHD. Since not only altered gene dosage but also other functionally relevant polymorphisms like SNPs may lead to changes in enzyme activity, we implemented a cost-effective approach to obtain information about both types of genetic variation. We therefore aimed to capture SNP variation across the gene by genotyping a minimal set of informative SNPs (for overview, see also Fig. 2): stretches of LOH across *BCHE* may be indicative for a heterozygous deletion of the gene. In a first step, we therefore reduced experimental effort by choosing 15 tag SNPs that represent the SNP variation in the *BCHE* region (gene −100 kb upstream/+8 kb downstream) in the HapMap CEU population. These 15 SNPs were genotyped in 700 individuals, thereof 348 aADHD patients. In total, 96 individuals displayed entirely homozygous genotypes in all 12 SNPs meeting stringent inclusion criteria, making them possible candidates to carry a heterozygous *BCHE* deletion. DNA from these 96 candidates was further analyzed by quantitative real-time PCR using a *BCHE*-specific CNV assay, but no deletion was found. Thus, apparent LOH was not the result from deleted *BCHE* alleles, but rather from homozygous haplotype combinations which is plausible given the LD structure of the gene (Fig. 1).

We next investigated whether allelic SNP variation in the *BCHE* region itself may go along with aADHD. Of the 12 tag SNPs that passed our inclusion criteria, two (rs4680612 and rs829508) were significantly associated with aADHD (Table 1). The polymorphisms' minor alleles (G and C, respectively) occurred at a higher frequency in cases than in controls and thus significantly (rs4680612; $p = 0.018$ and rs829508; $p = 0.039$, see Table 1) increase the risk for aADHD. However, neither association remained significant after Bonferroni correction for multiple testing (rs4680612; $p = 0.213$, rs829508; $p = 0.471$). Of note, the minor T allele of a further SNP, rs9832712, conveyed an almost significant increase in disease risk ($p_{marginal} = 0.051$). For replication of our strongest finding, we genotyped rs4680612 in a second independent AADHD sample and compared this to the same control sample. This however did not yield a significant result ($p = 0.663$).

SNPs were in considerable LD allowing the definition of three haplotype blocks (see Fig. 1 and Table 2). No associations could be

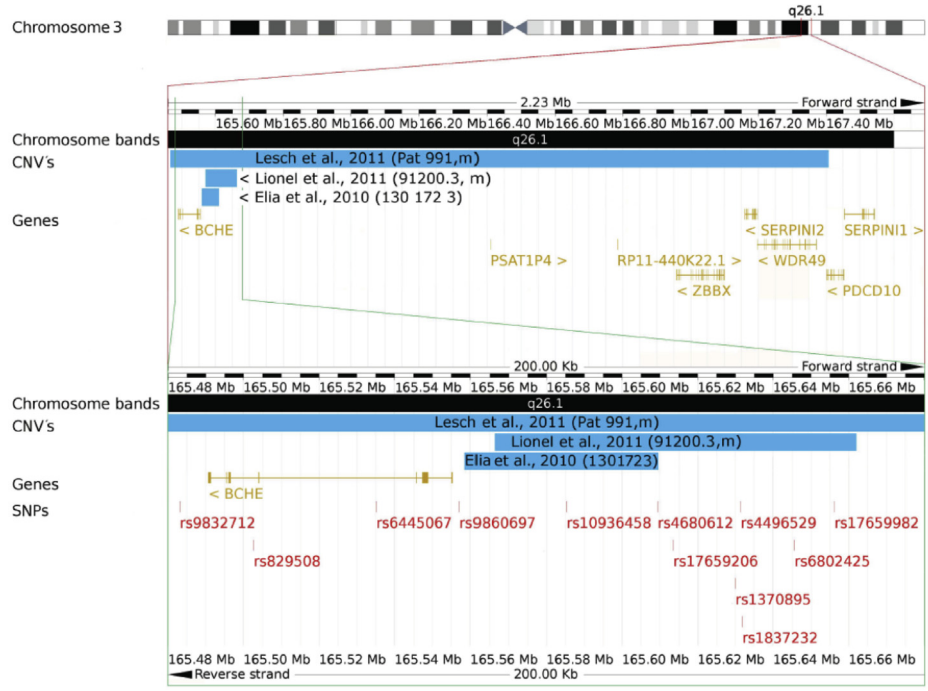

Fig. 2. Overview of the *BCHE* locus (chr3 [upper panel]: 165,460,000–167,690,000 according to Genome assembly GRCH37/hg19). The deletions on chromosome 3, detected in ADHD cases by Lesch et al, (2011) (165,462,265–167,413,570), Lionel et al., (2011) (165,565,929–165,662,040) and Elia et al., (2010) (165,558,900–16,560,950) display by blue bars, and neighboring genes are shown (middle panel). The lower panel indicates the position of the genotyped SNPs in relation to *BCHE* and the deletion region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detected for haplotypes in block one or block three; however block 2, consisting of the five SNPs rs4680612, rs17659206, rs1370895, rs4496529 and rs1837232 contained one haplotype significantly associated with disease (5′-GTAGG-3′). This is in line with our single marker results in that 5′-GTAGG-3′ contains the minor G (=risk) allele of rs4680612. This nominal haplotype association ($p = 0.023$) did however not withstand permutation testing ($p_{permutation} = 0.179$, Table 2).

In order to identify putative causal variants, perfect proxies ($r^2 = 1$ and $D' = 1$) of rs4680612 and rs829508 were searched for within a distance of 500 kb. This yielded 7 perfect proxies for rs4680612, which were like rs4680612 all located upstream of *BCHE* which may influence transcription factor (TF) binding sites (BS). Using bioinformatic tools, differential TFBS binding was predicted for rs4680612 and its two perfect proxies rs6796150 and rs957049. Focusing on TFs relevant in neuronal tissue, the minor alleles of rs6796150 and rs957049 delete binding sites for HOX_PBX.01 and MYT1.01, respectively. Only a single proxy (rs829500) was found to be in perfect LD with rs829508. Both variants are located in *BCHE*'s intron 3, therefore changes at splice junctions are possible functional consequences. The minor alleles of rs829508 and its proxy variant rs829500 are predicted to delete four putative binding sites for several splicing silencer (PESS, hnRNP A1, Sironi's Motif 1 and 3) and create one new binding site for the splicing enhancer 9G8 (Table 3).

4. Discussion

The results of three independent genome-wide CNV analyses in ADHD gave evidence for a chromosome 3q26.1 deletion (Elia et al., 2010; Lesch et al, 2011; Lionel et al., 2011). Among the involved genes, *BCHE* is of particular interest. The present data are still

Table 1
Association results for examined SNPs along with their minor (d)/major (D) alleles (converted to the coding strand), allele counts for cases and controls, the nominal and the Bonferroni corrected *p*-values. Bold indicates $p < 0.05$.

Chromosome	SNP	Alleles		Cases	Controls	Nominal	Bonferroni
		Minor (d) (d)	Major (D) (D)	n d/D	n d/D	*P*-value	*P*-value
3	rs9832712	T	A	199/439	168/472	0.051	0.611
3	rs829508	C	T	175/467	145/505	**0.039**	0.471
3	rs6445067	G	T	66/580	52/602	0.155	1
3	rs9860697	T	A	30/618	30/620	0.990	1
3	rs10936458	C	T	237/393	213/425	0.115	1
3	rs4680612	G	A	41/603	23/631	**0.018**	0.213
3	rs17659206	G	T	76/570	69/573	0.564	1
3	rs1370895	G	A	240/384	222/404	0.272	1
3	rs4496529	A	G	91/555	86/568	0.622	1
3	rs1837232	A	G	210/428	228/406	0.253	1
3	rs6802425	A	T	276/362	259/393	0.197	1
3	rs17659982	T	A	47/591	50/588	0.751	1

Table 2
Association of haplotypes (converted to the coding strand) with their frequencies in cases and controls, nominal *p*-value, as well as the corrected *p*-value after permutation. Bold indicates $p < 0.05$.

Block 1:			Frequency case/control	Nominal *P*-value	Permutation *P*-value
rs6445067	rs9860697	rs10936458			
T	A	T	0.619/0.661	0.117	0.679
T	A	C	0.277/0.257	0.397	0.998
G	A	C	0.056/0.036	0.078	0.498
G	T	C	0.040/0.042	0.798	1

Block 2:					Frequency case/control	Nominal *P*-value	Permutation *P*-value
rs4680612	rs17659206	rs1370895	rs4496529	rs1837232			
A	T	A	G	A	0.323/0.348	0.349	0.994
A	T	G	A	G	0.142/0.131	0.580	1
A	G	G	G	G	0.114/0.095	0.246	0.952
A	T	G	G	G	0.125/0.126	0.939	1
G	T	A	G	G	0.060/0.033	**0.023**	0.179
A	T	A	G	G	0.229/0.252	0.320	0.991

Block 3:		Frequency case/control	Nominal *P*-value	Permutation *P*-value
rs6802425	rs17659982			
T	T	0.069/0.069	0.961	1
A	A	0.428/0.391	0.130	0.865
T	A	0.498/0.534	0.129	0.896

inconclusive with respect to a possible contribution of common genetic variants in BCHE to the pathogenesis of ADHD. Results from our first discovery sample support a role of *BCHE* in the etiopathology of aADHD by revealing evidence for CNV, SNP and haplotype associations with disease. The latter results are however only suggestive and hence preliminary, because nominally significant *p*-values did not withstand Bonferroni-correction for multiple testing which might be overly conservative given the LD structure of the gene (Fig. 1). Additional data from a second independent aADHD sample did however not support a role of BCHE variants in ADHD, calling for even larger studies on both childhood as well as adulthood ADHD cases. Until confirmation, the association of both common variants as well as copy number variants in *BCHE* with ADHD has to be considered preliminary in nature.

Given that the deletion was not detected in 348 aADHD patients and 352 controls, it can be considered a rare event. Furthermore, the restriction to cases with an IQ > 80 due to our inclusion criteria might have even lowered the chance to pick up another deletion carrier. However, the association of hemizygous *BCHE* deletion and ADHD is still significant: including the present data and three previous studies (Elia et al., 2010; Lesch et al, 2011; Lionel et al., 2011), three deletions were identified in a total of 1030 patients (plus one relative), but not in 5787 controls ($p = 0{,}00004$). Most noteworthy, the deletion detected by Elia and associates (130_172_3) does not cover coding regions for *BCHE*, but the putative transcriptional control region including putative TFBS. Most interestingly, all of the three deletions harbour the rs4680612 risk variant (Fig. 2). Therefore, both a common variant as well as a rare variant converge to the same locus being associated with ADHD, although penetrance evidently varies between the SNP and the CNV. Nevertheless these preliminary findings strengthen the hypothesis that *BCHE* is associated with ADHD and also provide an example how the integration of different genetic models might produce meaningful results.

According to bioinformatic analyses, the minor risk allele goes along with disruption of a binding site for the MYT1 TF, as it is also the fact for the three described deletions. One can thus hypothesize that both common genetic variation, in the form of rs4680612

Table 3
Biometric analysis of rs4680612, rs829508 and proxy SNPs in high linkage disequilibrium ($D' = R^2 = 1$). Transcription factors expressed in the nervous system are given in bold.

(Proxy) SNP	Position (bp)	Alleles (d/D)	SNP function	Transcription factor (TF)	
				Minor allele (d)	Major allele (D)
5'-Promotor SNPs					
rs4680612	167092125	G/A	Changed		**HOX_PBX.01**
rs9881386	167077527	T/C	–		
rs1438582	167066058	A/G	–		
rs6796150	167062427	A/G	Changed		**MYT1.01**
rs957049	167056563	T/C	Changed		SP2.01
rs6445089	167055091	A/G	–		
rs6771817	167039794	A/C	–		
rs1398088	167038682	A/G	–		

Proxy SNP	Position (bp)	Alleles (d/D)	SNP function	Splice sites and splicing regulatory elements (SREs)	
				Minor allele (d)	Major allele (D)
SNPs (Intron 3)					
rs829508	166985418	C/T	Changed		Splicing Silencer (hnRNP A1)
			Changed		Splicing Silencer (Sironi's Motif 3)
			Changed		Splicing Silencer (Sironi's Motif 1)
rs829500	166985418	T/C	Changed	Splicing Enhancer (9G8)	Splicing Silencer (PESS Octamer)

(minor allele) as well as rare events (CNV) go along with disruption of MYT1-regulated *BCHE* expression. Most interestingly, MYT1 can only be found in differentiating embryonic neurons (Bellefroid et al., 1996; Kim et al., 1997) and has previously been associated with intellectual disability (Kroepfl et al., 2008), as has been its analogue MYT1L (Stevens et al., 2011; de Ligt et al., 2012). Dysregulation of *BCHE* in the developing brain due to compromised MYT1 binding therefore might be a genomic risk factor to develop later life ADHD. Whether or not changes in *BCHE* expression levels in the developed brain still contribute to ADHD pathogenesis is as yet unclear; however, it would be worthwhile to test for this as BCHE is a drugable target.

Variations in BCHE activity could influence the cholinergic system in general. Central cholinergic pathways are one of the most important modulatory neurotransmitter systems in the brain, with acetylcholinesterase (ACHE) as one of the most prominent constituents. In humans, ACHE is more abundant in the CNS, while BCHE is more abundant in serum which however nevertheless is present in all tissues, including nervous system (Mack and Robitzki, 2000). Surrogate ACHE and BCHE enzyme activity extended to all parts of the brain receiving cholinergic innervation, and hence it can be presumed that BCHE is involved in regulating the cholinergic tone in the CNS at least under some circumstances.

Interestingly, there are several links between cholinergic dysfunction and endophenotypes of ADHD. Cortical cholinergic neurotransmission and activity of thalamic pathways and regulate sensory gating and spatial attention (Sarter and Bruno, 1994; Perry et al., 1999). Findings from both animal and human studies therefore suggest that central muscarinic and nicotinic mechanisms are likely to contribute to the psychopathology of ADHD and cognitive deficits. In line with this assumption, polymorphisms and/or CNVs of neural nicotinic acetylcholine receptor α4 subunit (*CHRNA4*) and neural nicotinic acetylcholine receptor α7 subunit (*CHRNA7*), respectively, have been associated with ADHD (Kent et al., 2001; Wallis et al., 2009; Williams et al., 2012).

Furthermore, allelic variations of *BCHE* could influence the balance between cholinergic system and dopaminergic system. Activity of striatal principal neurons that play a major role in movement control is modulated by dopaminergic and cholinergic neurotransmission (Kaneko et al., 2000). The functionally significant relevance of the nicotinic system and its interaction with dopaminergic neurotransmission is supported by the fact that nicotine improves attention in adults with aADHD, smokers and non-smokers going along with promoting the release of dopamine.

In addition, there is evidence for gene × gene interactions of polymorphisms in the apolipoprotein E (*APOE*) and *BCHE* genes, which influence personality traits, markers of stress and cognitive function (Fiocco et al., 2009): Participants carrying the E4 variant of the APOE gene (APOE E4) and the K variant of the *BCHE* gene (*BCHE* K) (*APOE* E4-*BCHE* K group) report lower self-esteem and higher levels of depression. In addition, the *APOE* E4-*BCHE* K group featured increased basal levels of glucocorticoids when compared to non-carriers of these variants which might underlie depressive symptoms (Gałecka et al., 2013). Whether or not this also contributes to the association with AADHD, is unclear, however is possible as aADHD patients have increased co-morbidity with mood and anxiety disorders (Jacob et al., 2007). It is known that aADHD goes along with an increase in co-morbid conditions such as mood and anxiety disorders. While we have previously determined the rate of such co-morbid disorders in our discovery sample (anxiety disorders lifetime, 27.2%, mood disorders lifetime, 57.3%%; Substance use disorders lifetime, 45%) (Jacob et al., 2007), we did not assess whether BCHE conveys genetic risk to “pure” ADHD or rather co-morbid conditions. However, one might assume that an imbalance in the stress hormone system not only impacts on stress-related disorders in a narrower sense, but probably also on ADHD persistence (Corominas et al., 2012).

4.1. Limitations

Beside *BCHE*, the 3q26 deletion described by Lesch et al. comprising an interval of 2 Mb involves at least four more genes, namely *ZBBX* (B-box domain containing zinc-finger protein), *WDR49*, *SERPINI2* (serpin peptidase inhibitor) and *PDCD10* (programmed cell death protein 10, disrupted by deletion of 4 exons at the 3′ end). Therefore *BCHE* is not the only possible candidate gene, although the Elia et al. CNV dataset (as the CNV affecting the *BCHE* transcriptional control region does not affect other genes) and single marker analysis argue in favor of our hypothesis. However, *SERPINI1* displays decreased gene expression in schizophrenia (Vawter et al., 2004), and therefore changes in *SERPINI2* gene dosage still might affect ADHD risk in a similar manner.

Despite these limitations, the present study provides converging evidence for an implication of *BCHE* in the molecular pathology of ADHD. In order to refute the possibility of false positive associations, the presented results need to be independently replicated in larger samples, especially as we did not find association of our best finding in an independent sample of aADHD patients, probably owing to the “winner’s curse”, phenotypic heterogeneity or type-I error. Therefore, additional work is required to clarify the role of *BCHE* deletions and common genetic variations in the context of ADHD.

Contributors

C.J., H.W., K.-P.L. and A.R. planned and designed the study. H.W. and S.K.S carried out the genetic analyses. J.H., C.J. and W.R. diagnosed and characterized the participants. C.J, H.W., K.-P.L., and A.R. analysed and interpreted the data. C.J., H.W. and A.R. drafted the manuscript. All authors contributed to and have approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2013.08.006.

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