Functional Impact of An ADHD-Associated *DIRAS2* Promoter Polymorphism

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The *DIRAS2* gene is coding for a small Ras GTPase with so far unknown function. In a previous study, we described the association of *DIRAS2* rs1412005, as well as a haplotype containing this polymorphism and located in the promoter region of this gene with attention-deficit/hyperactivity disorder (ADHD). In the present study, we searched for rare variants within or near the *DIRAS2* gene that might be associated with ADHD using next-generation sequencing. As we were not able to detect any rare variants associated with the disease, we sought to establish a functional role of *DIRAS2* rs1412005 on the molecular or systems level. First, we investigated whether it has an influence on gene expression by means of a luciferase-based promoter assay. We could demonstrate that the minor risk allele goes along with the increased expression of the reporter gene. Next, we aimed to identify differences in response inhibition between risk-allele and non-risk allele carriers in children suffering from ADHD and healthy control individuals by analyzing event-related potentials in the electroencephalogram during a Go/NoGo task. Risk-allele carriers showed a changed NoGo anteriorization. Therefore, our results suggest an impact of the investigated polymorphism on the prefrontal response control in children with ADHD. These results imply that the promoter polymorphism is indeed the associated as well as in itself a causal variant. Further research is thus warranted to clarify the mechanisms linking *DIRAS2* to ADHD.

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INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is neurodevelopmental in nature and characterized by inattention, hyperactivity, and increased impulsivity. The disorder is common among children, with an estimated prevalence of up to 5% (Polanczyk *et al*, 2007), and over the last decades, it has become evident that a majority of the patients experience significant symptoms also in adulthood (Faraone *et al*, 2005). The disorder is frequently observed to aggregate in families, and the heritability of childhood ADHD (cADHD) has been estimated to around 76%, giving evidence of a strong genetic component (Faraone *et al*, 2005). Many genetic loci have been reported to be associated with ADHD, but only few of these findings have been successfully replicated. Accordingly, overall findings are inconsistent, and most of the genetic contribution remains to be explained (Franke *et al*, 2009; Gizer *et al*, 2009).

One of the genes that have shown promising results as ADHD risk candidates is the *GTP-binding Ras-like protein 2 gene (DIRAS2)*. Attention was first drawn to this gene when its locus on chromosome 9q22.2 was implicated in two cADHD-linkage scans from Germany (Romanos *et al*, 2008) and the UK (Asherson *et al*, 2008). Further corroborating evidence came from our previous genome-wide association study (GWAS) on adult ADHD (aADHD) patients, recruited from the same catchment area as the German linkage study (Lesch *et al*, 2008). Two of the 500 top ranking single nucleotide polymorphisms (SNPs) were located in an intergenic region on chromosome 9q22.2. The neighboring gene found most likely to be involved in the aetiopathogenesis of ADHD was *DIRAS2*, because, in contrast to the other neighbor (*SYK*), it is expressed in the brain (Kontani *et al*, 2002). On the basis of these reports, we further examined a potential association between tagging SNPs in *DIRAS2* and aADHD in a German sample of 600 patients and 420 controls, as well as European replication sample of 1035 patients and 1381 controls (Reif *et al*, 2011). Four SNPs and two haplotype blocks showed significant results ($p < 0.05$) in

the German sample. Meta-analyses with the replication sample showed consistent and significant effects for the promoter SNP rs1412005 (odds ratio (OR) = 1.12; $p = 0.04$), and for a haplotype comprising six SNPs including the risk allele of rs1412005 (OR = 1.45; $p = 0.0003$).

In the present study, we follow up these previous findings with the objective of coming closer to an understanding of the mechanisms behind the observed associations between ADHD and *DIRAS2*. Our first aim was to investigate whether the promoter SNP rs1412005 is indeed the causal variant, or if the reported association is rather a 'synthetic association' observed due to rare causal variants located in same haploblock as the common variant (Dickson *et al*, 2010). To search for unknown and rare (minor-allele frequency (MAF) < 0.05) variants, we used next-generation sequencing (NGS) technology on DNA from aADHD patients and controls. To test for a regulatory function of the SNP on gene expression, we performed a luciferase reporter assay. To study the functional impact of the polymorphism on the systems level, especially during neurodevelopment (and assuming a stronger functional effect in children), we explored whether the *DIRAS2* rs1412005 risk variant is also associated with the altered response inhibition, a putative endophenotype of ADHD. To do so, we measured inhibitory performance and alterations in brain activation patterns by analyzing event-related potentials (ERPs) in the electroencephalogram (EEG) of 62 children suffering from ADHD, as well as 36 matched controls during a Go/NoGo task (Rosvold *et al*, 1956).

MATERIALS AND METHODS

Next-Generation Sequencing

Samples. The patient sample consisted of 300 aADHD patients (99 males and 201 females; 176 combined, 97 inattentive, and 21 hyperactive-impulsive subtype) ascertained at the Department of Psychiatry, University of Würzburg. The age at inclusion ranged from 18 to 70 years (mean = 33 years; SD = 9.8). The inclusion criteria was aADHD according to the DSM-IV criteria; onset before 7 years of age via retrospective diagnosis, life-long persistence and current diagnosis, and additionally an intelligence quotient (IQ) level > 80 and absence of bipolar affective disorder. DNA from 300 healthy individuals (98 males and 202 females) recruited from the Lower Franconia Area of Germany served as the control sample. Controls were matched with the patients for sex and the mean age at recruitment was 24 years (SD = 4.2). All included individuals were Caucasians. Written informed consent was obtained from all participants and the study was approved by the Ethics Committee of the University of Würzburg. The overall sample has previously been described in greater detail (Franke *et al*, 2009; Jacob *et al*, 2007).

DNA amplification and sequencing. Equimolar amounts of DNA from either 50 patients or 50 controls were pooled prior to the amplification. The exons of the *DIRAS2* gene including exon–intron boundaries and untranslated regions including the promoter region, were amplified by polymerase chain reactions (PCRs) using either the iProof High Fidelity DNA Polymerase (Bio-Rad Laboratories, München, Germany) or the SequalPrep Long PCR Kit

from Invitrogen (Invitrogen; see Supplementary Table 1 for primer sequences). Equimolar amounts of the amplicons from two patient or control pools were combined. Samples were prepared for the sequencing run performed on the Life Technologies SOLiD 3+ sequencer following the manufacturer's instructions. Bead production and enrichment was carried out manually as a full-scale reaction. For the emulsion PCRs, a final concentration of 0.5 pM of each library was used as DNA template. Bead enrichment resulted in 87 to 110 million beads per library. The SOLiD sequencing run was performed using SOLiD Opti sequencing chemistry for a single F3 Tag with a read length of 50 bp.

Data analysis: variant detection and annotation. The raw reads generated by the SOLiD run underwent a two-step quality control (QC) procedure. First, reads were trimmed dynamically, eliminating all bases succeeding and including the fifth base of insufficient quality (phred-like quality value ⩽ 10). Second, trimmed reads consisting of < 30 bases in length were excluded. The reads overcoming the QC were aligned using BWA version 0.6.2 (Li and Durbin, 2009) to chromosome 9 of the human genome (hg19), allowing a maximum of four mismatches. For additional confirmation, we used SHRIMP version 2.2.2 (David *et al*, 2011) as the alternative alignment strategy. To perform single nucleotide variation (SNV) calling, we used vipR (http://sourceforge. net/projects/htsvipr/) (Altmann *et al*, 2011). vipR compares the number of base calls for the minor allele at a given position obtained from a given pool with the minor allele calls at the same position obtained from the remaining pools. Using the Skellam distribution, vipR assesses the likelihood of the base calls originating from sequencing errors. Only if the likelihood is sufficiently small, the minor allele at that position is reported as a SNV. Only positions with coverage of at least 5000 at a given base position in each pool were included for variant calling, resulting in an exclusion rate of 10% of the sequenced region. SNV annotation was performed using ANNOVAR (Annotation Variant; http:// www.openbioinformatics.org/annovar/) (Wang *et al*, 2010).

Genotyping and replication of sequencing results. To validate the results of the SOLiD sequencing, non-pooled DNA was genotyped using TaqMan Assays (Life Technologies) following the manufacturer's instructions and, in one case where this was not feasible (rs10993608), using Sanger sequencing (Eurofins, Ebersberg, Germany). Primers and sequences are available on request. Genotyping of the *DIRAS2* SNP rs1412005 was performed with a Taq-Man assay (assay ID **C___**3069317_10) according to the manufacturer's specifications (Applied Biosystems) with a call rate of 93.3%. We analyzed the data using a dominant model, comparing risk-allele carriers (genotypes TT and GT) and participants without the risk allele (genotype GG).

Luciferase Reporter Assay

Complementary oligonucleotides containing the risk or the non-risk allele of the promoter SNP, and a HindIII or a NheI restriction enzyme overhang (Supplementary Table 1 for the sequences) have been annealed and cloned into the pGL4.23 minimal promoter firefly luciferase 2 gene vector

(Promega, Mannheim, Germany). The human neuroblastoma cells SH-SY5Y (European Collection of Cell Cultures, Salisbury, UK) were seeded at a density of 5×10^4 cells/well into 24-well culture plates. Twenty-four hours after seeding, cells were transfected with 75 ng of the pGL4.74 vector (Promega, Mannheim, Germany) coding for the renilla luciferase under the control of a HSV-TK promoter and with 675 ng of the pGL4.23 constructs following the TransFast Transfection Kit protocol (Promega, Mannheim, Germany). Forty-eight hours post transfection, the luciferase activities were measured on the GloMax Multi+ Detection System luminometer (Promega, Mannheim, Germany). Measurements were carried out in triplicates. Each experimental assay was performed three times.

Background signals were subtracted from the raw data, and the quotients between firefly and renilla luciferase activity were calculated for normalization. Z-scores were calculated for the minor and major alleles, adjusting for daily variations between the measurements. Finally, a two-sided Welch's two-sample *t*-test was performed using the software R version 2.15.1 (www.r-project.org).

Transcription factor-binding sites that would be altered by the presence of the different nucleotides at rs1412005 were searched using the FuncPred tool (http://manticore.niehs.nih.gov). With the aim of examining if the reported transcription factors are actually expressed in SH-SY5Y cells, an expression study was performed. RNA was isolated from SH-SY5Y cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reversely transcribed into complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative real-time PCR was run on a CFX384 Real-Time PCR Detection System using iQ SYBR Green SuperMix (Bio-Rad Laboratories) and Quanti-Tect Primer Assays (Qiagen, Hilden, Germany).

Functional Correlates in the EEG

Sample. The investigated sample comprised 62 cADHD patients of which 55 had the combined subtype and 7 the inattentive subtype of the disease. Of these 62 patients, 30 were unmedicated (mean age = 10.06 ± 0.48 years; age range 5–17 years; 12 females and 20 males; 4 inattentive subtype; 26 combined subtype), whereas 32 received psychostimulants (mean age = 10.52 ± 0.42 years; age range 7–16 years; 6 females and 25 males; 3 inattentive subtype; 29 combined subtype). The patients were recruited from the Clinic for Child and Adolescent Psychiatry at the university hospital in Würzburg. The control group comprised 36 matched controls without psychiatric morbidity (mean age 10.86 ± 0.56 years; age range 6–17; 18 females and 18 males). Exclusion criteria were IQ < 70 for all the participants, and for controls, the presence of psychiatric disorders, whereas for ADHD patients, patients with conditions that interfered with EEG recordings, like Tourette's syndrome, severe motor tics, or epilepsy, where excluded.

Cued continuous performance task. Participants were seated in front of a computer screen in a quiet darkened room and were instructed to press a button as quickly as possible whenever the letter O was followed by the letter X (Go trials), but to not react to any other combination of the

stimuli. Stimuli were presented for 200 ms, with an interstimulus interval of 1650 ms.

EEG recording. We recorded a 21-channel EEG placed according to the international 10-20 system (Fp1, Fpz, Fp2, F3, Fz, F4, F7, F8, T3, T4, C3, Cz, C4, T5, T6, P3, Pz, P4, O1, Oz, O2; (Jasper, 1958)) with a 32-channel DC amplifier (Brain-Star System, Erlangen, Germany) and the software Vision Recorder (Brain Products GmbH, Munich, Germany) at a sampling rate of 1000 Hz, a bandpass between 0.1 and 100 Hz, and a 50 Hz notch filter. The recording reference was placed between Fz and Cz.

Data analysis. For offline data analysis, the Vision Recorder software (Brain Products GmbH, Munich, Germany) was used. A band-pass filter (0.1–50 Hz) was applied and the data were re-referenced to an average reference. Blink detection was accomplished with the algorithm by Gratton and Coles (Gratton and Coles, 1989). After segmentation into Go (correct response) and NoGo (successful inhibition) trials, segments containing signals exceeding $\pm 100 \, \mu V$ were rejected, leaving a minimum of 15 valid epochs. P300 peaks were semiautomatically determined at Pz for Go and at Cz for NoGo in the Global Field Power ERP. The location of the centre of the brain-electric field (centroid) was calculated with the weighted centroids method (Lehmann and Skrandies, 1980) for that precise point in time. NoGo anteriorization (NGA) was computed by subtracting the NoGo from the Go centroid location, with higher values indicating better anteriorization.

For statistical analyses, we used SPSS version 18. As preliminary results indicated strong effects of *DIRAS2* genotypes in an ADHD sample, we conducted separate analyses for functional *DIRAS2* effects on ADHD children and adolescents (mean age 10.38 ± 3.0 years), and matched healthy controls (mean age 10.81 ± 0.54 years) without psychiatric morbidity. As behavioral parameters were not normally distributed, they were analyzed nonparametrically with Kruskal–Wallis or Wilcoxon's tests, respectively, whereas all electrophysiological data were examined in analyses of variance with a significance level of $p = 0.05$.

RESULTS

Sequencing

Pooled NGS revealed eight variants within the *DIRAS2* coding sequences or flanking regions to differ significantly between ADHD patients and healthy controls ($0.047 < p < 4.2 \times 10^{-5}$). The positions of the variants on chromosome 9, minor and major alleles, MAFs in patients and controls, and *p*-values are given in Table 1. rs10993608 was especially intriguing, as the minor allele was exclusively present in ADHD patients according to the NGS data.

To verify the sequencing results, the DNA of ADHD patients and controls that had been sequenced, was individually genotyped using TaqMan probes specific for six of the eight identified variants (rs10993608, rs80274505, rs6479611, and variants at positions 93402517, 93409910, and 93410462; the remaining two were omitted owing to their borderline significance not surviving correction for multiple testing). The chromosomal positions of the investigated variants and

Table I Positions of the Variants that Differ Significantly Between ADHD Patients and Healthy Controls Within or Near the *DIRAS2* Gene on Chromosome 9, Minor and Major Alleles, Minor Allele Frequencies (MAF) in Patients and Controls, and *p*-values

Chromosomal position	Region	SNP ID	REF	ALT	MAF cases	MAF controls	P-value
93369678	Downstream	rs10993608	G	A	0.076	0.001	0.000042
93371890	Downstream	rs80274505	T	G	0.002	0.036	0.023378
93402517	Intronic	—	T	A	0.756	0.617	0.003869
93403987	Intronic	—	G	A	0.374	0.267	0.047137
93404249	Intronic	rs6479611	A	G	0.431	0.610	0.000253
93409910	Upstream	—	T	C	0.541	0.659	0.032950
93410462	Upstream	—	G	A	0.796	0.637	0.000325
93410463	Upstream	—	C	T	0.728	0.618	0.041002
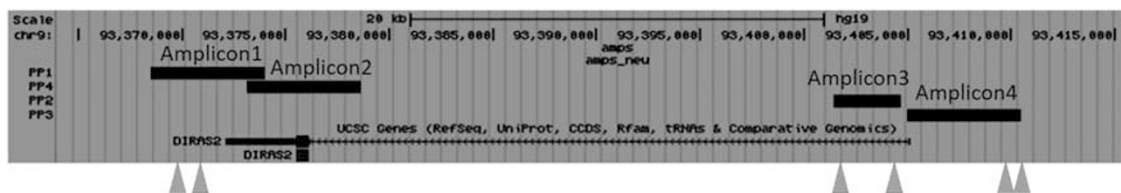
Figure I Schematic of the *DIRAS2* gene taken from the UCSC Genome Browser (http://genome.ucsc.edu/) showing the positions of the amplicons used for NGS (black bars) and the variants investigated in the replication (red triangles). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

an alignment of the amplicons generated for the NGS are shown in Figure 1. The TaqMan assays for rs10993608 and the variant at position 93410462 failed to produce any reliable results. The four assays genotyping rs80274505, rs6479611 and the variants at positions 93402517 and 93409910 however generated reliable data. In contrast to NGS results, genotypes were not significantly different between the cases and the controls. As the genotyping assay for rs10993608 failed, DNA fragments containing that variant were additionally investigated by Sanger sequencing. The minor allele suggested by NGS could not be detected. Also, the total count of minor alleles was not significantly higher for ADHD cases than for controls when performing individual genotyping (data not shown). As we could not detect rare variants associated with ADHD, we continued to study the functional effects of rs1412005 on the molecular and systems level.

Luciferase Assay

The firefly luciferase activity measurements were in the range 0.4×10^5–2.7×10^5 relative luminescence units after subtraction of the background signals. The *t*-test for the *z*-score of the luminescence by rs1412005 risk allele gave a *t*-value of -5.648, and the corresponding *p*-value for the difference between the constructs containing the major and minor allele was 4.2×10^{-5} (Figure 2).

Using the National Institute of Environmental Health Sciences homepage's SNP function prediction tool, rs1412005 was found to alter the binding sites of the androgen receptor, the hepatocyte nuclear factor 1 homeobox A (HNF1A), the cyclooxygenase-1 (COX1), the paired box protein Pax-6, cut-like homeobox 1 (CUX1), and the peroxisome proliferator-
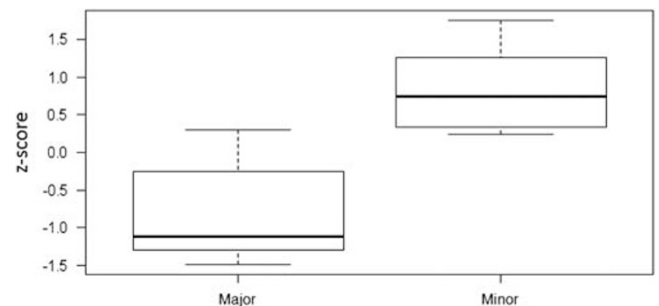

Figure 2 Boxplot showing the combined *z*-scores for the three luciferase activity measurements in SH-SY5Y cells of the *DIRAS2* SNP rs1412005. The luciferase activity is significantly higher ($p = 4.207 \times 10^{-5}$) in cells transfected with the constructs containing the minor allele of the *DIRAS2* promoter polymorphism.

activated receptor gamma (PPARγ). When analyzing cDNA from SH-SY5Y cells, we found that *PPARγ* and *CUX1* were both expressed in these cells, whereas no expression of the other transcription factors was found.

Response Inhibition: Behavioral and ERP Data

Behavior. *DIRAS2* genotype alone did not affect errors or reaction times in total sample or in individual diagnostic groups (all *p*-values > 0.240).

Centroids. The repeated measures ANOVA comparing locations of Go and NoGo centroids for control subjects only yielded a main effect for condition, signifying more anterior centroids for NoGo trials ($F_{1, 34} = 10.240$, $p = 0.003$). In the ADHD group, this main effect ($F_{1, 58} = 9.341$,
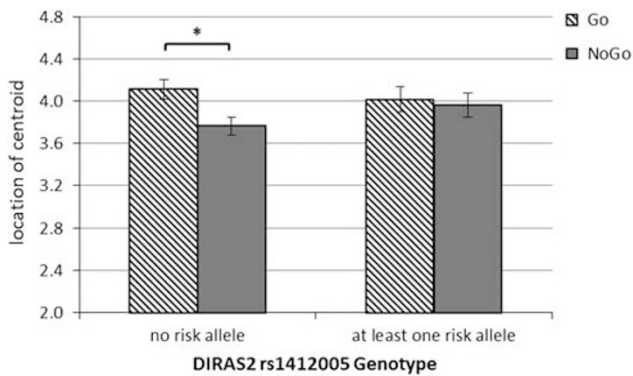
Figure 3 Position of the centroids in the Go/NoGo task in risk-allele carriers and non-risk allele carriers. In individuals lacking the risk allele of the *DIRAS2* promoter polymorphism, no NoGo anteriorization could be found. Statistically significant difference is indicated by an asterisk (p = 0.001).

$p = 0.003$) was a result of an interaction of *DIRAS2* genotype and condition ($F_{1, 58} = 5.917$, $p = 0.018$). The difference between conditions in terms of centroid location was only observed in rs1412005 non-risk allele carriers ($p = 0.001$; $n = 32$), but not in the risk-allele group ($p = 0.539$; $n = 30$; Figure 3). Testing for a group effect comparing medicated with unmedicated patients revealed no differences between groups ($p = 0.484$).

NoGo anteriorization. We found a main effect for *DIRAS2* genotype in ADHD children ($F_{1,58} = 5.917$, $p = 0.018$), but not in the control group ($p = 0.869$). ADHD individuals with at least one risk allele had a dampened NGA compared with those without the risk allele. The medication status did not influence NGA in patients ($p = 0.986$).

DISCUSSION

We here followed up on previous reports of *DIRAS2* as an ADHD candidate gene, with the aim of better understanding the underlying mechanisms of the observed associations, particularly regarding the promoter SNP rs1412005. Our results show that the rs1412005 risk allele results in significantly higher gene expression as measured by luciferase activity. We found no rare variants in neither the coding nor the flanking regions of *DIRAS2* likely to better explain the previously observed association signal. Further, we observed that the risk allele is associated with a diminished NGA in ADHD, despite normal behavioral performance.

Notwithstanding the results of our previous association studies, the possibility remains that there are rare variants in linkage disequilibrium with rs1412005, which are truly causal and thus represent what has been called 'synthetic associations' (Dickson *et al*, 2010). However, the idea that those synthetic associations caused by rare variants could explain the majority of GWAS results has been questioned (Wray *et al*, 2011) by reanalyzing real life GWAS data and by running additional simulations. Nevertheless, we first performed a NGS experiment yielding eight promising variants that were associated with disease. None of the association signals however survived replication attempts suggesting that these were false-positive results, emphasizing the need for verification of NGS data. As PCR amplification

was used to generate specific DNA fragments for the sequencing, false positives could be introduced due to misincorporation of bases during this process. Such problems could be overcome by designing overlapping amplicons and accepting only consensus-variant calls (Meldrum *et al*, 2011). However, this strategy would lead to a considerable cost increase. As the patient and the control sample consisted of only 300 individuals each, we can evidently not rule out that there could be even rarer variants or variants in other populations. Nevertheless, our SNP association finding on rs1412005 would still be reliable. Thus, with a high level of confidence, we conclude that indeed rs1412005 is the causal variant associated with aADHD in our previous studies.

To establish a functional role for rs1412005, we performed a luciferase assay and demonstrated increased reporter gene expression when the minor allele was present, suggesting that the risk allele may lead to higher *DIRAS2* expression. However, a luciferase promoter assay performed in a human cancer cell line only provides limited information of the processes in the human organism. Also, the association of *DIRAS2* with aADHD is not only based on the promoter polymorphism as such, but also on the haplotype block containing it (Reif *et al*, 2011). As this haploblock spans > 5 kb, it was not possible to clone the entire region into the luciferase reporter vector to test for expression changes owing to the full risk haplotype. As *DIRAS2* is not expressed in human blood cells, attempts to determine *DIRAS2* expression levels in human samples with the relevant genotypes did thus far not lead to reliable results.

According to the SNP function prediction tool used for detecting changes in transcription factor-binding sites, the promoter polymorphism may lead to altered binding sites of six transcription factors. However, only two of these, *PPARγ* and *CUX1*, were found to be expressed in the brain and also in the SH-SY5Y cells used for the luciferase assay, and therefore may be involved in the observed expression changes. To investigate whether *PPARγ* or *CUX1* are crucial for the regulation of *DIRAS2* expression, a knockdown of the transcription factors followed by luciferase assays will be performed in future experiments, thereby clarifying the molecular cascade leading to differential *DIRAS2* expression.

The impact of the possible changes in *DIRAS2* gene expression is difficult to predict, as the functions of the gene, or the Di-ras2 protein respectively, and the pathways via which they operate, are generally unknown. Di-ras2 is member of the Ras superfamily of small guanosine triphosphatases. Other members of the Ras superfamily are involved in the regulation of cell functions such as proliferation and differentiation (Wennerberg *et al*, 2005). If Di-ras2 possesses similar functions, this could be important in relation to ADHD being a neurodevelopmental disorder.

DIRAS2 has been found to be almost uniquely expressed in the brain. The expression is reported to be strongest in the cerebral cortex, the occipital pole, the frontal, and temporal lobes and the cerebellum (Kontani *et al*, 2002). Among these regions, for instance, the prefrontal cortex and the cerebellum are known to constitute core parts of the network regulating impulsive decision making and behavior, which are traits affected in ADHD. In line with this, several studies have suggested that Di-Ras2 is involved in cognitive processes and behavior. Seshadri *et al* (Seshadri *et al*, 2007) found *DIRAS2* to be associated with the performance on the

Boston naming test in a GWAS of the ageing brain and Wylie *et al* (Wylie *et al*, 2010) found a 13-fold increase in *Diras2* expression in serotonergic neurons from the caudal hindbrain of mouse embryos, which could be indicative of a role of Di-ras2 in the regulation of the caudal serotonergic system. The latter is intriguing, as serotonin has been found to be critically involved in brain development and to impact on ADHD-related traits such as aggression and impulsivity (Azmitia, 2001; Halperin *et al*, 1994; Lucki, 1998).

We also strived to study a potential functional role of the *DIRAS2* rs1412005 risk variant on the systems level, ie, to test for an association with functional deficits known to be frequently present in ADHD representing putative endophenotypes of the disease. Inhibition of inappropriate responses is an often reported deficit associated with ADHD (Willcutt *et al*, 2005) and has the potential to explain a range of overt behavioral symptoms. In the highly structured setting of a laboratory experiment, however, ADHD does not always manifest in behavioral changes in this experiment (ie, increased error rates (Draeger *et al*, 1986)). Nevertheless, analysis of brain functioning during neuropsychological tasks may reflect subtle deficits underlying response control also in the absence of behavioral consequences (Fallgatter *et al*, 2005).

The NoGo task requires inhibitory control from the prefrontal cortex and deficits should be reflected in the number of false alarms in the behavioral output (errors of commission). NGA may be an indicator of prefrontal functioning, and in ADHD, a diminished NGA and corresponding impaired inhibitory performance on the Continuous Performance Test has been reported in both children (Fallgatter *et al*, 2004) and adult samples (Fallgatter *et al*, 2005).

On the electrophysiological level, *DIRAS2* had functional implications in patients afflicted with ADHD. Carrying the risk genotype went along with impaired prefrontal response control, as indicated by the absent anteriorization of the brain's electrical field when a prepared response has to be actively inhibited (NGA). Notably, the *DIRAS2* genotype did not affect behavioral outcomes such as errors rates or reaction times in this group. This moderating influence of *DIRAS2* is absent in controls, where only the condition (Go *vs* NoGo), but not genotype (Figure 3) influenced brain activation in accordance with previous publications (Dresler *et al*, 2010). Response inhibition is closely linked to the right inferior frontal cortex (Aron and Poldrack, 2005), and the origins of the NGA as an electrophysiological correlate of prefrontal function have been traced back to the anterior cingulate cortex (ACC), a brain region that is known for its role in a wide range of cognitive and emotional tasks. Several studies could show structural as well as functional alterations in the ACC of individuals suffering from ADHD (Cubillo *et al*, 2012). As *DIRAS2* is prominently expressed in this brain region (Allen brain atlas, http://www.brain-map.org/) and the promotor polymorphism seems to have an impact on *DIRAS2* expression levels, its expressional changes in interaction with structural alterations associated with ADHD ultimately might lead to the development of ADHD.

Taken together, the results from the present study imply that rs1412005 in the *DIRAS2* gene is in itself a causal variant, and that it is not only functional on the molecular, but also the inhibition-related brain activation level, the latter specifically in patients suffering from ADHD. This warrants further investigations on the mechanisms mediating between *DIRAS2* genotype and ADHD.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)