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ORIGINAL PAPER

Genetic Evidence for the Association of the Hypothalamic–Pituitary–Adrenal (HPA) Axis with ADHD and Methylphenidate Treatment Response

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Abstract Exposure to stressors results in a spectrum of autonomic, endocrine, and behavioral responses. A key pathway in this response to stress is the hypothalamic–pituitary–adrenal (HPA) axis, which results in a transient increase in circulating cortisol, which exerts its effects through the two related ligand-activated transcription factors: the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Genetic polymorphisms in these receptors have been shown to influence HPA axis reactivity, and chronic dysregulation of the HPA axis has been associated with the development of several psychiatric disorders. The objective of the study was to test the association between four functional polymorphisms in *NR3C1* (encoding GR: ER22/23EK-rs6189, N363S-rs6195, *Bcl*I-rs41423247, A3669G-rs6198) and two in *NR3C2* (encoding MR: 215G/C-rs2070951, I180 V-rs5522) with childhood ADHD. Family-based association tests (FBAT) were conducted with the categorical diagnosis of ADHD, behavioral and cognitive phenotypes related to ADHD, as well as with treatment response assessed in a 2-week, double-blind, placebo-controlled trial with methylphenidate. A specific haplotype (G:A:G:G; ER22/23EK- N363S-*Bcl*I- A3669G) of *NR3C1* showed a significant association with behaviors related to ADHD (particularly thought and attention problems, aggressive behavior), comorbidity with oppositional defiant disorder, and executive function domains. An association was also observed with treatment response (assessed by the Conners'-Teachers and Restricted Academic Situation Scale). In contrast, MR gene polymorphisms were not associated with any of the variables tested. To the best of our knowledge, this is the first report showing an association between functional polymorphisms in *NR3C1* and ADHD, providing genetic evidence for involvement of the HPA axis in the disorder and treatment response.

Keywords Attention-deficit/hyperactivity disorder (ADHD) · HPA axis · Cortisol · Glucorticoid receptor · Mineralocorticoid receptor · Methylphenidate

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is the most common psychiatric disorder in children, occurring in 8–12 % of the population (Biederman and Faraone 2005).

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ADHD has a heterogeneous clinical expression, with core symptoms of poor sustained attention, impulsiveness, hyperactivity, and accompanying cognitive deficits. The disorder has a strong genetic component, with a mean heritability estimate of 76 % (Faraone et al. 2005). It has been suggested that multiple genes are involved in ADHD, each accounting for a small portion of increased risk (Biederman and Faraone 2005). Additionally, environmental factors and gene-environment interactions are thought to play a pivotal role in the disorder (Stergiakouli and Thapar 2010).

Several studies have suggested that ADHD is associated with dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, a cornerstone of the neuroendocrine system which controls reactions to stress (Corominas et al. 2012). The perception of a physical or psychological threat activates the HPA axis, resulting in the release of glucocorticoid hormones (mainly cortisol in humans) from the adrenal glands into the bloodstream. Cortisol then acts on the hypothalamus, pituitary, and hippocampus to inhibit its own production in a negative feedback loop. It has been reported that children with ADHD have a blunted cortisol response to psychosocial stressors, a decreased cortisol awakening response (CAR), or lower plasma daytime cortisol (King et al. 1998; Ma et al. 2011; Randazzo et al. 2008; Scerbo and Kolko 1994; van West et al. 2009), though there is considerable inconsistency between studies (Freitag et al. 2009; Hastings et al. 2009; Snoek et al. 2004).

Cortisol exerts its effects through the two related receptors: the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). GRs are widely expressed in the brain, in contrast with MRs, which are restricted to limbic areas (DeRijk and de Kloet 2008). It is well known that stress responsiveness is highly variable among humans (Kudielka et al. 2009), and an inadequate stress response may increase vulnerability for disease (de Kloet et al. 2005; Rohleder et al. 2003). As GR and MR mediate the cortisol signal, variations in the two genes coding for these receptors (*NR3C1* located at 5q31, and *NR3C2* located at 4q31, respectively) have been extensively studied. Four functional single nucleotide polymorphisms (SNPs) in *NR3C1* have been shown to change HPA axis reactivity after a dexamethasone suppression test (DST) or a psychological stressor; these include ER22/23EK -rs6189/6190 (van Rossum et al. 2002), N363S -rs6195 (Wust et al. 2004), *Bcl*I -rs41423247 (Kumsta et al. 2007), and A3669G -rs6198 (Kumsta et al. 2007) (Fig. 1, Table 1). Within *NR3C2*, the 215G/C (rs2070951) and I180V (rs5522) SNPs have been associated with differential response to cortisol in vitro *and* in vitro (Fig. 1, Table 1) (van Leeuwen et al. 2010, 2011). The negative feedback system of the HPA axis is generally tested in the DST wherein the synthetic glucocorticoid dexamethasone (0.25 or 1 mg) is administered at 23h00, and the cortisol levels are measured the next morning (Huizenga et al. 1998). The response of the HPA axis to a psychosocial stressor is traditionally measured using the Trier Social Stress Test (TSST), where an individual is asked to deliver a short speech and perform mental arithmetic in front of a small jury (Kirschbaum et al. 1993). The cortisol levels are measured at specific time points during the task.

The ER22/23EK polymorphisms have been associated with relative glucocorticoid resistance, assessed by non-suppression in DST, and low cortisol response in the TSST (Table 1) (Claes, 2009). Since these two SNPs are in strong linkage disequilibrium (LD), they are usually studied as one polymorphism (Koper et al. 1997). N363S and *Bcl*I, in contrast, show an association with increased cortisol response in the TSST (Table 1) (Claes 2009). With A3669G, the minor allele affects the expression and stability of GR-β isoform in vivo (a transcriptionally less active GR form), resulting in mild corticosteroid resistance (Table 1) (Manenschijn et al. 2009; Russcher et al. 2005). In addition, the ER22/23EK polymorphism has been shown to be in strong LD with A3669G (van den Akker et al. 2006). Although the DST and TSST have provided some insight into the in vivo function of these mutations, their role in complex disorders is still relatively unclear, particularly when these SNPs are considered in conjunction with each other. Due to the pleiotropic nature of regulation via GR, these functional polymorphisms have been associated with diverse disorders including obesity, diabetes, cardiovascular disease, depression, and post-traumatic stress disorder (Bachmann et al. 2005; Manenschijn et al. 2009; van Rossum et al. 2006).

Given the observation that ADHD has been associated with dysregulation of the HPA axis, the objective of this study was to test the association between the functional polymorphisms in *NR3C1* and *NR3C2* and childhood ADHD using a family-based design. To the best of our knowledge, these genetic factors have not been investigated in the context of ADHD. [Since the submission of the manuscript, a study by Kortmann and colleagues reported an association between the *NR3C2* rs5522 SNP and symptoms of inattention and hyperactivity, with *P* between 0.01–0.05 (Kortmann et al. 2012). This study compared adult patients with ADHD, $n = 478$, to healthy controls, $n = 597$]. In addition to DSM-IV diagnosis of ADHD, clinical dimensions of ADHD (child's behavior at home, in school and in the clinic) and measures of executive function (EF) domains were used as quantitative phenotypes in the genetic analysis. It has been suggested that this quantitative trait loci approach may be an important complement to the categorical diagnosis in molecular genetic studies (Thapar et al. 2006). Finally, association with treatment response was tested in a double-blind, placebo-controlled trial with a single dose of methylphenidate (0.5 mg/day).

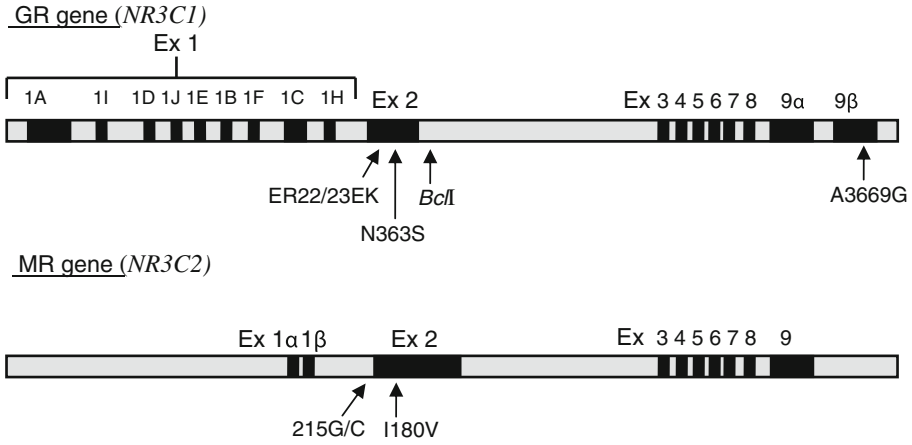


Fig. 1 Schematic representation of human GR and MR gene structure. Translation starts at the beginning of exon 2 and ends in exon 9. In the GR gene, the 5′ region of exon 2, which comprises 9 alternative first exons (exon1A1-1A3, 1B, 1C1-1C3, 1D, 1E, 1F, 1H, 1I, 1J), is part of the promoter region of the gene. It displays alternative splicing within two exon 9 producing either GRα or GRβ (adapted from deRijk and de Kloet 2008)

Table 1 Description of GR (*NR3C1*) and MR (*NR3C2*) SNPs most frequently linked to HPA-axis regulation

SNP	Gene	Location	rs number	Minor allele	Minor allele frequency (%)	In vitro effect of minor allele	Effect of minor allele on HPA-axis function
ER22/23EK	*NR3C1*	Exon 2	rs6189/90	A	±4	↓ trans-activating capacity	GC resistance (i.e., ↓cortisol suppression after DST)
N363S	*NR3C1*	Exon 2	rs6195	G	±4	↑ trans-activating capacity	GC sensitivity (i.e., ↑cortisol suppression after DST)
							↑cortisol after TSST
Bcl1	*NR3C1*	Intron 2	rs41423247	C	±33	Unknown	GC sensitivity (i.e., ↑cortisol suppression after DST)
A3669G	*NR3C1*	3′ UTR of exon 9β	rs6198	G	±19	↑ stability and expression of GR-β isoform	GC resistance (i.e., ↓cortisol suppression after DST) in males
							↑cortisol after TSST
215G/C	*NR3C2*	-2 from exon 2	rs2070951	C	±47	↑ trans-activating capacity	GC sensitivity (i.e., ↑cortisol suppression after DST in males)
I180 V	*NR3C2*	Exon 2	rs5522	G	±11	↓ trans-activating capacity	GC sensitivity (i.e., ↑cortisol suppression after DST in males)
							↑cortisol after TSST

Adapted from Claes 2009 (additional references in the text and in DeRijk et al. 2006 and van Leeuwen et al. 2010, 2011.) *DST* dexamethasone suppression test, *TSST* Trier Social Stress Test, *UTR* untranslated region, *GC* glucocorticoid

Methods

Subjects

Four hundred children between 6 and 12 years of age [mean = 9; SD = 1.8] with a diagnosis of ADHD and their families participated in the study (clinical details have been published earlier) (Grizenko et al. 2006; Sengupta et al. 2012). The study was approved by the Research and Ethics Board of the Douglas Mental Health University Institute (Montréal, Canada). All participating children agreed to take part in the study, and parents provided written consent.

Each child was diagnosed with ADHD according to DSM-IV criteria, based on clinical interviews of the child and at least one parent by a child psychiatrist. Of the total number of affected children, 77.9 % were male and 82.5 % were of Caucasian ethnicity. 54.1 % met DSM-IV criteria for the combined subtype, while 35.6 % and 10.3 % were diagnosed with the inattentive and hyperactive subtypes respectively. Among comorbid disorders, 40.6 % had oppositional defiant disorder, 22.9 % had conduct disorder, 46.2 % had anxiety disorder (including phobias), and 8.8 % had a mood disorder.

Evaluations

The behavior of the child at home and in the classroom was evaluated by the parent(s) and teacher using the Conners' Global Index-Parents (Conners'-P) and Conners' Global Index-Teachers (Conners'-T), respectively (Conners et al. 1998a, b). In addition, the parents were asked to evaluate the behavior of the child using the Child Behavior Checklist (CBCL) (Achenbach 1991). For the sample of

children assessed, the mean (standard deviation) for the total CBCL, CGI-P, and CGI-T scores were: 68.6 (8.9), 73.1 (11.4), and 69.5 (12.7) respectively (scores lower than 65 are considered to be in the normal range).

Besides clinical dimensions of ADHD, neuropsychological measures, particularly measures of EF, were included as quantitative traits in the genetic association analyses. Executive function encapsulates the range of cognitive abilities that are required for completing a given task and include response inhibition, sustained attention, working memory, set-shifting, planning and organization. Deficits in EF have been hypothesized to underlie some ADHD symptoms (Willcutt et al. 2005). The battery of neuropsychological tests included the Wisconsin Card Sorting Test (WCST; measure of cognitive flexibility and set-shifting) (Heaton et al. 1993), Tower of London test (TOL; planning, organization, and problem-solving capacity) (Shallice 1982), Self-Ordered Pointing Task (SOPT; visual working memory, planning and response inhibition) (Petrides and Milner 1982; Shallice 1982), Conners' Continuous Performance Test (CPT; attention, response inhibition, and impulse control) (Conners 1985) and Finger Windows (FW; visual-spatial working memory) (Sheslow and Adams 1990). The WCST, TOL, SOPT, and CPT were performed as described (Gruber et al. 2007; Taerk et al. 2004). FW is a subtest of the Wide Range Assessment of Memory and Learning (WRAML). In this test, the child is required to repeat the sequential placement of a pencil into a series of holes on a plastic card, as conducted by the examiner. When children were medicated prior to their inclusion in the study, the clinical and neuropsychological assessments were carried out at the end of a 1-week washout period to limit variability due to medication effects. In addition to the measures of EF, IQ (full scale, verbal, and performance IQ) was evaluated using the Wechsler Intelligence Scale (WISC-III/IV) (Wechsler 1991). In the IQ, WCST, FW and TOL tests, a higher score indicates a better performance, whereas in the SOPT and CPT, a higher score indicates a worse performance.

Response to treatment with methylphenidate (MPH) was assessed in a double-blind, placebo-controlled, within-subject (crossover) randomized control trial conducted over a two-week period, as described (trial registration number: NCT00483106) (Grizenko et al. 2006). Briefly, all subjects received 1 week of treatment with placebo (PBO) and 1 week of treatment with 0.5 mg/kg of MPH in a divided b.i.d. dose following a wash-out period. At the end of each week of treatment, the parents and teacher were asked to evaluate the behavior of the child using the Conners'-P and Conners'-T respectively. Clinical assessments were performed prior and after the administration of PBO and MPH. In addition, the clinical staff completed the Clinical Global Impression (CGI)-overall improvement scale based on their half day of behavioral observation while the child was completing various tasks in the clinic.

Task-oriented behavior was assessed using the Restricted Academic Situation Scale (RASS). This test was conducted at four time points (before and after administration of placebo and MPH during week 1 and week 2 of the trial); it was not conducted at baseline. RASS is a coding system designed to record the behavior of a child when assigned a set of math problems during a simulated independent academic situation within a clinic setting (Barkley 1990). It is an assessment of the child's ability for sustained attention to routine, repetitive academic work in the presence of potential distractions, with no adult supervision (Fischer and Newby 1998). The task has been described in detail elsewhere (Fischer and Newby 1998; Sengupta et al. 2008). We have previously reported results from principal component analysis of the RASS showing that off-task, out-of-seat, and playing with objects consist of one factor, while vocalizing and fidgeting appear to be independent factors (Karama et al. 2009). The RASS score is the total number of recorded behavioral events, and the difference score was obtained by subtracting the score after MPH administration from the score obtained after PBO.

Genotyping

The affected child, parents and siblings were invited to participate in the genetic component of the study. For each parent and child, DNA was extracted from either a blood sample or saliva sample. A saliva sample was acquired only if the subject refused the blood withdrawal. The study included 377 nuclear families having one or more child with a DSM-IV diagnosis of ADHD. Of the 377, 182 were trios with information from both parents, 51 were trios with information from one parent and one or more unaffected sibling, 121 were duos including the proband and one parent, 11 were families with two parents and two affected children, while 12 were families with two affected siblings and one parent.

A panel of four SNPs in *NR3C1* (rs41423247, rs6195, rs6189, rs6198) and two SNPs in *NR3C2* (rs5522 and rs2070951) was genotyped using SequenomiPlex Gold Technology (Ehrich et al. 2005). Every plate included duplicates of two reference samples used to estimate genotyping error. Genotypes for these samples were read with 100 % accuracy on each of the plates. The genotype frequency did not depart from Hardy–Weinberg equilibrium ($P > 0.01$). The four SNPs within *NR3C1* are in strong LD with each other (Supplementary table 1) as determined in Haploview v4.0 (Stephens et al. 2001) using genotype information in the pedigree file. In addition, a few reports have suggested that these SNPs may form functional haplotypes (van den Akker et al. 2006). These four SNPs were therefore analyzed as a haplotype block.

Statistical Analyses

Family-Based Association Tests (FBAT) were conducted using the FBAT statistical package (version 2.0.3). FBAT represents a unified approach for family-based tests of association (Laird et al. 2000; Rabinowitz and Laird 2000), built on the original Transmission Disequilibrium Test (TDT). The classical TDT considers parents who are heterozygous for a specific allele at a given genetic polymorphism and examines the frequency with which the "risk allele" (i.e., the allele associated with the disorder) is transmitted to the affected offspring (Spielman et al. 1993). For a quantitative trait, if a specific allele is associated with an abnormal level of a trait, it is expected to be transmitted more frequently to the child presenting an abnormal level of that trait. FBAT compares the genotype distribution in the affected offspring ("cases") to the distribution that would be expected given the null hypothesis of "no linkage and no association" (Laird et al. 2000). All analyses were performed under the assumption of an additive model. Given that all SNPs are functional, significance level was set at $P < 0.01$.

Results

First, the association between each of the SNPs in *NR3C1* and *NR3C2* was tested with ADHD as a diagnostic category. Trends for significance (P between 0.01 and 0.05) were noted between each of the four *NR3C1* SNPs and various behavioral and cognitive dimensions (Table 2; Supplementary table 2). Significant association was observed between ER22/23EK (rs6189) and social as well as attention problems on the CBCL, *Bcl*I (rs41423247) and WCST measures (perseverative errors and responses), A3669G (rs6198) and variability of standard error measured on the CPT. In contrast, the two *NR3C2* polymorphisms (rs5522 and rs2070951) did not show any association with ADHD, quantitative behavioral or cognitive measures, nor treatment response.

While the results with the individual SNPs have been presented, haplotype analysis likely provides a clearer picture of how the different functional SNPs interact with each other in children with ADHD. A specific haplotype G:A:G:G (ER22/23EK- N363S- *Bcl*I- A3669G or rs6189- rs6195- rs41423247- rs6198; haplotype frequency = 0.12) showed a trend for association with ADHD, as well as with number of hyperactivity, impulsivity and conduct disorder items on the DISC-IV (Table 2; Supplementary table 2). However, a highly significant association was observed between this haplotype and number of oppositional defiant disorder items recorded on the DISC ($P = 0.002$), total CBCL score ($P = 0.001$), and both the CBCL factor scores (internalizing behavior, $P = 0.009$ and externalizing behavior, $P = 0.0003$). When the different CBCL dimensions were examined, the most significant associations were noted for thought ($P = 0.002$) and attention problems ($P = 0.004$) as well as aggressive behavior ($P = 0.001$).

The haplotype G:A:G:G that appears to confer risk for ADHD also showed an association with quantitative measures of EF, as measured by the CPT, SOPT and WCST (Table 3; Supplementary table 2). The strongest association was observed with erratic behavior, as measured by "hit reaction time (RT) standard error (SE)" ($P = 0.001$) on the CPT, and the related measure "variability of SE" ($P = 0.0006$). The G:A:G:G haplotype was over-transmitted to the higher T scores, implying greater variability in reactions, a measure of inattentiveness or erratic behavior. Similarly, on the "hit RT ISI change" and the related "hit SE ISI change", a highly significant association was noted ($P = 0.006$ and 0.001, respectively), indicating that there is more erratic behavior as the time between targets is increased.

On the SOPT, which measures spatial working memory, planning, and interference control, trend for association (over-transmission of the risk haplotype) was observed with the total score ($P = 0.01$). In this evaluation, the higher scores are indicative of poorer performance on the task. On the WCST, a trend for association was observed with perseverative errors and responses as well as non-perseverative responses ($P = 0.03$–0.05), with a negative slope noted on the FBAT (i.e., under-transmission to the better scores), suggesting that children carrying the risk haplotype likely display problems in set-shifting and interference control. Conversely, a different haplotype G:A:C:A (ER22/23EK- N363S- *Bcl*I- A3669G; haplotype frequency = 0.36) was associated with better performance on the WCST, with lower perseverative errors and responses ($P = 0.008$, and $P = 0.004$, respectively).

Finally, an association was observed with overall treatment response, assessed during the two-week, double-blind, placebo-controlled trial with MPH (Table 4; Supplementary table 2). Significant association was observed with improvement on task-oriented behavior measured in the clinic using the RASS ($P = 0.001$). Congruent with this effect, a significant association was observed with the Conners'-T difference scores ($P = 0.006$). Interestingly, no association was observed with the Conners'-P difference score.

Discussion

These results provide genetic evidence for the involvement of the HPA axis in ADHD, given the pivotal role of the GR

Table 2 Association of *NR3C1* and *NR3C2* SNPs with ADHD and related behavioral traits (Color Table online)

SNPs/Haplotypes	PANEL A						PANEL B					
	rs6189	rs6195	rs41423247	rs6198	rs2070951	rs5522	G:A:G:A	G:A:C:A	G:A:G:G	A:A:G:G	G:G:G:A	G:A:C:G
Alleles	G	A	G	G	C	A	a1	a2	a3	a4	a5	a6
ADHD												
• **Total number DISC ADHD items**												
• Number of DISC inattention items												
• Number of DISC hyperactivity items												
• Number of DISC impulsivity items												
• Number of DISC conduct disorder items												
• Number of DISC oppositional defiant disorder items												
Conners' Parents												
Conners Teachers												
CBCL Total score												
• **CBCL Internalizing behaviour**												
- CBCL Withdrawn												
- CBCL Somatic complaints												
- CBCL Anxious/depressed												
- CBCL Social problems												
- CBCL Thought problems												
• **CBCL Externalizing behaviour**												
- CBCL Attention problems												
- CBCL Delinquent behaviour												
- CBCL Aggressive behaviour												

ADHD= ADHD diagnosis; ADHD items= number of ADHD items as assessed by the Diagnostic Interview Schedule for Children fourth edition (DISC-IV); Conners'= Conners' Global Index, CBCL= Child Behavioral Checklist T score. P values are provided according to a color code as indicated by the appended scale.

	0.01-0.05
	0.001-0.009
	0.0001-0.0009

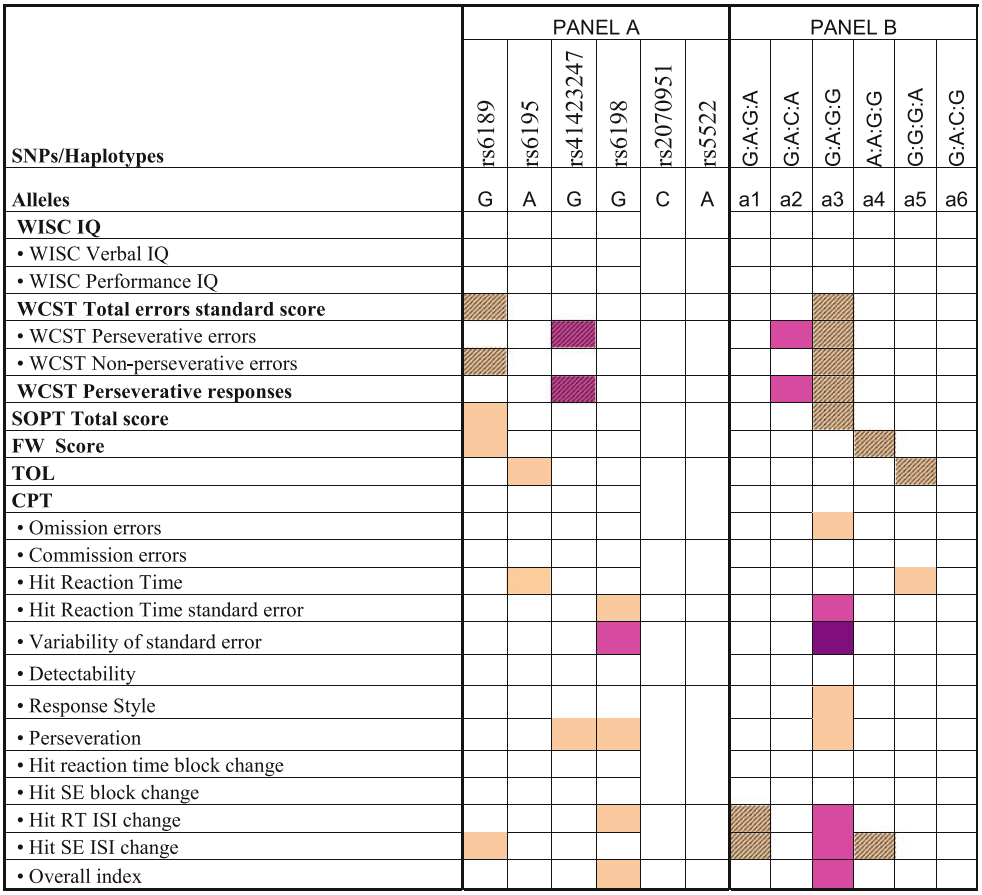
Under-transmitted allele

in this pathway. To the best of our knowledge, this is the first study to report an association between functional polymorphisms in *NR3C1,* which codes for the GR, and ADHD. In this study, an association was observed between the G:A:G:G haplotype (ER22/23EK- N363S- *Bcl*I-A3669G or rs6189- rs6195- rs41423247- rs6198) of *NR3C1* and ADHD. The same association was also observed in other domains related to ADHD, namely behavioral (particularly increase in thought and attention problems, aggressive behavior, comorbidity with oppositional defiant disorder) and EF (particularly increased erratic behavior). In addition, it was noted that carriers of the risk haplotype show better response to MPH. Since the risk haplotype is associated with greater number of behavioral problems at baseline, the positive effect of MPH treatment may be expected to be more pronounced.

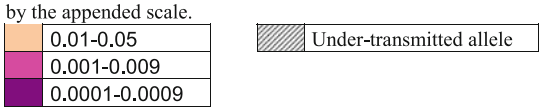
In contrast to the statistically significant association observed with *NR3C1,* no association was noted with the functional polymorphisms in *NR3C2,* which codes for the MR. This lack of association is noteworthy, given the observation that in the brain, MRs are present only within limbic regions (DeRijk and de Kloet 2008), which have not been associated with ADHD (Arnsten and Rubia 2012). [Since the submission of the manuscript, a recent case–control study reported an association between the *NR3C2* rs5522 SNP and symptoms of inattention and hyperactivity, with *P* between 0.01 and 0.05 (Kortmann et al. 2012). This association was not replicated in the current study, when the number of inattention and hyperactivity items of the CBCL was used as quantitative traits in the FBAT analysis. Independent replication in large samples is required to validate the findings of the current study and the one reported by Kortmann and colleagues].

Based on previous reports, when looking separately at each allele that composes the risk haplotype in this study (G:A:G:G), we observe that the G allele of rs6189 is

Table 3 Association of *NR3C1* and *NR3C2* SNPs with neurocognitive traits (Color Table online)



WISC= Wechsler Intelligence Scale for Children 3rd/4th edition scores; WCST=Wisconsin Card Sorting Test; SOPT= Self ordered pointing task raw scores; FW= finger windows standardized score; TOL=Tower of London standardized scores; CPT= Continuous Performance Test t scores; SE= standard error RT=reaction time; ISI= inter-stimulus interval. P values are provided according to a color code as indicated by the appended scale.



associated with glucocorticoid sensitivity, the A allele of rs6195, with glucocorticoid resistance, the G allele of rs41423247, with glucocorticoid resistance and the G allele of rs6198, with glucocorticoid sensitivity (Table 1). Together this creates a confusing picture, and the only approach that would give a satisfactory answer is to specifically test the children with the different haplotypes within the TSST paradigm. However in the absence of this information, the following model may be proposed. Close examination of the alleles in the risk haplotype shows that the *G*, *A*, and *G* alleles of the SNPs rs6189- rs6195-rs41423247 are in fact the most commonly observed in the population with allele frequencies of 0.96, 0.96, and 0.66 respectively (Table 1). Given their high frequency in the normal population, these alleles are unlikely to be directly involved in the etiology of ADHD, as the disorder occurs only in 8-12 % of the population. An important clue might be provided by A3669G (rs6198), where the *G* allele (population frequency of ~0.15) in the haplotype is associated with various clinical dimensions. Therefore, in the G:A:G:G haplotype ("normal": "normal": "normal": "risk"), the rs6198 *G* allele appears to be most relevant.

In vitro studies have shown that the *G* allele of the SNP rs6198 (A3669G) increases the stability of the GR-β splice variant, thereby altering the ratio of GR-α/GR-β so that the predominant activity is GR-β (Lu and Cidlowski, 2004). GRβ functions as the dominant negative isoform at GRE-containing, glucocorticoid-responsive promoters and is thereby a natural inhibitor of glucocorticoid actions. It may be suggested therefore that at a first level, the *G* allele may increase cortisol release (or "hyperactivity") as observed following the TSST (Table 1). This is due to a less-efficient

Table 4 Association of *NR3C1* and *NR3C2* SNPs with methylphenidate treatment response (Color Table online)

SNPs/ Haplotypes	PANEL A						PANEL B					
	rs6189	rs6195	rs41423247	rs6198	rs2070951	rs5522	G:A:G:A	G:A:C:A	G:A:G:G	A:A:G:G	G:G:G:A	G:A:C:G
Alleles	G	A	G	G	C	A	a1	a2	a3	a4	a5	a6
CGI - overall improvement (placebo -active)												
Conners P (placebo -active)												
Conners T (placebo -active)												
RASS total difference score (PBO time2 - Active time2)												
RASS fidgeting diff erence score												
RASS vocalization difference score												
RASS task disengagement difference score												

CGI= Clinical Global Impression scale overall improvement score; PBO= placebo; RASS total difference score = Restricted Academic Situation Scale score after placebo – score after active medication.
P values are provided according to a color code as indicated by the appended scale:

	0.01-0.05
	0.001-0.009
	0.0001-0.0009

Under-transmitted allele

negative feedback loop, given that GR-β is a dominant inhibitor of GR-α. However, in the brain, the *G* allele may generate a hyporesponsiveness, since GR-β is unable to bind cortisol. Once again, it is underscored that these models are speculative and will need to be formally tested in children with ADHD performing the TSST and genotyped for the risk haplotype.

Several studies have suggested that ADHD is associated with dysregulation of the HPA axis. It has been reported that children with ADHD have a blunted cortisol response to psychosocial stressors, a decreased CAR, or lower plasma daytime cortisol (King et al. 1998; Ma et al. 2011; Randazzo et al. 2008; Scerbo and Kolko 1994; van West et al. 2009). However, there is considerable inconsistency between studies, with some studies reporting a lack of association between ADHD and cortisol levels (Freitag et al. 2009; Hastings et al. 2009; Snoek et al. 2004). There are many possible explanations for the ambiguity in the results including small sample sizes in some studies, variation in the methodology used, association with specific subtypes and presence of comorbid disorders. Several recent studies have attempted to clarify the picture by examining the different subtypes and presence of comorbid disorders. In particular, recent studies have reported that the presence of comorbid oppositional defiant disorder with ADHD is associated with blunted CAR and/or lower stress-response salivary cortisol levels (Freitag et al. 2009; Hastings et al. 2009; Oosterlaan et al. 2005; Snoek et al. 2004). The results of the present study are consistent with these findings since the risk haplotype shows a strong association with number of oppositional defiant disorder items on the DISC, as well as aggressive behavior on the CBCL. It is likely that these children may have a blunted HPA axis function that is directly or indirectly involved in the etiology of the disorder.

Further insight on the association between the HPA axis and ADHD may be provided by the fact that glucocorticoids have an important influence on memory and cognition. Studies in rodents (de Kloet et al. 1999) and humans (Lupien et al. 2007) have demonstrated that an optimal level of these hormones is necessary for performance on tasks assessing memory formation. Both an elevated and diminished level of glucocorticoids lead to a decrease in performance. In a recent report, Shin and Lee reported that a large percentage (75 %) of the ADHD children tested did not show an increase in cortisol after an IQ test (Shin and Lee 2007). Interestingly, they noted a correlation between poor test performance and decreased cortisol level among these children. It is therefore conceivable that a blunted HPA axis function may prevent some children with ADHD from reaching optimal neurocognitive function.

While the GR is critical in the regulation of the HPA axis, it is an important mediator of a diverse range of physiological and developmental processes. In humans, glucocorticoids play an important role not just in the response to stress, but in the maintenance of basal-level homeostasis. They are involved in every organ system and regulate almost every physiological, cellular, and molecular network, playing a pivotal role in critical biological processes from reproduction, growth, and development to

central nervous system function (Chrousos et al. 2004). Given the important role of the *NR3C1* polymorphisms in regulating the function of GR, the association with ADHD is intriguing. While these results shed light on at least one of the pathways involved in the disorder, detailed molecular analyses are required to determine the function of the associated haplotype in vivo. Careful investigation of physiological measures of HPA axis function, as well as global analyses of gene expression, in the subgroup of children with the risk haplotype may help in clarifying the association with the disorder.

The principal limitation of this study is the sample size, particularly given the low heterozygosity of some of the SNPs, and the low number of informative families that results in the family-based analysis (Supplementary table 2). A second limitation is that the sample contained many more boys than girls, reflecting the fact that ADHD is more commonly observed in boys. Some studies have reported that gender modulates the association between *NR3C1* SNPs and altered HPA axis function, observing an effect primarily in males (Kumsta et al. 2007). A larger sample of girls would be required to determine whether there is a differential association based on gender.

Finally, the behavioral and cognitive tests performed in this study measure different facets or endophenotypes of ADHD and are thus correlated. This correlation would make a correction for multiple testing (such as the Bonferroni correction) overly conservative, as it would unduly increase type II errors. Furthermore, the widespread associations are observed with behavior and cognition relevant to ADHD as measured by different observers (parents, teachers, researchers) in different settings (school, home, clinic), all point in the same direction, which would be highly unlikely to happen by chance. Nonetheless, these results must be considered exploratory and may help to inform related studies with ADHD.

In conclusion, the results presented suggest that there is an association between a haplotype within *NR3C1* and ADHD-related phenotypes, thus providing initial genetic evidence for involvement of the HPA axis in the illness. Investigation of HPA axis function in relation to this haplotype in both normal and ADHD individuals is needed to understand its potential role in psychopathology.

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