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Glutamatergic Copy Number Variants and Their Role in Attention-Deficit/Hyperactivity Disorder

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Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neurodevelopmental disorder with a strong genetic component. The glutamate metabotropic receptor genes (*GRM*s) have been considered potential candidates for ADHD susceptibility. The aim of the present study was to investigate if copy number variants (CNVs) in *GRM1*, *GRM5*, and *GRM8* genes are overrepresented in ADHD subjects. A total of 1038 individuals with ADHD and 1057 subjects without this disorder were investigated. No significant difference in the total number of CNVs was found comparing the entire ADHD sample and the population sample without ADHD ($P = 0.326$, OR = 1.112, 95% CI = 0.762–1.624). The presence of CNVs was associated with lower intelligence quotient (IQ) scores in ADHD samples ($P = 0.026$, OR = 1.824, 95% CI = 1.066–3.121) but not in the sample of individuals without ADHD. CNVs in *GRM5* were associated with presence of anxiety disorders in ADHD cases ($P = 0.002$, OR = 3.915, 95% CI = 1.631–9.402), but not in individuals without ADHD. Taken together, our results suggest a role for glutamate in ADHD as CNVs in the glutamatergic genes investigated herein were associated with cognitive and clinical characteristics of ADHD individuals.

Key words: *GRM1*; *GRM5*; *GRM8*; IQ; anxiety

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INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a common childhood neurodevelopmental disorder characterized by impairing symptoms of inattention, hyperactivity and impulsivity [American Psychiatric Association, 2013]. The worldwide pooled prevalence is estimated at 5 to 7% in school-aged children [Polanczyk et al., 2007; Willcutt, 2012]. Although symptoms might remit, the ADHD persistence into adulthood is rather common [Faraone et al., 2006; Lara et al., 2009], with prevalence rates in adults estimated between 2.5 to 5% [Simon et al., 2009; Willcutt, 2012]. ADHD is a complex and heterogeneous disorder and its etiology is not yet completely understood [Genro et al., 2010]. Molecular genetic studies have shown that both common and rare variants are implicated in the susceptibility to this disorder [Akutagava-Martins et al., 2013].

An increased overall copy number variant (CNV) rate in ADHD children compared to controls has been reported [Williams et al., 2010] and replicated [Yang et al., 2013]. The higher CNV

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rate found in ADHD cases is even greater when ADHD cases that also presented mild intellectual disability (ID) were considered [Williams et al., 2010]. Langley et al. [2011] demonstrated an increased risk of large (>500 kb) and rare (<1% frequency) CNVs in ADHD children with comorbid ID. No other difference on symptom severity or type, comorbidity, developmental features, family history or pre- and peri-natal markers was reported, suggesting that CNV presence do not determine an atypical subgroup of ADHD children [Langley et al., 2011].

In Yang et al. [2013], a strategy of combining the investigation of CNVs and single nucleotide polymorphisms (SNPs) was used. ADHD was associated with both rare and common variants of genes involved in neurodevelopment and synaptic plasticity, especially those of glutamate synaptic development pathway [Yang et al., 2013]. Moreover, Elia et al. [2010] demonstrated that glutamate metabotropic receptor genes *GRM5* and *GRM7* were among the genes affected by CNVs [Elia et al., 2010]. In a subsequent study, these investigators again detected an overrepresentation of CNVs affecting glutamate metabotropic receptor genes *GRM1*, *GRM5*, *GRM7*, and *GRM8* in ADHD cases. Additional analyses showed that about 10% of ADHD cases where enriched for CNVs in genes interacting with the *GRM* gene family, forming a network. The most frequent CNVs were *GRM5* deletions; *GRM1* duplications and *GRM8* deletions were also associated with ADHD [Elia et al., 2012].

Glutamate is the major excitatory neurotransmitter in the brain and is involved in a number of processes relevant to ADHD: brain development, modulation of neuronal activity, bidirectional regulation of dopamine signaling, synaptic plasticity, memory formation, and learning [Lesch et al., 2013; Mukherjee and Manahan-Vaughan, 2013]. *GRM5* seems to be critical for inhibitory learning mechanisms because impaired receptor function results in inappropriate retention of aversive memories, which may result in anxiety disorders [Xu et al., 2009]. *GRM1* knockout mice demonstrated that this receptor is involved in associative learning [Aiba et al., 1994a; Gil-Sanz et al., 2008] and motor learning [Aiba et al., 1994b] due to reduced hippocampal long-term potentiation and deficient cerebellar long-term depression, respectively. Impaired motor coordination was also observed in adult mice without this receptor [Nakao et al., 2007]. The *GRM8* null mutant mice showed novelty induced hyperactivity and altered fear responses [Fendt et al., 2010; Gerlai et al., 2002]. Anxiety disorders, motor coordination problems and learning disorders are common features found in ADHD cases [Rommelse et al., 2009].

Considering these evidences and the genome wide screenings results that suggest a role for glutamatergic genes in ADHD, the aim of the present study was to determine the contribution of CNVs in glutamate metabotropic genes *GRM1*, *GRM5*, and *GRM8* to ADHD susceptibility based on a candidate gene approach.

MATERIALS AND METHODS

Sample

A total of 1038 ADHD cases were investigated. These samples came from three sources. The first sample comprises 528 ADHD children and adolescents and their biological parents. These patients were recruited at the ADHD Outpatient Program (ProDAH) from Hospital de Clínicas de Porto Alegre. The diagnostic procedures for ADHD and comorbidities followed the Diagnostic and Statistical Manual of Mental Disorders (4th ed, DSM-IV) criteria [American Psychiatric Association, 1994]. Children and adolescents were evaluated in a three-staged protocol which included clinical interview, the Schedule for Affective Disorders and Schizophrenia for School-Age Children, Epidemiological Version (K-SADS-E) [Orvaschel, 1985], and discussion of the derived diagnosis in a clinical committee. Further details are available elsewhere [Roman et al., 2001]. The second ADHD sample included 400 adults with ADHD that were also recruited at ProDAH. ADHD and oppositional defiant disorder diagnosis followed the same three-staged procedure described above for children and adolescents. Questions from K-SADS-E designed for children were adapted for adults [Grevet et al., 2005]. Axis I psychiatric comorbidities were evaluated using the Structured Clinical Interview for DSM-IV, research version (SCID-I-RV) [First et al., 1998]. The diagnoses of conduct disorder and anti-social personality disorder were obtained using the appropriate sections of the Mini International Neuropsychiatric Interview (MINI) [Amorim, 2003]. MINI has a previously validated Portuguese version [Amorim, 2003]. In primary health care in Brazil, MINI had kappas of 0.65–0.85, sensitivity of 0.75–0.92, and specificity of 0.90–0.99 when using Structured Clinical Interview for Diagnosis (SCID) applied by a psychiatrist as a parameter [de Azevedo Marques and Zuardi, 2008].Further details are available elsewhere [de Cerqueira et al., 2011].

The third ADHD sample is composed of 110 individuals from the 1993 Pelotas Birth Cohort. These subjects were evaluated at age 18 in a one-stage procedure. The ADHD assessment was performed with a structured interview based on the Diagnostic and Statistical Manual of Mental Disorders (5th ed, DSM-5) [American Psychiatric Association, 2013]. The DSM-5 defines ADHD in adults as the presence of at least five among nine symptoms of inattention and/or five among nine symptoms of hyperactivity/impulsivity. ADHD symptoms must cause clinical interference, several of them must be present in more than one setting, and their age of onset should be before 12 years of age. The DSM-5 ADHD symptoms were rated as present or absent. Considering that this is a large population study, we initially applied a screening questionnaire using the same structure of the six-question World Health Organization Adult ADHD Self-Report Scale Screener (ASRS) for all subjects. This instrument includes six questions about ADHD symptoms (four inattention items: "does not follow through", "difficulty organizing tasks", "forgetful", "reluctant to engage in 'mental' tasks"; and two hyperactivity items: "fidgets", and "on the go"), which were adapted to the DSM-5 wording. In a previous population study, ASRS had 68.7% sensitivity, 99.8% specificity, and 97.9% total classification accuracy, considering a cut-off of 4/6 screening symptoms [Kessler et al., 2005]. In our study, in order to enhance sensitivity, any subject with two or more positive questions among the six was considered screening positive, and answered questions about the 12 remaining ADHD symptoms, as well as about additional criteria (symptom pervasiveness, age of onset before 12 years old, and clinical impairment). Pervasiveness was assessed by questioning if the subject presented symptoms in at least two of the three main settings: home, social and work/school environments. Clinical impairment specifically related to ADHD was measured through a 0 (no impairment)

to 3 (severe impairment) scale answered by the subjects at the end of the ADHD assessment interview. Clinical impairment was defined as ADHD impairment scores of 2 (moderate) or 3 (severe).

For comparison purposes, a total of 1057 individuals were also selected from the 1993 Pelotas Birth Cohort. It includes all subjects with positive screening (i.e., at least two positive questions in the six question screening) who did not meet full ADHD diagnostic criteria in the subsequent evaluation (n = 187) and individuals with none of the six ADHD screening symptoms (n = 870). This strategy was chosen to maximize the chance of having at the same time a huge control group and individuals endorsing few ADHD symptoms, what is relevant in a dimensional disorder like ADHD.

Methodology of collection and demographic data from this Cohort are described fully in Victora et al. [2006] [Victora et al., 2006]. The general psychiatric assessment was performed with the MINI, a short semi-structured diagnostic interview for DSM-IV and ICD-10 psychiatric disorders, which provided prevalence estimates of common mental disorders. Due to logistic issues (i.e, the psychiatric interview was part of a larger follow-up assessment), only some MINI sections were used. The most prevalent mood (bipolar and major depression), and anxiety (agoraphobia, generalized anxiety, social phobia) disorders were assessed.

Intelligence quotient (IQ) weighted scores were estimated in all samples using either Wechsler Intelligence Scale for Children (WISC-III, [Wechsler, 1991]) or Wechsler Adult Intelligence Scale (WAIS-R, [Wechsler, 1981]) applied by trained psychologists.

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre and by the Ethics Committee of Universidade Federal de Pelotas. Adults were invited to participate and provided a written informed consent. Children and adolescents verbally agreed to participate and their parents provided a written informed consent.

Genotyping

A 5 mL blood sample was collected from each patient enrolled at ProDAH. Blood samples were collected from parents whenever possible. Genomic DNA was extracted from leukocytes by standard procedures. DNA samples from the 1993 Pelotas Birth Cohort were obtained from saliva, using Oragene® OG-250 DNA Self-Collection kit, following the manufacturer's recommended protocol (DNA Genotek Inc., Kanata, Ontario, CA). DNA from all samples was quantified by spectrophotometry using NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and diluted to a standard concentration of 5 ng/μL.

All CNVs affecting *GRM1*, *GRM5*, and *GRM8* described by Elia et al. [2012] were revised in order to identify overlapping regions [Elia et al., 2012]. Once these regions were identified, a TaqMan Copy Number Assay® pre-designed for genotyping was chosen. An assay for Ribonuclease P RNA component H1 gene (RNase P) was used as reference to determine copy number. Quantitative real time polymerase chain reactions (qRT-PCR) were performed in quadruplicates for each sample as recommended by the manufacturer. The amplification products were analyzed with the CopyCaller® software v2.0. Reactions were considered acceptable if confidence >95% and Z score <1.75. (Applied Biosystems Inc., Foster City, CA, USA). Once a CNV was identified, inheritance was assessed whenever DNA from the parents was available and the same genotyping procedure described above was followed.

Statistical Analyses

The CNVs frequencies were obtained by counting. Given the low frequency of CNVs, comparisons between different groups were carried out by Fisher's Exact Test. All tests were performed with SPSS for Windows, version 19.0 (IBM Corp., Armonk, NY, USA). Fisher's Exact Test statistical power was calculated by post hoc power analyses for given α levels, sample sizes, and effect sizes using G*Power v. 3.1 software [Faul et al., 2007]. A significance level of 0.05 was accepted in all analyses.

RESULTS

Demographic and clinical characteristics of each sample are presented in Table I. The adult ADHD sample from the 1993 Pelotas Birth Cohort presented the lowest IQ mean: 72.4 ± 10.3. Disruptive behavior disorders, anxiety disorders, and mood disorders were the most common comorbidities found in the ADHD samples from ProDAH. CNV frequencies were estimated for each gene in each group (Table II). CNVs affecting *GRM1* were the most common, except for the adult ADHD sample from ProDAH. These CNVs were primarily deletions, although two duplications were also identified. Inheritance investigation demonstrated that these CNVs were all inherited, most of them from the mother (82.3%). *GRM5* CNVs were all *de novo* deletions. CNVs affecting *GRM8* were the rarest. Only two were identified: a deletion and a duplication in the adult ADHD sample from ProDAH; for this CNV it was not possible to determine the inheritance pattern. When all CNVs were considered, deletions were far more common than duplications (97.4%). Only three duplications were identified, all in the adult ADHD sample from ProDAH.

First, the total number of CNVs was compared between the entire ADHD sample and our controls. No significant findings emerged ($P = 0.326$, OR = 1.112, 95% CI = 0.762–1.624). Second, we performed the same analyses contrasting each ADHD sample and the comparison sample of individuals without ADHD. The adult ADHD sample from the 1993 Pelotas Birth Cohort presented a significantly higher CNV frequency compared to the population sample, from the same Cohort ($P = 0.002$, OR = 2.928, 95% CI = 1.562–5.488). However, this was not replicated in the ADHD samples from ProDAH, even though these samples present a 99% power to detect an association of the same magnitude, at $P < 0.05$.

Given that a higher CNV rate was reported in ADHD cases with ID, we investigated a possible association with IQ scores. The scores were divided into two categories: ≤ 79 (below average) and ≥ 80 (average and above). The presence of CNVs was associated with lower IQ scores in ADHD cases ($P = 0.026$, OR = 1.824, 95% CI = 1.066–3.121) but not in the individuals without ADHD (Table III).

Since *GRM5* has been linked to anxiety in animal models, we assessed the possible association between *GRM5* and anxiety disorders comorbid with ADHD. The presence of *GRM5* CNVs was associated with comorbid anxiety disorders in ADHD cases ($P = 0.002$, OR = 3.915, 95% CI = 1.631–9.402) but not in subjects

TABLE I. Demographic and Clinical Characteristics of Each Sample[a]

	ADHD children and adolescents ProDAH	ADHD adults ProDAH	ADHD adults 1993 Pelotas Birth Cohort	Population sample without ADHD 1993 Pelotas Birth Cohort
Age	10.6±3.2	33.9±11.1	18	18
Male	77.2	50.8	38.2	48.6
IQ	92.4±13.9	101.3±8.4	72.4±10.3	75.4±11.5
Disruptive behavior disorders	49.5	48.1	-	-
Anxiety disorders[b]	16.9	32.5	49.5	10.4
Mood disorders	15.4	59.2	20.9	2.5

[a]Data are given as number (percentage) or mean (±standard deviation).
[b]Agoraphobia, generalized anxiety disorder, social phobia.

TABLE II. CNVs of Glutamatergic Genes and Their Frequency in Each Sample

			ADHD children and adolescents ProDAH		ADHD adults ProDAH		ADHD adults 1993 Pelotas Birth Cohort		Population sample without ADHD 1993 Pelotas Birth Cohort	
Gene	**Probe location[a]**	**Type[b]**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
GRM1	Chr6:146666882	Del	21	4.0	4	1.0	10	9.1	40	3.9
		Dup	0	0	2	0.5	0	0	0	0
GRM5	Chr11:88310296	Del	7	1.3	10	2.5	4	4.1	14	1.4
GRM8	Chr7:126473038	Del	0	0	1	0.2	0	0	0	0
		Dup	0	0	1	0.2	0	0	0	0
			28	5.3	18	4.6	14	14.1	54	5.3

[a]Based on National Center for Biotechnology Information build 37.
[b]CNV type: 'del' deletion; 'dup' duplication.

TABLE III. IQ Scores in ADHD Cases[a] and Comparison Sample Without ADHD According to the Presence of CNVs[b]

	IQ score ≤ 79		IQ score ≥ 80				
	n	**%**	**n**	**%**	***P* value**	**OR**	**95% CI**
ADHD cases							
CNV							
Yes	17	10.5	42	5.7	0.026	1.824	1.066 - 3.121
No	145	89.5	688	94.3			
Population sample without ADHD							
CNV							
Yes	35	5.5	18	4.8	0.385	1.136	0.653 - 1.976
No	602	94.5	354	95.2			

[a]ADHD cases from both ProDAH and 1993 Pelotas Birth Cohort.
[b]Fisher's exact test.

TABLE IV. Frequency of Anxiety Disorders[a] in ADHD Cases[b] and in the Population Sample According to Presence of *GRM5* CNV[c]

	With anxiety disorders		Without anxiety disorders				
	n	%	n	%	*P* value	OR	95% CI
ADHD cases							
GRM5 CNV							
Yes	12	4.5	9	1.2	0.002	3.915	1.631 – 9.402
No	252	95.5	740	98.8			
Population sample without ADHD							
GRM5 CNV							
Yes	2	1.9	12	1.3	0.431	1.461	0.323 – 6.619
No	103	98.1	903	98.7			

[a]Agoraphobia, generalized anxiety disorder, social phobia.
[b]ADHD cases from both ProDAH and 1993 Pelotas Birth Cohort.
[c]Fisher's exact test.

without ADHD (Table IV). About 57% of the ADHD patients with a *GRM5* CNV also present at least one anxiety disorder, mainly phobias (75%).

DISCUSSION

Elia et al. [2012] demonstrated, in a large genome-wide CNV study, an increased rate of CNVs affecting *GRM1*, *GRM5*, and *GRM8* genes in ADHD children. In the present study, we investigated these rare variants in ADHD based on a candidate gene approach. A higher CNV frequency was detected in the adult ADHD sample from the 1993 Pelotas Birth Cohort, which, in turn, is the sample with the lowest IQ mean. Despite methodological differences, our results, although not strictly comparable points to the same direction as those presented by Williams et al. [2010] and Langley et al. [2011], which demonstrated that ADHD cases with mild ID have an increased risk of carrying a CNV [Langley et al., 2011; Williams et al., 2010].

The first study to investigate CNVs in ADHD reported no difference in CNV rate between cases and controls corrected for IQ [Elia et al., 2010]. Based on this, the increased CNV rate in ADHD cases and in ADHD cases with ID reported by Williams et al. [2010] was questioned. It was argued that the CNV enrichment seen in ADHD cases was in fact due to the known causal relation between large CNVs and ID [Elia et al., 2011; Williams et al., 2010]. However, a study of both fluid and crystallized intelligence in a large non-clinical sample following the same methodology of Williams et al. [2010] found no evidence of association between these large (>500 kb) and rare (<1% frequency) variants and general cognitive ability [MacLeod et al., 2012; Williams et al., 2010]. These results are in agreement with the idea that ADHD and IQ share genetic influences.

In a twin study, Kuntsi et al. [2004] demonstrated that ADHD and lower IQ scores co-vary and the co-occurrence is largely attributed to shared genetic components. In this sense, genes associated with ADHD could influence IQ and vice versa [Kuntsi et al., 2004]. The ataxin 1 gene (*ATXN1*) has been associated with ADHD susceptibility in a meta-analysis [Neale et al., 2010]. In another study, the same gene was associated with IQ in an ADHD sample, but not in population-based samples. These authors concluded that genes associated with IQ in a psychiatric context may not necessarily influence IQ in a non-psychiatric population [Rizzi et al., 2011]. Our results support this hypothesis: the presence of *GRM* CNVs was associated with lower IQ scores in the ADHD sample, but not in the population sample without ADHD. These genes are involved in learning processes, which are impaired in ADHD cases, as a large portion of ADHD patients present comorbid learning disorders [Czamara et al., 2013], which could in turn affect IQ [Kuntsi et al., 2004].

Anxiety disorders are among the most frequent comorbidities found in ADHD patients [Rommelse et al., 2009]. It was seen in more than 15% of ADHD samples (Table I). In these samples, *GRM5* CNVs were associated with presence of anxiety disorders, mostly phobias. The mechanism whereby *GRM5* underlies anxiety disorders is complex. The knockout mice lacking the metabotropic glutamate receptor 5 (mGluR5) exhibits a partially impaired fear acquisition, which could be initially interpreted as a less anxious behavior. However, this learning process is only partially affected. Once fear was acquired in a conditioning task, the reversal learning process aiming the extinction of fear was abolished [Xu et al., 2009]. It seems reasonable to suggest that CNVs affecting *GRM5* impact heavily in mGluR5 receptor function. The stimulation of these receptors is clearly required for proper fear extinction through plasticity in the infralimbic prefrontal cortex in rodents. A defective receptor could lead to inappropriate modulation of fear expression [Sepulveda-Orengo et al., 2013]. The ability to reverse or extinct a previously acquired task is an important adaptation mechanism to a different environmental context. The adaptive learning processes, or inhibitory learning, are involved in retasking, being particularly relevant to anxiety disorders in humans such as phobias and post-traumatic stress disorder [Barad, 2005; Bouton, 1993; Xu et al., 2009).

Our analyses of anxiety disorders focused on agoraphobia, generalized anxiety, and social phobia since they represent those

anxiety disorders with higher prevalence and clinical significance [Gadermann et al., 2012]. For the same reason, only data on these disorders were collected in the 1993 Pelotas Birth Cohort. In addition, they represent more than 50% of the cases of anxiety disorders in all our clinical samples and present a huge comorbid rate with ADHD in the 1993 Pelotas Birth Cohort. The high comorbid rate between ADHD and anxiety disorders added to the evidence of association with *GRM5* gene only in the ADHD group reported here suggests a possible shared genetic component between these disorders.

The results presented herein should be interpreted in the context of some limitations. First, the genotyping methodology employed here is restricted to the gene region analyzed and does not determine CNV size. CNVs affecting other portions of these genes would pass unnoted. However, the genotyping probes were selected based on CNVs previously described and validated by the same technique [Elia et al., 2012]. The CNV frequencies showed here are higher than those previously reported [Elia et al., 2012], probably reflecting the genotyping methodology employed because qRT-PCR is highly sensitive for CNV genotyping [D'Haene et al., 2010], while the ability to detect CNVs from genome-wide association data, particularly the smaller ones, depends largely on SNP coverage and the algorithm employed for CNV calling [Zhang et al., 2011].

Second, as we have the WISC-III and WAIS-R translated to Portuguese and the validation of the translation assessed but do not have Brazilian norms for WISC-III and WAIS-R validated in representative populations, we use the American norms to derive IQ scores for all samples. Although this strategy does not affect the comparability among groups, our individual IQ estimates might be lower than expected. The possible influence of learning disorders on lower IQ scores was not investigated as information on these disorders is not available. Langley et al. [2011] suggest that CNVs are associated with IQ scores only in individuals with intellectual disability. Given the role of *GRM*s in learning processes, future studies should further examine the association with IQ scores reported here in ADHD groups with and without learning disorders.

Third, the analysis of *GRM5* and anxiety disorders did not consider age and gender, as potential confounders due to sample size limitations. Fourth, children and adult ADHD cases were pooled and different developmental stages may have influenced our results by increasing heterogeneity. Finally, the results were not corrected for multiple testing as we consider this study as preliminary and exploratory. Further investigation of *GRM*s on IQ and anxiety in both ADHD and non-ADHD samples are clearly warranted to determine the role of *GRMs* on these characteristics.

In conclusion, our results should be viewed as a preliminary evidence and suggest a role for glutamate in ADHD. Although it was not possible to detect an association between the presence of CNVs affecting *GRM1*, *GRM5*, and *GRM8* and ADHD susceptibility, we observed that these variants were associated with lower IQ scores. The *GRM5* gene, specifically, was associated with the presence of comorbid anxiety disorders. Taken together, our results suggest that CNVs in the glutamatergic genes investigated herein are associated with cognitive and clinical characteristics of ADHD individuals. Such characteristics are clinically important and impact on disease treatment and outcome. Future studies are needed in order to explore and replicate these findings.

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