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Synergistic effects between *ADORA2A* and *DRD2* genes on anxiety disorders in children with ADHD

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ABSTRACT

The prevalence of anxiety disorders in patients with Attention Deficit/Hyperactivity Disorder (ADHD) is around 15–40%, three times higher than in the general population. The dopaminergic system, classically associated with ADHD, interacts directly with the adenosinergic system through adenosine A_{2A} receptors (A_{2A}) and dopamine D_2 receptors (D_2) forming A_{2A}-D_2 heterodimers. Both dopaminergic and adenosinergic systems are implicated in anxiety disorders. Therefore, the aims of this study were: a) to investigate the main effects of *ADORA2A* and *DRD2* gene variants on anxiety disorders in an ADHD sample of children and adolescents; b) to test potential synergism between *ADORA2A* and *DRD2* genes on the same outcome; c) to explore *ADORA2A* variants functionality using an *in silico* approach. The sample consists of 478 children and adolescents with ADHD and their parents, totalizing 1.239 individuals. An association between the *ADORA2A* rs2298383 TT genotype with the presence of anxiety disorders ($P = .004$) and an interaction between *ADORA2A-DRD2* risk haplotypes with the same outcome ($P = .005$) was detected. The *in silico* analyses showed that rs2298383 has the highest score for regulatory function among all variants in the *ADORA2A* gene described up to date. Altogether, the present findings suggested that the *ADORA2A* gene and the interaction of *ADORA2A* and *DRD2* genes may play a role in anxiety disorders in children and adolescents with ADHD.

1. Introduction

A relevant aspect of Attention-Deficit/Hyperactivity Disorder (ADHD) is its high phenotypic heterogeneity, mainly due to the presence of comorbidities, which may determine the scarcity of replicable molecular genetic findings. Epidemiological studies have shown that 50–90% of ADHD patients present another psychiatry disorder (Spencer et al., 1999; Wilens et al., 2002). This fact has significant consequences on ADHD presentation, diagnosis, and prognosis (The MTA Cooperative, 1999). The prevalence of current anxiety disorders in ADHD patients is around 30–40% (Sobanski et al., 2006; Uchida et al., 2015) which is three times higher than that observed in the general population (Remes et al., 2016). Moreover, the presence of anxiety disorders in patients with ADHD was associated with an increase of ADHD related impairments compared to ADHD patients without comorbidities (O'Rourke et al., 2017; Reimherr et al., 2017), suggesting that anxiety could act as a mediator between ADHD severity and functional impairment (Oh et al., 2018).

The dopaminergic system is directly implicated in ADHD, as well as in many other psychiatric phenotypes, including anxiety disorders. This system is closely related to adenosinergic system (Ferré et al., 1994; Díaz-Cabiale et al., 2001), which has an essential role in the status of

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fear, anxiety, memory, learning, mood, aggression and motivation (Krugel et al., 2001; Burnstock, 2008). The physical interaction between adenosine A_{2A} receptors (A_{2A}) and dopamine D_2 receptors (D_2) strongly supports the relationship between dopaminergic and adenosinergic systems, showing complex effects mediated by A_{2A}-D_2 heterodimers (Torvinen et al., 2004; Fuxe et al., 2005).

Statistically significant findings have been reported for genetic associations between the A_{2A} gene (*ADORA2A*) and different psychiatric disorders (Freitag et al., 2010; Turčin et al., 2016; Janik et al., 2015), including ADHD (Molero et al., 2013). However, the most robust association results are related to anxiety disorders, mainly with panic disorder (Deckert et al., 1998; Hamilton et al., 2004; Hohoff et al., 2010). Anxiety symptoms in psychiatric and non-psychiatric subjects (Boulenger et al., 1984; Rogers et al., 2010; Renda et al., 2015), as well as in animal models (Bhattacharya et al., 1997; Jain et al., 2005; El Yacoubi et al., 2000; Braun et al., 2011), have been consistently associated with caffeine consumption. Caffeine is one of the most known A_{2A} receptor antagonists, and *ADORA2A* gene has been associated with caffeine/amphetamine-induced anxiety responses in individuals with no history of psychiatric disorders (Childs et al., 2008; Alsene et al., 2003; Hohoff et al., 2005; Domschke et al., 2012; Gajewska et al., 2013). These findings were recently summarized in a systematic review, reinforcing the relevance of the A_{2A} receptor gene in the anxiogenic consequences of caffeine (Fulton et al., 2018).

Regarding dopamine D_2 receptor gene (*DRD2*), its variants have been associated with anxiety phenotypes (Lawford et al., 2006; Kulikova et al., 2008; Hayden et al., 2010) and extensively explored in ADHD. For ADHD, the studies were summarized in two meta-analyses with negative results (Gizer et al., 2009; Wu et al., 2012). Most of the association studies focused on a variant located in a closer gene (*ANKK1*), which plays an essential role in *DRD2* expression regulation (Neville et al., 2004).

The mentioned evidence points to a relevant role of *ADORA2A* and *DRD2* in the biological mechanisms shared between anxiety and ADHD pathophysiology. A significant interaction effect between these genes has been shown in anxiety parameters after caffeine intake in a healthy sample (Childs et al., 2008). Despite the close biological relationship between A_{2A} and D_2 receptors, to date, no study investigated the interaction between these genes in psychiatry outcomes. Therefore, the present study aimed to examine the main effects of *ADORA2A* (rs2298383 and rs3761422) and *DRD2* (rs1076560 and rs2283265) variants on anxiety disorders in an ADHD sample of children and adolescents and to test potential synergism between *ADORA2A* and *DRD2* genes on the same outcome. We also explored *ADORA2A* variants functionality using an *in silico* approach, since the literature has already described the functional effects of the *DRD2* variants.

2. Materials and methods

2.1. Sample

The study sample consisted of 478 children and adolescents with ADHD (437 with both genotypic and comorbidity data), between 4 and 17 years old, and their biological parents, comprising 1.239 individuals. The sample recruitment occurred at the ADHD Outpatient Program (ProDAH) from Hospital de Clínicas de Porto Alegre (HCPA).

2.1.1. Demographic characterization

Eighty percent of the individuals included in our sample were White Brazilians. We adopted the skin color as a way to analyze ethnicity since the Brazilian Institute of Geography and Statistics (IBGE) use this classification in the national census. In Brazil, people can classify themselves as White, Black, Yellow, Pardo or Indigenous. The sample enrollment occurred in Porto Alegre, a city located in Southern Brazil where the population is formed mainly by European immigrants. Genetic admixture analyses have shown that the proportion of European ancestry in this location ranges from 90 to 99.9% (Ruiz-Linares et al., 2014; Kehdy et al., 2015).

2.1.2. Clinical characterization

The diagnosis of ADHD and comorbidities followed the Diagnostic and Statistical Manual of Mental Disorders-4th edition (DSM-IV) criteria (American Psychiatric Association, 1994), in a three-step protocol: 1° a semi-structured interview (Schedule for Affective Disorders and Schizophrenia for School-Age – Present and Lifetime Version), 2° a diagnostic discussion by a clinical committee, and 3° a clinical evaluation of the patients and their parents. One hundred thirty-three (30.4%) children and adolescents presented at least one kind of anxiety disorder. Among them, the anxiety disorders diagnosed were simple phobia (33.7%), generalized anxiety disorder (20.9%), separation anxiety disorder (16.3%), social phobia (15.8%), agoraphobia (9.2%), obsessive-compulsive disorder (3.1%), posttraumatic stress disorder (0.5%), and panic disorder (0.5%). The Ethics Committee of HCPA approved this study protocol. Parents provided written informed consent and children and adolescents provided verbal permission to participate.

2.2. Genotyping

All patients enrolled at ProDAH as well as their biological parents donated blood samples. DNA was extracted from whole blood lymphocytes as previously described (Lahiri and Numberger, 1991). This study focused on two *ADORA2A* polymorphisms (rs2298383 and rs3761422), and two *DRD2* polymorphisms (rs1076560 and rs2283365). The *ADORA2A* polymorphisms were chosen based on previous ADHD studies (Molero et al., 2013) and other anxiety outcomes (Childs et al., 2008; Rogers et al., 2010), minor allelic frequencies, and on the technical viability. *DRD2* polymorphisms (rs1076560 and rs2283365) were selected based on functionality evidence (Bertolino et al., 2009; Moyer et al., 2011). Polymorphisms were genotyped using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA), according to the manufacturer's recommended protocol for the allelic discrimination system (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA).

2.3. Statistical analysis

Allele frequencies estimated by direct counting were used to calculate Hardy-Weinberg equilibrium by chi-square tests. The linkage disequilibrium (LD) calculation was performed using the Plink software v.1.07 (Purcell et al., 2007). Sociodemographic and clinical characteristics of the patients with and without anxiety disorders were compared using chi-square and *t*-tests.

2.3.1. Single-marker and haplotype analyses

Plink v.1.07 software was used to perform logistic regression analyses to assess the main effects of *ADORA2A* and *DRD2* polymorphisms on anxiety in different genetic models (additive, dominant, and recessive). Logistic regression was also used to perform haplotype-based associations between *ADORA2A* and *DRD2* with anxiety.

2.3.2. Interaction analyses

The interaction method described by Andersson et al. (2005) was used to test if the prevalence of anxiety in the context of the presence of *ADORA2A* and *DRD2* risk haplotypes departures from a simple additive model. There are two known measures of interaction that can be calculated following the method used. Since they are related to each other, we decide to show the attributable proportion (AP), that is the proportion of the combined effect that is due to interaction. Under the null hypotheses of no interaction, the AP due to interaction is equal to 0. We considered the T/C haplotype (rs2298383/rs3761422) of the *ADORA2A* gene and the A/T haplotype (rs1076560/rs2283365) of the *DRD2* gene as the risk haplotypes based on association findings and the

functionality data from the literature. Therefore, the reference group included those individuals not carrying any risk haplotype for the two genes. Sex, skin color, and age entered as covariates. Besides, the presence of mood disorders was also included as a covariate, considering the association between mood and anxiety disorders in the ADHD sample ($P < .010$). Interaction analyses were carried out using SPSS v.18.0 (SPSS Inc., Chicago, IL, USA).

2.3.3. Family-based analyses

Secondary analyses using transmission disequilibrium test (TDT) allowing for missing genotypes included in the UNPHASED software v.3.1.7 (Dudbridge, 2008) were carried out in the ADHD total sample and anxiety disorders subsample (Supplementary Table 1). TDT uses un-transmitted alleles as internal controls, avoiding the population stratification effects. This method determines if there is a preferential transmission (T) or not transmission (NT) of a risk allele from biological parents to affect offspring.

2.4. In silico analysis of ADORA2A gene

HaploReg v4.1 (Ward and Kellis, 2012) and Regulome DB (Boyle et al., 2012) databases tested the role of rs2298383 and rs3761422 single nucleotide polymorphisms (SNPs) in the *ADORA2A* gene regulation. HaploReg (Ward and Kellis, 2012) is a tool for the exploration of annotations of the noncoding genome at variants on haplotype blocks, such as candidate regulatory SNPs at disease-associated loci. Information on chromatin state and protein binding annotation from the Roadmap Epigenomics and ENCODE projects are among the functional evidence integrated into this dataset. RegulomeDB (Boyle et al., 2012) is a database of SNPs with known and predicted regulatory elements in the intergenic regions of the *H. sapiens* genome, integrating data from Gene Expression Omnibus (GEO) database, the ENCODE project, and published literature. We also used theSuRFR (SNP Ranking by Function R package) software (Ryan et al., 2014), which integrates functional annotation and prior biological knowledge to prioritize candidate functional variants. This software provides as output a rank of given SNPs based on functional information. We used SuRFR to assess the functional score/rank position for rs2298383 and rs3761422 concerning other *ADORA2A* (± 5 kb) common variants (MAF ≥ 0.1). The European continental population dataset phase 3 of the 1000Genomes project was used as a reference for SuRFR analyses. This study adopted the model specific for complex disease variants (DFP). A total of 54 SNPs were analyzed (Supplementary File 1).

3. Results

Demographic and clinical data are shown in Table 1. The minor allele frequencies (MAF) in ADHD patients were 0.48 (C) for the rs2298383 and 0.40 (T) for the rs3761422, in the *ADORA2A* gene. For *DRD2* gene, MAF were 0.17 (A) for rs1076560, and 0.18 (T) for rs2283265. The allele frequencies observed in our study are very similar to those frequencies reported for European populations by the 1000 genomes project (Supplementary Table 1). Polymorphisms were in accordance with the expected values for Hardy-Weinberg equilibrium (*ADORA2A* rs2298383: $P = .286$; rs3761422: $P = .951$; and *DRD2* rs1076560: $P = .3192$; rs2283265: $P = .3558$). The polymorphisms investigated are in strong LD for both genes *ADORA2A* ($r^2 = 0.694$; D' = 0.983) and *DRD2* ($r^2 = 0.886$; D' = 0.962).

Table 1
Sociodemographic and clinical characteristics of the sample.

Characteristics[a]	Without anxiety disorders ($n = 304$)	With anxiety disorders ($n = 133$)	*P*-value
Age (years)	10.48 (0.18)	10.37 (0.27)	.729
IQ	92.92 (0.77)	93.87 (1.15)	.493
Gender (male)	241 (78.24%)	99 (72.26%)	.106
Skin color (white)	254 (82.46%)	112 (81.75%)	.476
Other comorbidity			
Oppositional defiant disorder	112 (36.36%)	52 (37.95%)	.414
Conduct disorder	34 (15.32%)	21 (11.03%)	.133
Mood disorders	25 (8.11%)	27 (19.70%)	.001

[a] Data are given as number (percentage) or mean (standard deviation).

3.1. Single-marker, haplotype, and interaction analyses

Table 2 shows the associations of *ADORA2A* and *DRD2* genes with the presence of anxiety in patients with ADHD. A statistically significant association between *ADORA2A* rs2298383 TT genotype and the presence of anxiety disorders was observed ($P = .004$, $P_{corrected} = 0.006$). Moreover, T/C *ADORA2A* risk haplotype (rs2298383/rs3761422) was significantly more frequent in the group of patients affected by anxiety disorders ($P = .012$, $P_{corrected} = 0.030$, Table 3). *DRD2* polymorphisms or the *DRD2* risk haplotype did not associate with anxiety disorders in the sample with ADHD.

An interaction effect was also tested to further explore the role of *ADORA2A* and *DRD2* genes in anxiety disorders. Fig. 1 shows a significant positive interaction when we consider *ADORA2A* and *DRD2* haplotypes risk jointly. *ADORA2A* T/C and *DRD2* A/T carriers were significantly more frequent in the group of patients presenting anxiety (OR = 2.7, IC95% = 1.3–5.3; $P = .005$). The proportion of the combined effect of *ADORA2A* and *DRD2* that is due to interaction was 40% (AP = 0.40).

Regarding the family-based association tests, no preferential transmission of alleles was observed from parents to ADHD children and adolescents in both the total sample and in the subgroup of patients with anxiety disorders, as described in Supplementary Table 2.

3.2. In silico analyses

In silico analyses were performed to predict the functionality of the *ADORA2A* variants to explore our results better. Table 4 presents the results of *in silico* analyses. Considering that *DRD2* variants functionality has mainly been investigated by *in vitro* studies (Bertolino et al., 2009; Moyer et al., 2011), the focus of the present analysis was *ADORA2A* gene variants. Available HaploReg data indicated histone marks (H3K4me1_Enh, H3K4me3_Pro, H3K27ac_Enh, and H3K9ac_Pro) at both rs2298383 (Supplementary File 2) and rs3761422 (Supplementary File 3) loci based on histone modification feature data in many brain-related cell lines (primary Brain Hippocampus Middle and Brain Anterior Caudate). The *ADORA2A* rs2298383 variant affects transcription factor binding according to Regulome DB (Table 4; Supplementary File 4). It is in a DNAse hypersensitive site (DHS), regions of accessible chromatin commonly associated with regulator binding, for several tissues from the Roadmap Epigenomics Consortium (2015), and cells from ENCODE (ENCODE Project Consortium, 2012), such as placenta, fetal adrenal gland, digestive system, thymus, blood cells and epithelial and astrocytes primary cells. ChIP-Seq experiments from ENCODE Project also showed that this variant is in a region of interaction with Regulatory Factor X5 (RFX5) transcription factors in lymphocytes, (Table 4; Supplementary Files 2 and 4) suggesting that this variant may exert a functional effect in several cell lines. Finally, SuRFR software analysis indicates that this variant has the highest score for regulatory function among all the analyzed variants (Table 4; Supplementary File 1).

Regarding the rs3761422 variant, RegulomeDB and HaploReg suggest it has minimal binding evidence (details in Supplementary Files 3 and 5). Although it is placed in an enhancer region, as indicated by the Roadmap Epigenomics Consortium, there is only elusive evidence of DNAse hypersensitivity near the SNP, and no evidence of binding through ChIP-Seq experiments is observed (details in Supplementary

Table 2
Logistic regression analyses of individual SNPs on anxiety in patients with ADHD.

SNP	Gene	Allele	Additive model			Dominant model			Recessive model		
			OR	CI95%	*P*-value*	OR	CI95%	*P*-value*	OR	CI95%	*P*-value*
rs2298383	*ADORA2A*	T	1.43	1.07–1.92	.015[a]	1.31	0.83–2.49	.242	2.22	1.29–3.84	.004[b]
rs3764122	*ADORA2A*	C	1.29	0.95–1.75	.094	1.36	0.90–2.13	.140	1.47	0.80–2.70	.213
rs1076560	*DRD2*	A	1.17	0.78–1.76	.438	1.21	0.78–1.90	.396	1.03	0.25–4.33	.969
rs2283265	*DRD2*	T	1.36	0.92–2.01	.123	1.42	0.92–2.21	.118	1.40	0.38–5.21	.617

N = 437. CI = Confidence interval.
* All analyses were adjusted for sex, skin color, age, and the presence of mood disorders. *P* corrected after 10,000 permutations [max(T) procedure]: [a]*P* = .046; [b]*P* = .006.

Table 3
DRD2 and *ADORA2A* haplotype effects on anxiety in patients with ADHD.

ADORA2A					
rs2298383	rs3764122	Frequency	OR	Statistics	*P*-value
C	T	0.397	0.78	2.570	.109
C	C	0.084	0.61	2.870	.090
T	C	0.517	1.45	6.260	.012[a,b]
DRD2					
rs1076560	rs2283265	Frequency	OR	Statistics	*P*-value
A	T	0.167	1.23	0.997	.318
C	G	0.817	0.77	1.710	.191

P corrected after 10,000 permutations [max(T) procedure]: [a]*P* = 0.030. [b]Compared to all other haplotypes.

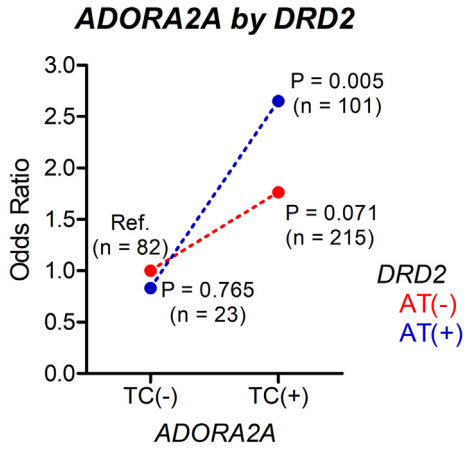

Fig. 1. Interaction effects of haplotypes on anxiety in patients with ADHD.

files 4 and 5). SuRFR software also suggests an overall small role in gene controls, providing this SNP the classification 47 of 54 SNPs in the ranking (Supplementary File 1).

3.3. Additional analyses

Considering that mood and anxiety disorder share a genetic component (Anttila et al., 2018; Otawa et al., 2016) and we detected an association between these disorders in our sample (Table 1), we decided to test the specificity of our interaction findings with anxiety. We rerun the same interaction analysis exploring mood disorders as the outcome (prevalence in our sample ≈12%). Sex, skin color, age, and anxiety disorders entered as covariates. There were no significant interaction effects. The joint prevalence of *ADORA2A* T/C and *DRD2* A/T risk haplotypes was similar in the group of patients presenting or not presenting mood disorders (OR = 0.5, IC95% = 0.2–1.3; *P* = .155).

4. Discussion

In the present study, we observed associations of rs2298383 and T/C haplotype (rs2298383/rs3761422) of *ADORA2A* gene with anxiety disorders in children and adolescents with ADHD. We also demonstrated a positive gene-gene interaction effect of *ADORA2A and DRD2* on the presence of anxiety disorders. These findings seem to be specific for anxiety disorders since no significant effects were detected when we tested the same model using another very prevalent comorbid condition (mood disorders). Moreover, our *in silico* analyses showed that the only SNP presenting main effects on anxiety disorders (rs2298383) has the highest score for regulatory function among all variants in the

Table 4
SNPs functional information collected by RegulomeDB, SuRFR and Haploreg v4.1.

SNP ID	Gene position	RegulomeDB score	SuRFR Ranking[a]	Promoter histone marks (Tissues)	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	Selected eQTL hits
rs2298383	intronic	2b	1st	Blood, Digestive	15 tissues	32 tissues	RFX5	19 altered	18 hits
rs3761422	intronic	5	47th	Blood	10 tissues	2 tissues	–	3 altered	15 hits

Detailed functional information can be found in supplementary material. RFX5: Regulatory Factor X5.
[a] Ranking considering 54 valid *ADORA2A* variants.

ADORA2A gene described up to date.

It is important to note that, to the best of our knowledge, no studies investigated the *ADORA2A* gene in anxiety outcomes in a sample with ADHD. However, the literature has consistently shown associations between rs5751876 polymorphism and anxiety disorders (Deckert et al., 1998; Hamilton et al., 2004; Hohoff et al., 2009). Additionally, some studies have also shown an association between the same polymorphism and anxiety responses followed by caffeine and amphetamine administrations in individuals with no psychiatric disorders (Alsene et al., 2003; Hohoff et al., 2005; Childs et al., 2008; Rogers et al., 2010; Domschke et al., 2012; Gajewska et al., 2013). Regarding other psychiatric disorders, Turčin et al. (2016) investigated five variants in this gene and showed an association of rs2298383 polymorphism with general symptoms in schizophrenic patients. In the same direction of our results, the authors showed that individuals carrying the T allele have the higher scores in a general psychopathology scale (Positive and Negative Symptoms Scale General – PANSSG) that include anxiety assessment (Turčin et al., 2016).

Different from rs5751876, a silent SNP in exon 2, the SNP studied here rs2298383, located in the promoter region, has been suggested to play an essential regulatory role in *ADORA2A* gene (Yu et al., 2004; Conde et al., 2006). The *in silico* analyses performed herein, assessing functional information from ENCODE and RoadMap databases, also suggested that this variant is the most relevant for *ADORA2A* gene regulation. It is possible to speculate that the association observed with rs5751876 may be an indirect effect of rs2298383, putatively the causal one, due to linkage disequilibrium. Our *in silico* analyses also suggested that rs3761422, the other variant investigated in this study, has probably a minor role in *ADORA2A* gene regulation compared to rs2298383, which corroborates our association findings with rs2298383.

Although we did not find an association between the *DRD2* gene and anxiety in the present sample, an interaction effect between *ADORA2A* and *DRD2* genes was observed. Our results showed that the simultaneous presence of *ADORA2A* T/C (rs2298383/rs3761422) and *DRD2* A/T (rs1076560/rs2283365) risk haplotypes is associated with anxiety in this ADHD sample. The present results suggest that 40% of the effect of these variants on anxiety was attributable to synergism. Only one study analyzed the interaction of these two genes with anxiety traits but in a non-psychiatry sample. That study showed interactions between two *ADORA2A* variants (rs5751876 and rs2298383) and several *DRD2/ANKK1* variants. For the rs2298383 they observed interactions with four *DRD2/ANKK1* polymorphisms (rs1799978, rs1079597, rs1110976, rs1800497), and, for all the analyses, the CC genotype of rs2298383 combined with different *DRD2* SNPs was associated with higher caffeine-induced anxiety (Childs et al., 2008). Our findings corroborate the relevance of this interaction in anxiety, despite the difference in risk alleles. Childs et al. (2008) investigated a smaller sample size composed of healthy adults and used caffeine-induced anxiety as an outcome, which could explain the divergence.

Regarding *DRD2/ANKK1* variants, other studies have already reported associations with anxiety phenotypes (Lawford et al., 2006; Kulikova et al., 2008; Hayden et al., 2010). Mota et al. (2015) found an association between rs2283265 and harm avoidance and an association involving haplotypes spanning *DRD2* and neighbor genes with Generalized Anxiety Disorder (GAD) in an ADHD adult sample, corroborating the rational to explore the *DRD2* gene in anxiety phenotypes in ADHD. It is important to note that both *DRD2* polymorphisms studied have functional effects described. The *DRD2* rs1076560 and rs2283265 polymorphisms modulate D_2 signaling through alternative splicing of exon 6, resulting in long (D_2L) and short (D_2S) isoforms (Montmayeur et al., 1991). Both sites minor alleles lead to an increased expression of the D_2 long isoform while reducing the expression of the D_2 short isoform (Zhang et al., 2007; Moyer et al., 2011). The spliced region of the receptor (the third intracellular loop) is critical for G proteins interactions as well as with other proteins, thus suggesting that each isoform might be coupled to distinct pathways (Montmayeur et al., 1993; Guiramand et al., 1995; Shioda, 2017).

The interaction of *ADORA2A* and *DRD2* genes is supported by *in vitro* experimental evidence since there is a robust antagonistic interaction between adenosine and dopamine in the central nervous system. As previously mentioned, adenosine A_{2A} and dopamine D_2 receptors are co-localized and physically interact forming heterodimers (Torvinen et al., 2004; Fuxe et al., 2005). As a consequence of this interaction, the stimulation of the A_{2A} receptor decreases the affinity of D_2 receptor for their agonists (Ferré et al., 1991). Hillion et al., 2002 suggested in an experiment in the human neuroblastoma cell line (SH-SY5Y) that A_{2A}-D_2 heteromers are formed even in the absence of agonists indicating that it would be constitutive. Besides the well-described interaction mechanism, it was also suggested that the excision led by rs1076560 and rs2283265 *DRD2* variants mentioned above might play an additional role on A_{2A}-D_2 heteromerization signaling and modulation (Navarro et al., 2009).

Considering the critical role of *ADORA2A* and *DRD2* genes in psychiatry phenotypes, we also used a family design association study to test the effect of these genes in ADHD. Regarding *ADORA2A*, only one study has investigated this gene in this disorder demonstrating an association between one variant (rs35320474) and inattention symptoms (Molero et al., 2013). However, in agreement with our results, they did not find an association with the variants analyzed in the present study. We also did not see an association between *DRD2* polymorphisms and ADHD. Mota et al. (2015) also did not observe an association with the rs2283265 and ADHD, but as mentioned before they found associations with comorbidities and personality profiles of the sample. Taking together our findings corroborate the idea that these genes could play a role in phenotypic heterogeneity of psychiatry diseases, and gene-gene interactions could help to explain the conflicting results for *ANKK1/DRD2* cluster gene in ADHD (Mota et al., 2015).

This study presents some limitations. First, our sample is relatively small and may be underpowered for the statistical analyses performed. Second, we classified the outcome as the presence of at least one anxiety disorder, which may have increased the heterogeneity of our group. Therefore, larger samples are required to confirm and refine our results. Third, most of the studies that investigated *ADORA2A* and *DRD2* genes on anxiety phenotypes evaluated adult samples. Replicating our findings in other children samples would be necessary.

Another point to be considered is that although we tested the functionality of *ADORA2A* variants, we used *in silico* approach. Although we can speculate that rs2298383 may play a role in the availability of the receptor in the membrane, further interpretation of functional consequences regarding specific alleles is not possible to be inferred with the methodology used. Further, *in vitro* studies are needed to validate and better explore our results. As a final point, *ADORA2A* and *DRD2* genes have not been associated with anxiety and ADHD in a genome-wide significant level. However, the currently explained heritabilities of anxiety and ADHD in GWASs are very small (Demontis et al., 2019; Otawa et al., 2016). Since it is expected genetic variants of small effect to be involved in these complex disorders, it is reasonable to think that these genes may become significant in a GWAS level for anxiety or ADHD in the future.

Finally, it is necessary to keep in mind the large clinical heterogeneity of psychiatric disorders that, at least in part, is due to the frequent occurrence of comorbidities. This heterogeneity is probably related to the polygenic architecture of psychiatric disorders but, despite the advances of molecular studies, there is still a considerable gap between the heritability estimated by the twin/family-based studies compared to the SNP based ones (Manolio et al., 2009; Geschwind and Flint, 2015). In this context, Faraone and Larsson (2018) suggested some strategies to address the ADHD “missing” heritability, such as the investigation of gene-gene interactions, gene-environmental interactions, and gene-environmental correlations. Here, we focused on an interaction of genes that encode for receptors that exhibits a relevant and well-described protein-protein interaction. Our results corroborate

the importance of the gene-gene interaction approach and the relevance of considering the comorbidity profile of the sample to understand the complex genetic architecture of psychiatric disorders better.

5. Conclusion

In conclusion, our findings suggest that the *ADORA2A* gene and the interaction of *ADORA2A* and *DRD2* genes may play a role in anxiety disorders in children and adolescents with ADHD. It is possible that these genes have an effect on anxiety shared among different psychiatric disorders, as well as anxiety traits in the general population. Future studies should test this hypothesis.

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Declaration of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2019.03.021.

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