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ORIGINAL RESEARCH

A Drosophila Model for Attention Deficit Hyperactivity Disorder (ADHD)

No Evidence of Association with PRKG1 Gene

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a prevalent psychiatric condition in children and follow up studies have indicated that 22–33% of patients continue to suffer from ADHD during late adolescence and adulthood. The action of psychostimulant drugs may be determined by additional mechanisms beyond the dopamine transporter and receptors. We are exploring new methodology for discovering these mechanisms. For example, in Drosophila, such an additional determinant of psychostimulant action could be protein kinase G (PKG) that affects food-search behavior. Here we initiated studies with the human homologue of PKG, the PRKG1 gene. The aim of this study was to investigate for the presence of linkage disequilibrium between the protein kinase G gene (PRKG1) and adult ADHD in a sample of nuclear families. Genotyping data for the C2276T polymorphism were analyzed using the Transmission Disequilibrium Test (TDT). Sixty three nuclear families were informative for the TDT on C2276T polymorphism, which showed no preferential transmission of either allele (chi-square = 0.778, df = 1, $p = 0.316$). These findings exclude a direct involvement of this genetic marker of the Protein kinase G gene in the pathogenesis of ADHD.

Index Entries: Adult ADHD; PRKG1 (protein kinase G gene); PKG (CGMP-dependent protein kinase); polymorphism; linkage disequilibrium; TDT (transmission/disequilibrium test); genetics.

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Introduction

ADHD is a chronic psychiatric condition that affects approximately 3–5% of school age children and it is characterized by severe impairment in attention span, or marked hyperactive and impulsive behavior not appropriate for age *(1)*. Several follow up studies have shown that ADHD is more rare in late adolescence and early adulthood than in childhood, however the disorder cannot be considered to be only a childhood disorder. *(2)*.

Convincing evidence has pointed out that the pathogenesis of child ADHD has a strong genetic component *(3)*. The adult, or persistent form of ADHD appears to have an even stronger genetic component than the child form based on the significantly higher genotype relative risk of 19 to 26 *(4)*. However, the percentage of retained diagnosis in adulthood varies across studies. The relative lack of specific validated criteria for adult ADHD and the different definitions used to evaluate the persistence of the disorder may have contributed to these discrepancies. In the fourth edition of DSM, the criteria for ADHD are applicable to both adults and children. However, because the phenomenology in ADHD varies across the life span in quality of symptoms as well as in degree of severity *(5,6)*, specific criteria for adults appear to be necessary. In several studies, the phenotype of adult ADHD was derived from the Brown Attention Deficit Disorder Rating Scale (BADDS) *(7)* and the Wender Utah Rating Scale (WURS) *(8)*. The BADDS measures core symptoms of ADHD, and WURS retrospectively assesses childhood ADHD symptoms.

Compounds acting on the dopaminergic system are very efficacious in the treatment of ADHD *(9)*. For these reasons, genes in the dopaminergic system have been considered good candidates for ADHD. To date, a relatively large number of replicating results from these studies are in agreement suggesting the involvement of DRD4 *(10)* and DAT *(11)* in ADHD. There are very few published studies on the molecular genetics of specifically adult ADHD. We have published positive results for the DRD4 gene *(12)* and negative results for DRD3 *(13)*. Faraone et al. *(14)* also report a positive association for DRD4 in adult ADHD proband families.

The risk factors for ADHD may be determined by additional mechanisms beyond the dopamine transporter and receptors. Convincing evidence has indicated synaptosomal-associated protein of 25 KD (SNAP-25) in the pathogenesis of attention deficit hyperactivity disorder (ADHD). The coloboma mouse mutant, considered to be a good animal model of hyperactivity, is deleted for this gene *(15)*. Based on this animal model, Barr et al. *(16)* and Mill et al. *(17)* have shown an association between the SNAP-25 gene and ADHD. We are exploring similar methodology for discovering candidate genes in ADHD. Drosophila models provide valuable analysis tools of the nervous system and behavior based on the significant number of fly homologs of human neurological disease loci *(18)*. An additional gene for ADHD could be protein kinase G (PKG) which affects food-search behavior in Drosophila. Osborne et al. *(19)* assigned behavioral functions to this relatively unknown member of the serine/threonine kinases and showed that small differences in PKG can lead to naturally occurring variants in Drosophila food-search behavior: *rover* and *sitter*. Individuals with a rover allele move greater distances while feeding than do those homozygous for the sitter allele. Although Osborne et al. *(19)* indicate that locomotion in the variants is similar during absence of food; when they are presented with multiple patches of food, *rovers* move from patch to patch visiting a food patch for a short time and then moving to the next patch, and then the next patch repeating this behavior again and again *(20)*. In addition, differences in a physiological measure of habituation have been shown in rovers and sitters as a result of differences in PKG *(21)*.

The cyclic guanosine monophosphate (cGMP) dependent Protein Kinase (PKG) is a transduction pathway enzyme. PKG is operative in a variety of cell responses *(22)* that are also typical of signal transduction components *(23)*. Neurophysiologial studies have shown that injected kinase affects neuronal membrane conductance in snails and mammals *(24,25)*, that PKG-inhibitors block long-term potentiation in mammalian hippocampus *(26)*, and that PKG is involved in prejunctional long-term potentiation in cultured hippocampal neurons *(27)*.

Here we initiated studies with the human homolog of PKG, the PRKG1 gene. Orstavik et al. *(28)* found surprisingly high homology between drosophila and human in that 5 of the 7 splice sites in the drosophila DG2 gene, which encodes a PKG, are also present in the human PKG gene (PRKG1). PRKG1 is located on chromosome 10q11.2 *(28a)*. PKG is ubiquitously expressed in the nervous system and outside the CNS, in neutrophil cells and

smooth muscle cells *(29,30)*, and has a variety of pleiotropic cellular regulatory functions.

There are no polymorphisms in the exons but many Single Nucleotide Polymorphisms (SNP) exist in intron regions of this gene, and currently there is one known in the 3′ UTR. To our knowledge there are presently no data regarding the allele frequencies and function of these SNPs in humans.

Case-control genetic association tests may be affected by problems of population stratification and heterogeneity of confounding factors, while family-based association studies are considered better strategies in the identification of linkage between genes and susceptibility to complex disease. This is particularly true when the risk genes are of small effect *(31)*, as is likely the case for psychiatric traits.

To test for the presence of linkage disequilibrium between genetic markers and ADHD, we employed the transmission disequilibrium Test (TDT) *(32)* in a sample of 125 nuclear families with adult ADHD patients. We tested the 3′ UTR polymorphism of the PRKG1 gene, C2276T (dbSNP accession number 1881597). This SNP is useful because it is the only one represented in the mature mRNA of PRKG1 and thus may be more biologically relevant than the others that are present only in the pre-mRNA.

Materials and Methods

Sample

For this study, 125 nuclear families identified through an adult ADHD proband were recruited from the Adult and Adolescent ADHD Research Program of the Centre for Addiction and Mental Health (CAMH). The ethnic makeup was 98% Caucasian, 1% Asian, and 1% African American.

The diagnosis of ADHD was determined by criteria from the Diagnostic and Statistical Manual, 4th Edition, (DSM-IV) *(1)*. Additional criteria were: >46 on the WURS, >55 on the BADDS, >60 on the Conner's Adult ADHD Rating Scale (CAARS) *(33)*, >10 on the Conner Continuous Performance Test (CPT) *(34)*, and >80 on Block Design Subtests of Weschler Adult Intelligence Scale, 3rd Edition (WAIS-III) *(34a)*. The Structured Interview for DSM-IV (SCID-I) *(1)* was employed to document other psychiatric symptoms and comobid diagnoses. If a comorbid diagnosis emerged as predominating and possibly accounted for the ADHD symptoms, then the subject was excluded. These rating scales were administered by trained interviewers not informed as to the genotypes of the probands. From all participants and their parents, written informed consent to participate in the study was obtained.

Laboratory Methods

Genomic DNA was extracted from white blood cells using a high-salt extraction method *(35)* in the Neurogenetics Laboratory of the Centre for Addiction and Mental Health (CAMH), Toronto, Ontario, Canada. The genotyping of each patient's DNA was performed with the laboratory staff unaware of the psychiatric rating.

To determine the presence of AciI-polymorphism in PRKG1, amplification of a 221 bp fragment of the 3′UTR (2129 through 2349) encompassing position 2276 (GenBank accession number NM_006258) of the PRKG1 gene was performed by polymerase chain reaction (PCR) with 125 ng of genomic template DNA, 1.5 m*M* $MgCl_2$, 1 µM of each oligonucleotide primer (P1 5′ TTA CCT GCT TCT GCC TTG CT 3′, and P2 5′ CAG GAC CAC CAT GTC AAC TG 3′), 200 µM of each nucleotide dATP, dGTP, dCTP, dTTP; and 1 unit of Amplitaq DNA polymerase in a final reaction volume of 25 µL. After an initial denaturation stage of 5 min at 95°, the PCR amplification profiles consisted of the denaturation at 94° for 30 s, primer annealing at 56° for 30 s, and extension at 72° for 30 s, for 35 cycles. A final extension step of 72° was added for 4 min after the last cycle. 25 µL of the PCR product was digested with 5 units of the restriction enzyme AciI, a restriction enzyme specific for the sequnce CCGC; this mixture of 30 µL was incubated at 37° overnight. The alleles were detected after separation by electrophoresis on a 2.5% agarose gel in TBE at 150V for 1 h and stained with ethidium bromide for UV visualization with the lengths estimated by standard markers. Allele 1(T) is not cut with the restriction enzyme and is seen as a 221bp band. Allele 2 (C) is cut into two bands of 151 and 70bp. The smallest of these fragments (70bp) is too small to be resolved on the gel.

Statistical Analyses

We tested the genotypes from the nuclear families for the non-random transmission of C2276T alleles of the PRKG1 gene to ADHD offspring with the Transmission Disequilibrium Test *(32)*. Analy-

Table 1
Results of the TDT on the 3'UTR Polymorphism
of PRKG1 Gene in Informative Triads

Alleles	C	T
Transmitted	28	35
Non-transmitted	35	28
Chi-square	0.778	0.778
P values	0.316	0.316

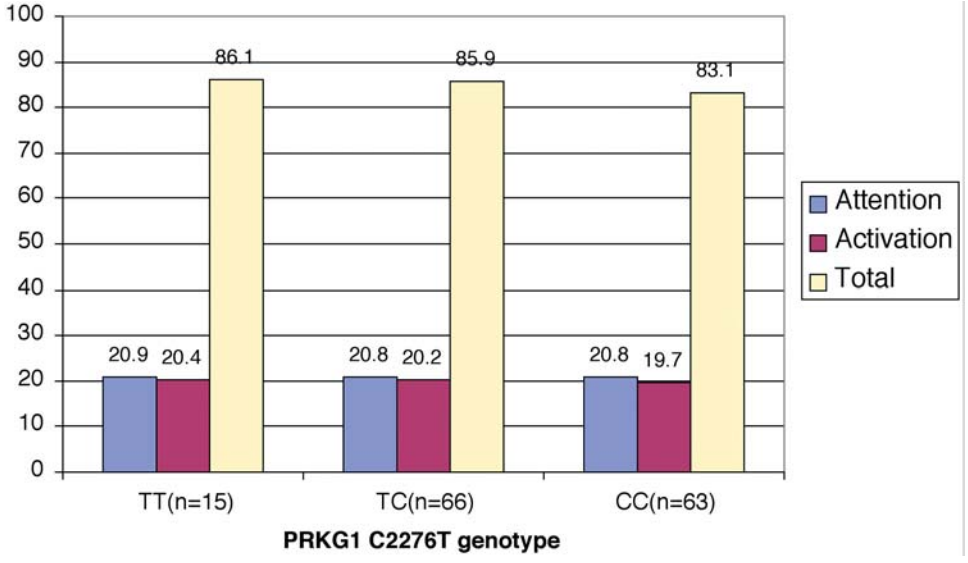

Fig. 1. ANCOVA analyses with BADDS mean scores. Color image available for viewing at **www.humanapress.com**

sis of covariance (ANCOVA) was used to compare the mean BADDS scores for each of the genotypic classes. The ANCOVA is, under most circumstances, more powerful than the non-parametric chi-square statistics.

Results

The genotype counts in the entire sample for the C2276T polymorphism were: CC = 269, CT = 246, and TT = 61 while allele frequencies were 68% and 32% for the C allele and T allele respectively. For this sample there was no deviation from Hardy-Weinberg equilibrium (chi-square = 0.181, 1df, p=0.67). With respect to this polymorphism, out of the 125 triads comprising the total sample, 63 were informative for the TDT, while the other 62 were triads with homozygous parents. The TDT showed that C and T allele were transmitted with similar frequency to the affected subjects (chi-square = 0.778, 1df, p= 0.316) (*see* Table 1). ANCOVA analyses with BADDS total scores, where covariates age and sex revealed no significant difference across the three genotypic classes (F[2,139] = 0.39, p = 0.68) (*see* Figure 1). Also, mean attention and activation subscales scores, respectively, did not differ significantly between the patients grouped according to genotype (F[2,139] = 0.015, p = 0.98; F[2,139] = 0.262 , p = 0.77).

Discussion

The main results from our study appear to exclude the direct involvement of C2276T variants of the PRKG1 gene in the pathogenesis of adult ADHD, as no biased transmissions were observed. The number of triads with heterozygous parents suitable for the TDT analysis was relatively small. Consequently, the sample we studied may not have sufficient power to detect association to ADHD. A limitation in the interpretation of our results is that

we have only used a single polymorphism for PRKG1 and it is a large gene (220 Kb) *(28)*. Thus large areas of the gene were not directly tested. Ideally, additional polymorphisms in PRKG1 should be genotyped to increase the possibility of detecting linkage by analyses of the haplotypes using several SNPs across the gene. While the use of haplotypes may increase the information at the DNA level, the increased degrees of freedom created by the use of haplotypes requires a larger sample size in order to have sufficient statistical power. Considering these limitations, there can be no definitive conclusions regarding the role of PRKG1 in ADHD.

There are no other known studies investigating the role of PRKG1 polymorphisms in ADHD. Although the C to T substitution of the C2276T variant does not change the amino acid sequence of the enzyme protein, the different alleles could induce a different mRNA secondary structure, affecting the stability, processing, or subcellular targeting of the mRNA transcript with changes in splicing, transcription, and efficiency of translation. However the gain-of-function or loss-of-function in humans of the C2276T naturally occurring polymorphism in PRKG1 remains unknown. There is evidence of the involvement of PKG in learning derived mostly by data from animal models. The protein kinase system is complex and future systematic investigations combining clinical pharmacological and genetic approaches will be quite valuable in elucidating the hypothesized involvement of protein kinase and second messenger systems in conferring risk to ADHD.

Finally, although our results are not suggestive of a direct involvement of PRKG1 in adult ADHD, it could still be hypothesized that the gene is involved in child ADHD. To this end, we are currently planning to genotype C2276T on a collaborative basis, in a collection of childhood ADHD patients. Further genetic investigations on this marker considering alternative quantitative phenotypes related to ADHD are warranted. The results of the analyses we did on the BADDS as quantitative traits related to ADHD with respect to the C2276T polymorphism of the PRKG1 deserve some comment. The analysis of quantitative traits continuously distributed within the population, as opposed to the study of categorical traits, has been identified as a more powerful strategy in detecting genetic susceptibility to complex diseases *(36–38)* with the additional benefit of minimizing the confounding effect of phenotypic heterogeneity in psychiatric diagnoses. However, in the alternate strategy that uses a parametric statistic (ANCOVA) which can remedy the problem of population stratification, we did not find any association between the BADDS scores and C2276T polymorphism of the PRKG1.

In particular, as PKG is a ubiquitous key component of second messenger cascades, it is possible that this variant could perturb the normal phenotype toward ADHD in one of several different ways. To detect this perturbation, more complex investigations may be required, such as using the phenotype of response to medication in ADHD.

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Electronic-Database Information

The accession numbers and URL for data in this article are as follow:

Database of Single Nucelotide polymorphisms (dbSNP), http://www.ncbi.nlm.nih.gov/SNP/ (for NCBI-assay ID number for C2276T SNP in this study [accession number 1881597])

Online Mendelian Inheritance in Man (OMIM), http:/www.ncbi.nlm.nih.gov/Omim/ (for *prkg1* [MIM 176894]).

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